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Sathyanarayanan Sevilimedu Veeravalli

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BIOLOGICAL HYDROGEN PRODUCTION FROM LIGNOCELLULOSIC BIOMASS IN AN UP-FLOW ANAEROBIC SLUDGE BLANKET REACTOR USING MIXED MICROBIAL CULTURES

By

Sathyanarayanan Sevilimedu Veeravalli

A Dissertation
Submitted to the Faculty of Graduate Studies through the Department of Civil and Environmental Engineering in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy at the University of Windsor

Windsor, Ontario, Canada

2014

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Biological Hydrogen Production From Lignocellulosic Biomass in an Up-flow Anaerobic Sludge Blanket Reactor using Mixed Microbial Cultures

by

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8 May 2014
Declaration of Co-Authorship / 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 D. Heath. The contribution of co-authors is limited to the provision of microbiological results which are included in Chapters 4, 5, 6.2, 6.3, 7 and 8 of the dissertation.

In all cases, the experimental design was sent to Dr. Jerald A. Lalman by the author, based on his suggestion and recommendations made, the 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 qualifications, this dissertation, and the research to which it refers, is the product of my own work.

II. Previous Publication:

Chapter 6: This chapter was published under title ‘Effect of furans and linoleic acid on hydrogen production’ in *Int J Hydrogen Energy*, 2013, Volume 28, Issue 18, 12283-1293 ([http://dx.doi.org/10.1016/j.ijhydene.2013.07.035](http://dx.doi.org/10.1016/j.ijhydene.2013.07.035)). The initial draft was written by Mr. Veeravalli, for which Dr. Lalman provided significant changes for publication. The additional recommendations and suggestions given by Dr. Lalman were incorporated in the dissertation Chapter. I would like to thank Dr. Lalman, for his contribution.

Chapter 7: This chapter was published under title ‘Optimizing hydrogen production from a switchgrass steam exploded liquor using a mixed anaerobic culture in an upflow
anaerobic sludge blanket reactor’ in *Int J Hydrogen Energy*, 2014, Volume 39, Issue 7, 3160-3175 (http://dx.doi.org/10.1016/j.ijhydene.2013.12.057). The initial draft was written by Mr. Veeravalli, which was reviewed by Dr. Lalman and Dr. Chaganti who provided additional recommendations for the improvement. Dr. Lalman provided significant changes for the publication, the additional recommendations and suggestions given by Dr. Lalman were incorporated in the dissertation Chapter. I would like to thank Dr. Lalman and Dr. Chaganti, for their contribution.

**Chapter 8:** This chapter was published under title ‘Fermentative H$_2$ production from a switchgrass steam exploded liquor fed to mixed anaerobic cultures: Effect of hydraulic retention time, linoleic acid and nitrogen sparging’ in *Int J Hydrogen Energy*, 2014 (In Press) (http://dx.doi.org/10.1016/j.ijhydene.2014.04.114). The initial draft was written by Mr. Veeravalli, which was reviewed by Dr. Lalman who provided additional recommendations for the improvement. Dr. Lalman provided significant changes for the publication, the additional recommendations and suggestions given by Dr. Lalman were incorporated in the dissertation Chapter. I would like to thank Dr. Lalman, for his contributions.

I hereby certify that no other part of this dissertation has been published or submitted for publication.

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 current research investigated hydrogen (H₂) production potential from lignocellulosic biomass via dark-fermentation in upflow sludge blanket reactors (UASBRs) using mixed anaerobic culture. The effects of hydraulic retention time (HRT) and organic loading rate (OLR), on H₂ production were examined under mesophilic conditions using linoleic acid (LA), as a methanogenic inhibitor. The dynamics of the microbial community were explored using terminal restriction fragment length polymorphism analysis.

Studies with pure glucose revealed that high H₂ yield ≥ 2.1 mol mol⁻¹ glucose was obtained in control cultures operating at HRTs ranging from 12 h to 20 h with OLRS corresponding to 16 g L⁻¹ d⁻¹ and 10 g L⁻¹ d⁻¹, respectively. Species belonging to Clostridia was observed under these conditions. A further decrease with the HRT in control cultures reduced H₂ yields up to 1.3 mol mol⁻¹ glucose, while addition of LA showed improved H₂ yields ≥ 2.0 mol mol⁻¹ glucose at HRTs ranging from 6 to 12 h.

A maximum H₂ yield of 303±20 mL g⁻¹ COD was obtained from switchgrass-derived sugars under the optimal conditions (pH 5.0, HRT 10 h and 1.75 g L⁻¹ of LA) determined using response surface methodology. The microbial characterization under optimal conditions showed dominance of Ruminococcaceae and Clostridiaceae with efficient suppression of methanogens. Nitrogen sparging of the UASBRs under the optimal conditions, increased H₂ yield by 15% in comparison to unsparged cultures. Sparging the bioreactors increased the abundance of Clostridium sp. and Bacillus sp. under LA treated conditions.

A stable H₂ yield of 274±40 mL g⁻¹ COD was obtained by the control cultures fed corn stover hydrolysate and operating at 18 and 24 g COD L⁻¹ d⁻¹, suggesting furans and phenols could serve as methanogenic inhibitors at low levels. The dominance of Clostridium sp., Flavobacterium sp. and Eubacterium sp., were observed under these H₂-producing conditions.
The results from current research suggest that H₂ production from lignocellulosic biomass is feasible and could be applied on a large scale by maintaining proper operational conditions.
DEDICATION

I dedicate this dissertation work to my mother Geetha, other family members and friends.
ACKNOWLEDGEMENTS

First I would like to thank the ALMIGHTY, for imparting moral support towards the completion of my doctoral work at Windsor, Ontario, Canada.

With a deep sense of gratitude, I wish to express my sincere thanks to my guide Dr. Jerald A. Lalman for his invaluable guidance at every stage of this research project and helping me get out the publications.

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<td>CH$_4$</td>
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<td>CO$_2$</td>
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<td>COD</td>
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<tr>
<th>Abbreviation</th>
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<td>Continuous stirred tank reactor</td>
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<tr>
<td>DF</td>
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<td>HPG</td>
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<td>MS</td>
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<td>Volatile solids</td>
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CHAPTER 1: INTRODUCTION

1.1 Background

The greatest energy challenge of the 21st century is to develop sustainable energy resources which will meet future demands. Currently, fossil fuels are the primary energy sources used to satisfy the global energy demand. Following the oil crisis in 1973, the need for developing alternative energy sources was initiated to reduce the dependency on fossil fuels and assist with mitigating environmental problems due to the usage of fossil fuels. Changes in global environmental conditions due to greenhouse gases (GHG), especially increasing CO$_2$ levels, can be linked to increasing use of fossil fuels (Bockris, 2002; Das and Veziroglu, 2001). In order to prevent increasing climatic issues, the need for researchers to develop economical and renewable fuels is evident. In response to these economic and environmental drivers, research on the use of hydrogen (H$_2$) as an energy carrier was initiated in 1977 by the International Energy Agency (Luzzi et al., 2004).

Hydrogen is considered a strategically important energy carrier because of its high energy content (142 kJ g$^{-1}$). Hydrogen is an alternative energy source because it can be produced from renewable feedstocks and H$_2$O the combustion byproduct of H$_2$ is a carbon neutral species (Momirlan and Veziroglu, 2002). A variety of feedstocks such as methane, natural gas, coal, water, alcohols, lignocellulosic biomass, glycerol, sugars, organic acids, wastewater and food waste can be used to produce H$_2$ using biological and non-biological methods (Levin and Chahine, 2010; Veziroğlu, 1975). Among these biological processes of H$_2$ production is preferred for using renewable sources such as organic waste or lignocellulosic biomass, which is more energy efficient in solving the growing energy needs (Claassen et al., 1999).

Hydrogen is commercially produced using non-biological methods such as steam reforming, thermal cracking, gasification and pyrolysis of fossil fuels. Among the various non-biological methods, steam reforming of natural gas is the most widely used commercial method for producing H$_2$. According to the United States Department of Energy, the steam reforming method accounts for 90% of the H$_2$ (USDOE, 2001). The United States Energy Information Administration (EIA) (2008) has reported that approximately 10-11 million metric tonnes of H$_2$ are produced each year and can fuel 20-
30 million cars. However, the H\textsubscript{2} produced from non-biological sources is mainly used as a feedstock by chemical industries for the synthesis of ammonia, alcohol (methanol) and a variety of organic chemicals (Rand and Dell, 2008).

Steam reforming is an energy intensive process because of the high temperature (above 800 °C) required for the reaction of steam with natural gas containing methane to produce H\textsubscript{2}. H\textsubscript{2} production from water is considered a favorable renewable process, since H\textsubscript{2} is produced by the electrolysis of water. The disadvantage associated with electrolysis of the water is attributed to high capital cost and the operating cost which accounts for 80% of the production cost (Armor, 1999). Other production methods which include thermal decomposition and photolysis are also energy intensive (Das and Veziroglu, 2001).

In order to avoid an undesirable scenario of using depleting resources to generate fuel with the energy intensive and GHG-contributing methods, H\textsubscript{2} production from renewable feedstocks has emerged as an alternative energy source. Bio-H\textsubscript{2} may prove to be a sustainable fuel as H\textsubscript{2} can be produced from organic waste. In comparison to the potential negative environmental impacts associated with the use of fossil fuels as well as the increase in fuel prices, the commercial production of H\textsubscript{2} from biomass has become very important (Nath and Das, 2003). Although pyrolysis, gasification and steam gasification techniques are available for converting biomass to a useful form of energy, increasing concern about global warming has increased, research interest in the development of environmentally friendly biological methods for H\textsubscript{2} production from biomass (Das and Veziroglu, 2001). The contribution to global warming potential (GWP) by Pyrolysis, gasification and steam gasification process was reported to be on average of 1.8E+02 kg CO\textsubscript{2}-equivalent. In each case the GWP contribution was majorly from the energy required at start up (Khoo, 2009).

Biological H\textsubscript{2} production can be achieved by photolysis, photo-fermentation and dark-fermentation using green algae, Cyanobacteria, photosynthetic bacteria and anaerobic fermentative bacteria, respectively (Levin et al., 2004). Photo fermentation from different waste materials requires a light source and low photochemical conversion efficiency results in low H\textsubscript{2} production rates (HPR) (Levin et al., 2004), while bio-photolysis from water produces O\textsubscript{2} in addition to H\textsubscript{2}. A major disadvantage of the bio-
photolysis process is the inhibitory effect of O$_2$ on hydrogenase leads to reduced HPRs (Das and Veziroglu, 2001). Dark-fermentative H$_2$ production using a variety of carbon sources offers high HPR and produces high-value liquid metabolites (e.g. acetic, butyric and lactic acid) and lower alcohols (e.g. ethanol and butanol), which have some commercial value (Benemann, 1996; Chang et al., 2010; Levin et al., 2004; Williams et al., 2013). A major issue for commercial implementation of dark fermentation is the cost of feedstock that is required for H$_2$ production (Kapdan and Kargi, 2006). Avoiding high cost in the production process can be accomplished using low value feedstocks or organic wastes. Dark fermentation can utilize a wide variety of renewable biomass sources which could be used in larger scale systems. Biomass sources include agricultural waste, woody and non-woody biomass (Antonopoulou et al., 2008; Panagiotopoulos et al., 2011; Saratale et al., 2008), municipal solid waste (Gomez et al., 2006) and food waste (Redondas et al., 2012).

Several reports have described efficient production of H$_2$ from biomass (de Vrije et al., 2009; Kapdan and Kargi, 2006; Panagiotopoulos et al., 2011). Important factors to be considered for using fermentative H$_2$ production include econonics, process efficacy and reliability of the H$_2$ production process. Using mixed culture inoculum from wastewater treatment facilities for H$_2$ production systems is a means of minimizing operational costs. This is because using mixed cultures does not require feedstock and nutrient media sterilization, which accounts for major operational cost on a larger scale (Hawkes et al., 2007). The present study is focused on assessing the impact of operational parameters such as pH, HRT and OLR and addition of LA on H$_2$ production by dark-fermentation using mixed anaerobic cultures and lignocellulosics as a feedstock.

1.2 Problem statement

Despite significant progress in fermentative H$_2$ production research and development, still issues remain before the technology can be adopted for producing H$_2$ from lignocellulosic biomass. In general, existing concerns for fermentative H$_2$ production are related to optimizing H$_2$ production by manipulating various factors affecting the performance of mixed anaerobic cultures (Antonopoulou et al., 2010; Lay, 2000; Shin et al., 2004).
A major issue affecting fermentative H₂ production using mixed anaerobic cultures is the syntrophic association between H₂-producers and H₂-consumers (e.g. homoacetogens and hydrogenotrophic methanogens) as well as non-H₂ producers (such as acetoclastic methanogens, ethanoligenes and acidogens) which are linked to undesirable byproducts (Angenent et al., 2004; Dinopoulou et al., 1988; Oh and Logan, 2005; Schink and Stams, 2006). In order to achieve optimal H₂ yields, pretreatment techniques are employed to enrich H₂-producers. A pretreatment technique that could be feasible and applied on a large scale to combat the H₂ consumers without affecting H₂ producers is required. Physical treatment such as heat and chemical inhibitors such as acetylene (Sparling et al., 1997), 2-bromoethanesulfonate (BES) (Zhu and Beland, 2006) and long chain fatty acids (LCFAs) (Ray et al., 2010) to suppress the activity of H₂ consumers is widely used. The use of heat shock is economically less feasible on large scale, while synthetic inhibitors such as BES could cause environmental effects if effluents from bioreactors are discharged into receiving water bodies. In comparison, LCFAs are biodegradable and preferred for selective enrichment of H₂-producers and suppression of methanogens in full-scale bioreactors (Chaganti et al., 2013; Hwu et al., 1998).

Selective large-scale enrichment involves a combination of several operational parameters as well as culture pretreatment. Furthermore, commercialization of dark-fermentation techniques for bio-H₂ production requires the achievement of high HPR in order for the technology to be economically feasible. Factors affecting HPRs are mainly the organic loading rate (OLR) and the hydraulic retention time (HRT). Although several studies have examined the optimization of operational parameters and culture enrichment for maximal H₂ production, conflicting experimental outcomes have been reported (Danko et al., 2008; Fang and Liu, 2002; O-Thong et al., 2011). Linking the impact of multiple factors on the performance of reactors and diversity of microbial populations in full-scale reactors using low value feed stocks is lacking in the literature.

A detailed characterization of the process (i.e., the culture and byproducts formed under different operating conditions) will assist in optimizing H₂ production and developing an economically feasible full-scale process. Future research challenges to develop economically feasible bio-H₂ production methods will include effective conversion of lignocellulosics to consumable sugars during biomass pretreatment and
increased substrate conversion efficiency in terms of \( \text{H}_2 \) production to overcome the inhibition caused by toxic substance (furfural, 5-hydroxymethylfurfural (5-HMF) and phenols. The performance of microbial communities is affected by environmental factors (pH, temperature), operational factors (OLR, HRT), substrate feed (type and concentration) and inoculum treatment. Assessing the impact of these factors on the performance of mixed microbial communities using genomic tools is important in optimizing \( \text{H}_2 \) production.

This study examined the production of \( \text{H}_2 \) from lignocellulosics using mixed anaerobic cultures. The work is also focused on investigating the effects of operational parameters on continuous bio-\( \text{H}_2 \) production from a steam exploded hydrolysate fed to microbial cultures pretreated with linoleic acid (LA) in an upflow anaerobic sludge blanket reactor (UASBR).

1.3 Objectives

The overall objective of the present research was to assess the biohydrogen production at mesophilic temperature (37 °C) and acidic pH as well as metabolite distribution under different fermentation conditions and correlating these outcomes with the microbial diversity. Pure and lignocellulosic sugars were used as the substrate in the \( \text{H}_2 \) production studies using the mixed anaerobic communities. To accomplish these objectives, the research experiments were divided and conducted in five different phases.

1) To evaluate continuous \( \text{H}_2 \) production in upflow reactors from glucose using granular and flocculated cultures treated with linoleic acid.

The objective of phase I (Chapter 4) was to examine different culture types (granulated and flocculated) for producing \( \text{H}_2 \) in continuous systems operating at pH 5.0. The impact of LA treatment on granulated and flocculated cultures to produce \( \text{H}_2 \) from glucose was also examined. Principal component analysis (PCA) was used to analyze trends between the metabolic byproducts and cultures at different operating conditions. Microbial variation within the granulated and flocculated cultures and addition of LA was studied using a principal co-ordinate analysis (PCoA) using terminal-restriction fragment length polymorphism (T-RFLP) data.
2) To evaluate the key parameters of a continuous H₂ producing system in a UASBR fed pure glucose using mixed anaerobic communities

The objective of phase II (Chapter 5) was to evaluate the effect of OLR in two different stages (under constant HRT (stage I) and with decreases in HRT (stage II)) on H₂ production using a mixed anaerobic culture maintained at pH 5.0. The changes in H₂ yield and HPR were monitored in a continuous flow reactor. The impact of OLR on methanogenic suppression and a shift of metabolic pathway between solventogenic and acidogenic phase is discussed. The effect of OLR on hydrogenase (evolution and uptake) activity was also studied. Further work was conducted to determine if there was any effect of LA on the H₂ yield and HPR (stage III). Statistical analysis using PCA and flux balance analysis were conducted to elucidate the metabolic performance of the microbial culture. Diversity in the microbial profile correlating to the metabolic products produced was accomplished by characterizing the microflora using 454-pyrosequencing and T-RFLP. Statistical analysis on the microbial profile was performed using a PCoA and diversity among the cultures at different operating conditions was studied using different indices.

3) Optimization of process parameters for continuous H₂ production from a mixture of pentoses and hexoses derived from switchgrass in UASBR using a Box-Bhenken design

In phase III, preliminary studies on H₂ production from glucose and fermentation inhibitors (furans) was performed in batch reactors using LA as methanogenic inhibitor (Chapter 6). The batch studies were followed by investigation of H₂ production using steam-exploded hydrolysate in laboratory-scale continuous flow reactors.

Hydrogen production using LA treated cultures and fed a resin treated switchgrass (SWG) hydrolysate in UASBRs was examined at 37°C. Optimizing pH, HRT and LA concentration for maximum H₂ production was performed using response surface methodology (RSM) (Chapter 7). A PCA was used to examine the relationships between the process parameters, culture conditions and the fermentation byproducts.
Characterization and variation in the microbial profile were obtained from the T-RFLP data and studied using a multivariate cluster analysis.

4) Effect of N\textsubscript{2} purging and linoleic acid treatment with a change in HRT, on H\textsubscript{2} production using a mixture of pentoses and hexoses derived from switchgrass in UASBR

In phase IV (Chapter 8), the objective was to investigate the effect of HRT, LA treated inoculum and N\textsubscript{2} purging on H\textsubscript{2} production using cultures fed a resin treated steam exploded SWG hydrolysate. Control cultures (without LA) fed a resin treated steam exploded SWG hydrolysate were operated in parallel to examine the effect of N\textsubscript{2} purging. A flux balance analysis was conducted to examine the effect of the different operating conditions on the H\textsubscript{2} yield. The shift in the metabolic pathway was studied using the PCA. A PCA was conducted to study the relationships present in the microbial dataset obtained from the T-RFLP and the H\textsubscript{2} yield.

5) Using steam exploded corn stover liquor for bio-H\textsubscript{2} generation using mixed anaerobic cultures – a sustainable approach

In phase V (Chapter 9), the effects of OLR, LA, furans and phenols on H\textsubscript{2} production from steam exploded corn stover (CS) hydrolysate was examined using cultures at pH 5.0 and a continuous flow reactor operating at 37°C. The study demonstrated the potential of using a CS steam exploded liquor as a feed for H\textsubscript{2} production in laboratory-scale UASBRs. The treatability efficiency of a feedstock containing furans and phenols was examined in the study. In addition, a canonical correspondence analysis (CCA) was used to assess the association of microorganisms with fermentation byproducts under different conditions.

1.4 Thesis organization

The thesis is focused on H\textsubscript{2} production from lignocellulosics. The research objectives included share a common aim of investigating the effect of process variables on fermentative H\textsubscript{2} production. This topic is introduced with a general description of relevant background material and related research findings on using biological methods
for \( \text{H}_2 \) production followed by a brief note on the dark-fermentation and its associated problems. This introductory framework is followed by a detailed literature review of research findings in this area. The next section provides an overview of the methodology used in the research presented in this thesis, including description of the experimental setup and chemical, analytical, enzymatic and microbial methods employed and a description on data/statistical analysis. The experimental goals were accomplished using glucose followed by using mixed sugars derived from lignocellulosic material (SWG) to feed cultures in UASBR while varying parameters for operating the reactor. Finally, the \( \text{H}_2 \) production potential of steam exploded hydrolysate obtained from lignocellulosic material (corn stover) is presented for a long test run of 80 days with varying sugar loading over the course of the test. The results are presented and discussed in separate chapters for each study, along with the engineering and the genomic data obtained during that particular study. The overall conclusions from these studies are presented with suggestions for future research.

1.5 References


CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

With increasing population and economic growth, rising demand for goods and transportation services and as well as energy supplies is expected in the near future. The world population is expected to reach 9.1 billion by the 2050’s (Zlotnik, 2005) and global energy demands are expected to increase 44% by 2060 (Starr et al., 1992). This suggest an urgent need for the plentiful supply of energy. Unlike energy from non-renewable sources, such as fossil fuels that adversely affects the environment, renewable fuels are carbon neutral and environment friendly. Energy from renewable sources do not negatively affect the environment and are preferred over fossil fuels (Klass, 2004). However, the major energy sources for fuel consumption are petroleum and natural gas. Figure 2.1 represents energy consumption in the United States (U.S.) by energy source for the year 2010 and the increase in renewable fuel source consumption expected by 2035 (US Energy Information Administration (EIA), 2012).

Figure 2.1 U.S. Energy consumption by energy source
Hydrogen ($\text{H}_2$) has been identified as a potential source of energy because of its high energy content per unit mass (William, 2004). However, most of the $\text{H}_2$ produced is from natural gas and when the demand for $\text{H}_2$ increases, the costs of natural gas and the processes involved in $\text{H}_2$ production from this source is expected to rise. Moreover, the use of natural gas for $\text{H}_2$ production leads to increasing GHG emissions and consequently, using biomass rather than natural gas to produce $\text{H}_2$ aids in attenuating this problem. According to research reports, agricultural and forest residues contribute 20-40% of the total global energy consumption (Perlack et al., 2005). Biomass sources include wood and wood wastes, agricultural crops and their residues, waste generated from the food and paper industries as well as municipal solid waste. Energy consumption from biomass accounts for only 3% of energy usage in industrialized nations, while in developing countries biomass serves as a major source of energy (35%) and accounting for approximately 14% of the world’s energy usage (Demirbas, 2005). Biomass can be used to generate both liquid and gaseous fuels; liquid fuel refers predominately to biofuels such as ethanol (EtOH), butanol and methanol while gaseous fuels includes methane, $\text{H}_2$ and carbon-monoxide. Figure 2.2 represents the biomass available for biofuel production and shows that nearly 148 million tonnes of available biomass are wasted or burnt and used for other purposes. Converting these biomass sources into $\text{H}_2$ will be very important in solving the problems of environmental pollution and future energy shortages.

![Figure 2.2 Biomass available for biofuel production (Adapted from Milbrandt and Overend (2008))](image-url)
Two major mechanisms of utilizing the biomass feedstocks for H\textsubscript{2} production includes the following: 1) thermochemical processing and 2) biological processes. The thermochemical processes involving pyrolysis and gasification are utilized on a wide scale. Briefly, in gasification the biomass is heated by steam with limited air available to produce syngas (a mixture of H\textsubscript{2} and other products) while in pyrolysis, the biomass is heated to a high temperature to produce oils, which can be steam reformed to H\textsubscript{2} (Kersten et al., 2010; Ni et al., 2006). The biomass gasification process is problematic because of the high temperature requirement, which uses the energy derived from the combustion of fossil fuels. In comparison, pyrolysis is also problematic because in addition to H\textsubscript{2}, the other products such as tar and aerosols are produced are unfavorable for H\textsubscript{2} production. The subsequent steam reforming followed by pyrolysis requires higher power input (Ni et al., 2006). Another setback associated with gasification or combustion is the formation of ash that causes deposition, sintering, fouling and agglomeration (Wornat et al., 1995). Although technologies are available for dealing with these issues such as using additives and catalysts during pyrolysis and imposing fractionation in reactors to reduce the formation of ash during gasification (Corella et al., 1999), biological processes are preferred over thermochemical processes.

Four types of biological processes which are able to produce H\textsubscript{2} includes: biophotolysis, biological water–gas shift reaction, photo-fermentation and dark fermentation (Ensign and Ludden, 1991; Eroglu et al., 2000; Khanal et al., 2004; Kondo et al., 2002; Park et al., 2004a). Briefly, in direct biophotolysis the adsorbed water is split into oxygen and H\textsubscript{2}. The photosystems generate reduced ferrodoxin, which a hydrogenase uses to produce H\textsubscript{2} from protons. The major problem associated with biophotolysis is that the oxygen produced in the process is inhibitory to the hydrogenase enzyme responsible for H\textsubscript{2} production (Flynn et al., 2002; Ghirardi et al., 2000). The low conversion efficacy of solar energy to H\textsubscript{2} also adversely affects the process (Hallenbeck et al., 2009). Indirect biophotolysis involves photosynthesis followed by H\textsubscript{2} production in which sugar and water yield H\textsubscript{2} and CO\textsubscript{2}. The major disadvantage of the indirect biophotolysis process is the optimal conversion is low (10%) (Prince and Kheshgi, 2005); however, in practice lowerer efficiencies have been reported (i.e., less than 2%) (Lindblad et al., 2002; Liu et al., 2006b).
Photo-fermentation is the biological process where H$_2$ is produced from organic acids (such as acetic acid (HAc), propionic acid (HPr) and butyric acid (HBu)) by photosynthetic bacteria in the presence of light. Although higher conversion can be obtained with this method, the H$_2$ production rate is low, since high light intensity and effective diffusion are important for this process (Shi and Yu, 2006; Uyar et al., 2007). Applying these aspects of photo-fermentation to large systems may require larger surface areas and light from many directions is required to ensure uniform intensity (Hallenbeck et al., 2009).

In the biological water-gas shift reaction system, photoheterotrophic bacteria use carbon-monoxide (CO) as the carbon source and produce H$_2$ by the reduction of protons from H$_2$O (Kerby et al., 1995). The water-gas shift reaction can occur under both light or dark conditions. However, it is in the dark that the classes of micro-algae (e.g. species belonging to *Rhodospirillaceae* such as *Rhodospirillum rubrum*) have the properties of splitting water into H$_2$ and oxygen, and oxidizing CO to CO$_2$. Selected microbial species (e.g. *Rubrivivax gelatinosus* and *Rhodopseudomonas gelatinosa*) in biological water-gas shift reaction show substrate conversion efficiency reaching approximately 90% of the stoichiometric equivalent (Maness and Weaver, 2002; Uffen, 1983). The process uses CO as the substrate which is not readily available in nature. The other disadvantage of the process includes formation of the CO$_2$ as the byproduct, a greenhouse gas. In spite of these disadvantages, this process is preferred over the processes described previously, as the process of water splitting is carried out through biological means at ambient temperature (e.g. in comparison to the steam reforming of methane, which performs the same reaction at the second step of its process mechanism).

Dark fermentation is the process where the substrate (typically comprised of sugars) is consumed by anaerobic bacteria, which converts the feedstock to H$_2$ under dark conditions. The process can occur across a wide temperature range (30-80 °C), depending on the organism used. Typically, mixed anaerobic cultures are studied under a wide range of mesophilic conditions. The dark fermentation process appears promising because of higher production rates and lower space requirements, leading to increasing development of commercial full-scale systems (Levin et al., 2004; Ni et al., 2006; Sen et al., 2008). The other main advantage of this process is that dark fermentation allows the use of a
wide range of substrates. Several studies have reported that mixed microbial consortia can feed on a variety of feedstocks, such as agricultural waste material and carbohydrate feeds (Hay et al., 2013; Rittmann and Herwig, 2012). The major problem associated with the use of dark-fermentation is that a lower yield of H₂ may be obtained (Benemann, 1996; Hallenbeck and Benemann, 2002). The maximum conversion that can be achieved through dark fermentation is 33% of the combustion energy of glucose (4 mol H₂·mol⁻¹ glucose) (Thauer et al., 1977). This is due to the limitations imposed by the thermodynamic and metabolic processes involved in dark-fermentation. As discussed earlier, the dark-fermentation process involves the production of H₂ by mixed anaerobic consortia from natural environments, which degrade the available carbohydrate (substrate) source within the biomass (e.g. agricultural waste). Nevertheless, the dark-fermentation method has been preferred for its ease of operation, cheaper and abundant source of substrate supply and also for the potential in treating the waste biomass (Hallenbeck et al., 2009).

2.2 Feedstock for dark-fermentation

2.2.1 Lignocellulosic waste material

A variety of complex organic materials, containing proteins, lipids and carbohydrates can be used as feedstock for microbial fermentation-based H₂ production. However, substrates that are rich sources of carbohydrates are preferred because of their degradation rates in comparison to proteins and lipids. H₂ produced from carbohydrates makes up a higher percent composition of the biogas content compared to biogas produced from substrates rich in proteins and lipids (Okamoto et al., 2000). Hydrogen production from dark-fermentation of cellulose and starch-rich wastes have also been studied, along with carbohydrate-rich wastes, such as molasses. The most widely studied substrates for bio-H₂ production are glucose and sucrose (Chang and Lin, 2004; Chang et al., 2001; Van Ginkel and Logan, 2005). Other waste material has been studied as a substrate for H₂ production, which includes palm oil mill effluent wastewater (POME) (Atif et al., 2005); wastewater from the food processing industry, such as potatoes, apple, mango pulp and other waste from the sugar processing industry (Jin and Jin, 2010; Van Ginkel et al., 2005) and wastewater from paper mill effluent (Valdez-Vazquez et al., 2005b). Though carbohydrates containing sugars and other complex organic carbons are
widely used, recent research activities have focused on using hemicellulosic sugars. This is because crop residues and other lignocellulosic biomass sources have more hemicellulosic content, which can be a suitable alternative to pure sugars and can be grown in abundance for energy purposes.

Lignocelluloses are preferred over other available biomass sources because of global availability and lower cost of these feedstocks. Appreciable levels of H₂ production have been obtained from lignocellulosic feedstock (Cheng et al., 2011; Sparling et al., 2006). Studies of lignocellulosic biomass for bio-H₂ production have reported that depending on the crop harvested, 0.8 to 2.3 GJ per dry Mg biomass⁻¹ is the expected output (Borjesson, 1996). Crop residues and forestry waste containing sugars, instead of sent to landfills or left on farm lands as waste, can instead be used for energy production. This action could assist in resolving environmental problems associated with landfilling (Mussatto and Teixeira, 2010).

In North America and especially in Canada, potential sources of biomass includes wheat and other grain straws, switchgrass (SWG), corn stover (CS) and other agricultural residues. On average, Canadian farmers produces wheat, rye, rice straw and corn grains with yields ranging from 4 to 9 dry tonnes ha⁻¹ y⁻¹ of (Graham and Perlack, 2009). The increased availability of agricultural biomass through changes in technology includes the residual from major crops and other perennial grasses suitable for bio-fuel production.

### 2.2.2 Potential feedstocks for fermentative hydrogen production in North America

A wide variety of lignocellulosic materials available as substrates for bio-fuel includes crops such as CS, SWG, maize, sorghum, and poplar. Based on the energy content of the crop (i.e., SWG has 170 GJha⁻¹ and CS have 154 GJha⁻¹ of energy value) (Samson, 2008; Zych, 2008) and local availability, SWG and CS might be selected as substrates for H₂ production in a larger scale systems. Apart from using SWG for heating and as fodder, the crop can also be used for ethanol (EtOH) production. The energy yield from SWG EtOH is five times greater than that of corn EtOH, which is a more widely used substrate for energy (EtOH) production. In addition, for SWG, the output energy is about 14.6 times of the input energy used for SWG production (Samson et al., 2004). SWG has reportedly been used for biofuel production (e.g. EtOH used to fuel automobiles) (MacLean et al., 2005), biogas production (Ahn et al., 2010), and H₂
production through thermochemical generation (Brown et al., 2004) or through catalytic gasification systems using supercritical water (Gupta et al., 2011). Very little research has been conducted on biological H₂ production from SWG. Other types of grass (e.g. rye grass) have been studied for bio-H₂ production. Rye grass yields reaching 82 ml H₂ g⁻¹ dry mass under continuous operation used mixed anaerobic cultures has been reported by Kyazze et al. (2008).

Another crop residue that is widely available in North America and could be studied as a potential feedstock for continuous H₂ production is CS, which accounts for 50% of the corn grain produced on a dry weight basis. Approximately 68 Tg y⁻¹ of CS is produced in the U.S. (Perlack et al., 2005). Supportingly, usage of CS as a substrate has been reported for bio-H₂ production using pure cultures (Cao et al., 2009) as well as mixed cultures (Zhang et al., 2011). Liu and Cheng (2010) reported an H₂ production rate of 8.5 ml H₂ g⁻¹ TS h⁻¹ (1.53 mol mol⁻¹ hexose) from CS using mixed microbial cultures available in natural environment. Figure 2.3 presents the energy yield for many lignocellulosic crops. The data show that the high energy value for CS and SWG is likely and indication for the H₂ production potential of these carbon sources.

![Energy yield per hectare of various crops](image)

**Figure 2.3** Energy yield per hectare of various crops (Adapted from Samson (2008))
2.3  Biomass characteristics and pretreatment methods for lignocellulosic sources

2.3.1  Physical and chemical characteristics of lignocelluloses

The composition of lignocellulosics is configured with cellulose, hemicellulose and lignin. Lignin forms the outer covering of the layers that protects biomass from fungal attack and it also prevents effective fermentation by bacteria (Stroeve et al., 2009). Hemicellulose, a co-polymer, is present beneath the lignin layer and contains C5 and C6 sugars. Cellulose, containing C6 polymeric units, is the major component of the feedstock, and is part of the cell-wall composition, which provides mechanical strength and chemical stability to plant biomass. Over $7.5 \times 10^{10}$ tonnes of lignocellulosic biomass are produced and utilized each year (French et al., 2003). The composition of these materials is highly dependent on the source from which they are derived such as hardwood, softwood and agricultural crop residues. The composition of various types of lignocellulosic biomass is shown in Table 2.1. In order to extract the sugars from these materials, an understanding of the linkage between the layers of these components of the lignocellulosic biomass is essential.

### Table 2.1 Composition of lignocellulosic biomass adapted from (Saha, 2003)

<table>
<thead>
<tr>
<th>Raw materials</th>
<th>Cellulose (D-glucose units)</th>
<th>Hemi-cellulose (Pentoses and hexoses)</th>
<th>Lignin (Phenyl propane units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn fiber a</td>
<td>15</td>
<td>35</td>
<td>8</td>
</tr>
<tr>
<td>Corn cob</td>
<td>45</td>
<td>35</td>
<td>15</td>
</tr>
<tr>
<td>Corn Stover</td>
<td>40</td>
<td>25</td>
<td>17</td>
</tr>
<tr>
<td>Rice straw</td>
<td>35</td>
<td>25</td>
<td>12</td>
</tr>
<tr>
<td>Wheat straw</td>
<td>30</td>
<td>50</td>
<td>20</td>
</tr>
<tr>
<td>Switchgrass</td>
<td>45</td>
<td>30</td>
<td>12</td>
</tr>
<tr>
<td>Coastal Bermuda grass</td>
<td>25</td>
<td>35</td>
<td>6</td>
</tr>
<tr>
<td>Softwood (Glucomannans)</td>
<td>45</td>
<td>30</td>
<td>25</td>
</tr>
<tr>
<td>Hardwood (Xylans)</td>
<td>47</td>
<td>31</td>
<td>22</td>
</tr>
</tbody>
</table>

a 20% of starch

Cellulose is a poly-acetyl link of cellulobiose units, consisting of two glucose units linked by an oxygen molecule. The $\beta$-1,4 glucosidic linkage between the glucose units causes the polymer to form long straight chains. This polymeric arrangement with hydroxyl groups evenly distributed on the sides of glucose units, supports hydrogen bonding between the cellulose units (Faulon et al., 1994). The integration of the polymer
chain assists in forming micro-fibrils, which in turn, form fibers. This feature of cellulose that makes them insoluble in water. Solubility of cellulose depends on the degree of hydrolysis, which requires a concentrated acid to solubilize the cellulose. The decomposition of cellulose is complex, and requires high temperatures (240-350 °C) that can produce the energy required to split the hydrogen bonds linked to the oxygen bonds on the neighbouring chain (Finnish Thermowood Association, 2003).

The hemicellulose component contains hexoses (glucose, mannose and galactose) and pentoses (xylose and arabinose) as the major sugars, linked by 1→4 and 1→6 linkages. The hemicellulose component is noncrystalline, and contains acid/acetyl units such as D-galacturonic acid and 4-O-methyl-D-glucuronic acid. Hemicellulose decomposition begins with heat at 150-200 °C and the release of acetic acid (HAc) leads to increase hydrolysis of hemicellulose in water. Note adding dilute acid at temperatures below 150 °C enhances the solubility of hemicellulose in the case of soft lignocellulosics.

Lignin, the dark outer covering of the biomass material, is comprised of phenylpropane units joined by ether, acetyl and carbon-to-carbon bonds. The phenylpropane linkages are broken in the presence of heat. The thermochemical reactions at the temperature of 120 °C or above affect the allylic side chains and also affects β-ketone and carboxylic acid groups following long exposure at temperatures greater than 120 °C. The most common reactions affecting the color, dissolution, and lignin properties of the biomass, occur at this temperature range with diphenylmethane condensation. Polymers of lignin, containing different functional groups in their degradation compounds that cause them to be soluble are present in the liquor obtained upon pretreatment.

2.3.2 Fermentation inhibitors

Achieving higher yields of bio-H₂ production requires pretreatment of lignocellulosic materials. However, pretreatment along with the conversion of polymeric substances into simple sugars, induces the formation of inhibitory degradation products from the lignocellulosics. The major type of inhibitor detected with pretreatment are furfural and hydroxyl methyl furfural (HMF), phenolic compounds, and other acidic compounds such as HAc, formic and levulininc acid (Palmqvist and Hahn-Hagerdal, 2000).
Furfural and HMF are produced from hemicellulosic sugar degradation at high temperatures or by high concentrations of the acids involved during pretreatment. Furfural is formed by the loss of a water molecule from pentose degradation while HMF is formed by the loss of a water molecule from hexose degradation. These compounds inhibit fermentation and microbial growth, so may adversely affect the desired high H₂ production rates (HPR) (Quemeneur et al., 2012). Phenolic compounds, consisting of poly-aromatic or aldehydic compounds, are released during lignin degradation at high temperatures. These compounds are considered more toxic (even at low levels) than furfural and HMF as they impede bacterial growth and adversely affect cell physiology resulting in decreased viability and productivity (Palmqvist and Hahn-Hagerdal, 2000). Common monomeric phenols includes syringaldehyde, vanillin, and ferulic acid (Mussatto and Roberto, 2004).

Acetic and levulinic acid are produced from organic acid derivatives of hemicellulose. Other organic acids, such as formic and terpene acids, are also produced from woody biomass. The toxicity of these acids depends on the fermentation process. For example, at concentrations up to 1 g L⁻¹ of acetic acid, xylitol production from xylose containing liquor is stimulated (Felipe et al., 1995) and for bio-H₂ production, the presence of acetic acid stimulates the HPR at threshold concentrations. For example, concentrations of HAc acid showed 50% inhibition in growth and H₂ production at 130 mM (de Vrije et al., 2009), whereas for ethanol production, Ethanoligenes were able to tolerated up to 10 g L⁻¹ in the absence of other inhibitors (Larsson et al., 1999a). Table 2.2 summarizes the toxicity levels reported for biological H₂ production from lignocellulosic hydrolysate.

Minimizing the formation of inhibitor compounds is a primary objective of biomass pretreatment because of the synergistic effect of these compounds on different microorganisms (Mussatto and Roberto, 2004). During pretreatment, the formation of toxic chemicals can be reduced by maintaining low residence times at high temperatures or by decreasing the acid concentration. Four factors which should be considered in selecting a pretreatment method are as follows: 1) maximum the quantity of sugars in the liquid phase; 2) reducing the quantity of toxic compounds generated; 3) minimizing the
cost of the pretreatment process and 4) the treatment process must be environmentally friendly.

### Table 2.2 Concentration of inhibitors after pretreating biomass

<table>
<thead>
<tr>
<th>Biomass material</th>
<th>Pretreatment condition</th>
<th>Inhibitor concentration (g L(^{-1}))</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rice straw</td>
<td>135 °C, 20 min, H(_2)SO(_4) 1.5%</td>
<td>Furfural 0.25, HMF 0.15; Acetic acid 1.43</td>
<td>(Baek and Kwon, 2007)</td>
</tr>
<tr>
<td>Corn stover</td>
<td>121 °C, 180 min, 4% H(_2)SO(_4)</td>
<td>Furans 0.94; Phenolics 0.15; Acetic acid 1.96</td>
<td>(Cao et al., 2009)</td>
</tr>
<tr>
<td>Switchgrass</td>
<td>195 °C, 7.5 min, SO(_2) catalyzed 3% wt/wt</td>
<td>Furfural 1.42, HMF 0.21; Acetic acid 1.43</td>
<td>(Ewanick and Bura, 2011)</td>
</tr>
<tr>
<td>Sugarcane bagasse</td>
<td>205 °C, 10 min, SO(_2) catalyzed 3% wt/wt</td>
<td>Furfural 0.72, HMF 2.52; Acetic acid 1.43</td>
<td>(Ewanick and Bura, 2011)</td>
</tr>
<tr>
<td>Spruce</td>
<td>203 °C, 5 min, SO(_2) sparging</td>
<td>Furfural 1.2 ± 0.1; HMF 3.2 ± 0.1; Phenolics 0.38 ± 0.1, Acetic acid 4.7 ± 0.3</td>
<td>(Alriksson et al., 2011)</td>
</tr>
</tbody>
</table>

#### 2.3.3 Pretreatment process

Pretreatment is broadly classified into mechanical, thermal, chemical, thermochemical and biological processes. The process involves disruption of the lignocellulosic structure and release fermentable sugars within the biomass. The removal of lignin increases the surface area porosity in order for hydrolysis to act on the biomass and hence, improves the efficiency of the pretreatment process.

Mechanical pretreatment primarily involves size reduction to increase the surface area. The milling process is carried out in a ball or hammer mill or with any other type of shredder, which is able to reduce the size and also create a less dense structure. Mechanical pretreatment produces a structure which can be further treated using chemical, heat or enzymes. A thermal pretreatment involves breaking down the hemi-cellulosic component of lignocellulosics at high temperatures (150-200 °C). During thermal hydrolysis, organic acids (e.g. HAc) are produced and this leads to hydrolysis and the subsequent release of sugars from hemi-cellulose structure.

#### 2.3.3.1 Hydrothermal pretreatment

Hydrothermal pretreatment involves treatment with liquid hot-water at high temperatures. Kim et al. (2009) reported that treatment with liquid hot water at 160-190...
°C improved the accessibility of enzymes into the biomass structure for hydrolysis. Pretreatment using hot water between 190-230 °C is able to recover 37 to 90% pentosans with limited contact time (Walsum et al., 1996).

2.3.3.2 Steam-explosion

The steam-explosion process is carried out at high temperatures (ranging from 190 to 234 °C) and pressures (up to 3.3 MPa) with short residence times (less than 10-15 min), after which the vessel is depressurized and cooled (Boussaid et al., 1999; Taherzadeh and Karimi, 2008). During steam treatment, the biomass is exploded, which disrupts the hemi-cellulosic components and solubilizes approximately 80-100% of the hemicellulose in the biomass (Grethlein and Converse, 1991). The flashing of water into steam followed by the rapid pressure drop caused by the expansion of steam which explodes the biomass. In addition to the hemicellulose fraction of biomass, a fraction of the lignin is disrupted which dissolves in the steam exploded liquor as well. Long retention times may cause the degradation of hemicellulose sugars and subsequently, causing increasing inhibitor levels in the liquor. Steam explosion is the method most preferred because of the practical application of the process on a large scale with low energy consumption and low usage of chemicals in the process. However, low concentrations of acid are used (depending on the type of lignocellulosic material) in order to catalyze the process and recover major sugars (Zimbardi et al., 2007).

2.3.3.3 Acid and alkali pretreatment

Acid pretreatment includes both weak and strong acid treatments. The weak acid treatment involves treatment with a dilute acids such as phosphoric, acetic, hydrochloric and sulfuric at temperature > 150 °C. The temperature selection depends on both the acid concentration (0.5-3.0% v/v) and retention times (Baboukani et al., 2012). Weak acid pretreatment involves controlling the pH, temperature and pressure. Under these conditions, the lower levels of lignin and other sugar degradation products which are formed during pretreatment may not adversely affect the fermentation activity or any other enzymatic process in a subsequent stage (Lee et al., 1983) when compared to other pretreatment methods utilizing harsh conditions. Dilute acid hydrolysis increases the porosity and accessibility of the biomass for further hydrolysis through enzymatic
hydrolysis. Weak acid hydrolysis is suitable for low lignin-containing biomass, such as agricultural crop residues (Torget et al., 1991). Although acid hydrolysis involves more downstream processing steps for sugar recovery, the process is widely preferred for the high conversion rate and ease of operation (Esteghlalian et al., 1997). This process is used on a commercial scale for sugar extraction from biomass (Bergius, 1937). Strong acid pretreatment involves high concentrations of sulfuric and hydrochloric acids with shorter retention times and lower temperatures when compared to the dilute acid pretreatment process. However, usage of strong acids is not feasible on a large scale, as the process would require a corrosion resistant reactor for operation, additional expenses for the recovery cost of extracting acid from the reaction process and also the need to neutralize spent steam before releasing them into the environment (Brown and Brown, 2014).

Alkali pretreatment involves saponification of the ester cross linkage between the lignin and hemi-cellulose, thereby disrupting the lignin and crystalline structure (Sun and Cheng, 2002). This pretreatment method also removes acetyl linkages and various uronic acid groups present in the hemi-cellulose (Chang and Holtzapple, 2000; Tarkov and Feist, 1969). Usually alkali pretreatment is carried out with sodium hydroxide, lime or aqueous ammonia solution. Treatment with NaOH and ammonia require an additional recovery stage, which involves high costs for large scale use (Chaturvedi and Verma, 2013). Although lime pretreatment involves relatively low costs, the salt component formed during pretreatment needs to be removed and recycled.

2.3.3.4 Other physiochemical pretreatment methods

There are a variety of pretreatment methods for handling the conversion of the lignocellulosic biomass to fermentable sugars. The most commonly used pretreatment includes acid hydrolysis, delignification with an organo-solvent, steam explosion, wet air oxidation (WAO), gas treatment, AFEX, CO₂ explosion. The CO₂ explosion method is the least preferred of the physiochemical treatments because of the process inability to modify the lignin or hemicelluloses. Delignification with organo solvents is problematic because of the cost of the solvents used and the cost of chemicals used for the recovery. The WAO seems to be effective when compared to other methods, and the cost of production is also similar to others but the initial capital investment required is higher.
Pyrolysis also yielded a 70-80% recovery of cellulose, but did not prove to be very effective compared to torrefaction and also produced residual char at the end of the process (Stroeve et al., 2009). The AFEX process is not suitable for high lignin-containing biomass, yielding below 40% of the sugars (Mcmillan, 1994).

2.3.3.5 **Enzymatic or biological pretreatment**

During biological pretreatment, microorganisms that produce enzymes are used in the conversion of lignocellulose to fermentable sugars (Chaturvedi and Verma, 2013). The biological pretreatment method has advantages, such as mild operating conditions and hence, no formation of inhibitors. However, the hydrolysis rate of the biological system is slow, which makes the biological treatment less preferable than other pretreatment technologies (Kumar et al., 2009).

2.3.3.6 **Choice of steam-explosion over other pretreatment methods**

Among the pretreatment methods available, steam explosion seems to be less expensive with steam being generated at a lower operating cost. The steam explosion process is a more promising technology because of high sugar conversion levels (Ren et al., 2009). Use of an acid catalysis during steam pretreatment has been widely studied and applied on a pilot scale as well, especially for woody biomass (Galbe and Zacchi, 2007). Steam explosion offers the advantage that the hemi-cellulose fraction contains xylans with fewer gluco-mannans and is easily susceptible to acid hydrolysis or any extraction or selective removal process. There are various pilot/full scale plants that have implemented the steam explosion process for biomass pretreatment. For example, in Canada, Iogen uses a modified steam explosion process at a commercial level for ethanol production, in the U.S., Verenium uses mild acid hydrolysis with a steam explosion in ethanol production and in Spain, Abengoa has a demonstration facility for acid-catalyzed steam explosion for ethanol production from wheat straw (Harmsen et al., 2010). All of these operational facilities demonstrate the use of steam explosion as a pretreatment process that is suitable for use on a larger scale.

2.3.4 **Reduction of fermentation inhibitors**

The principal problem of using SE with acid catalysis would be the production of furfural and HMF which would inhibit the microbial population during fermentation
(Mackie et al., 1985). There are several technologies available for overcoming inhibition by these substances. **Table 2.3** summarizes the different physical, chemical and biological methods available for detoxification that could remove these fermentation inhibitors and enhance recovery of a larger sugar fraction from liquid hydrolyzate.

<table>
<thead>
<tr>
<th>Hydrolyzate Material</th>
<th>Detoxification method</th>
<th>Removal of Inhibitors</th>
<th>Removal of Sugars</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn Stover</td>
<td>Steam Stripping (120 min)</td>
<td>Removal of 58.79% formic acid, 80.83% Acetic acid, 33.33% HMF and 100% Furfural</td>
<td>3.9% reduction in total sugars</td>
<td>(Zhu et al., 2009)</td>
</tr>
<tr>
<td>Corn Stover</td>
<td>Vacuum evaporation 11.13 times</td>
<td>Removal of 59.89% formic acid, 77.72% Acetic acid, 45.45% HMF and 100% Furfural</td>
<td>17.3% reduction in total sugars</td>
<td>(Zhu et al., 2009)</td>
</tr>
<tr>
<td>Corn Stover</td>
<td>Membrane extraction with Solvents</td>
<td>Removal of 50% formic acid, 86% Acetic acid, 40% HMF and 75% Furfural (approx)</td>
<td>No significant adsorption of sugars</td>
<td>(Grzenia et al., 2012)</td>
</tr>
<tr>
<td>Sugarcane bagasse</td>
<td>Over liming at pH 10-11</td>
<td>54% removal of total Furans, &lt; 1% in removal of Acetic acids and phenols</td>
<td>15.6% reduction in sugars</td>
<td>(Martinez et al., 2000)</td>
</tr>
<tr>
<td>Synthetic hydrolysate</td>
<td>Adsorption to steam-activated biochars</td>
<td>99% removal of furans, Phenol and Acetic acid adsorption not studied</td>
<td>No sugar adsorption noticed</td>
<td>(Klasson et al., 2011)</td>
</tr>
<tr>
<td>Northern US hardwood chips</td>
<td>Polymeric adsorbent resin (XAD-4)</td>
<td>Removal of 60% Acetic acid, 90% Phenols, 40% Formic acid and 90% of Furans</td>
<td>25% of total sugars</td>
<td>(Schwartz and Lawoko, 2010)</td>
</tr>
<tr>
<td>Spruce hydrolyzate</td>
<td>Biological abatement (T. reseei)</td>
<td>Removal of 85% of Furfural, 6% of Phenols, 25% of HMF</td>
<td>65% of total sugars</td>
<td>(Larsson et al., 1999b)</td>
</tr>
</tbody>
</table>
2.4 Hydrogen production through dark fermentation

2.4.1 Microbial fermentation of lignocellulosic material

Dark fermentation is the conversion of organic substrates to bio-H\textsubscript{2} through a series of biochemical reactions by anaerobic bacteria in the absence of light (Figure 2.4). In comparison to other bio-H\textsubscript{2} production methods, dark fermentation is a promising technology (Levin et al., 2004). Dark fermentation is an intermediate step in the anaerobic digestion process and involves multiple series of oxidation and reduction reactions (Pavlostathis and Giraldo, 1991). Anaerobic digestion involves 4 major steps: hydrolysis; acidogenesis; acetogenesis; and methanogenesis (Figure 2.4).

![Figure 2.4 Schematic representation of anaerobic digestion pathway (Erickson and Fung, 1988)](image)

2.4.1.1 Hydrolysis

During this step, all of the macro-molecules of lignocellulosic or carbohydrate polymers are broken down into monomeric or fermentable sugars. The rate of hydrolysis is mainly dependent on the particle size, the composition of the biomass material and the conditions under which hydrolysis take place (Sanders, 2001). Hydrolysis is carried out by obligate or facultative anaerobes, which convert the biomass into a soluble form that can be assimilated by the fermenting organisms (Gerardi, 2003).
2.4.1.2 Acidogenesis

Fermentable sugars are degraded into liquid byproducts such as volatile fatty acids (VFAs), alcohols and gaseous products including H$_2$ and carbon-dioxide (CO$_2$). The VFAs produced at this stage are diverse and typically include succinic acid, lactic acid (HLa), HAc, HPr, and HBu. There are a variety of factors that may affect acidogenesis, such as: pH, temperature, substrate composition, inoculum source and type, and HRT in the case of continuous operating systems (Banerjee et al., 1998; Zhang et al., 2005). The acidogenesis reaction is carried out strictly by anaerobes that are not tolerant to oxygen; however, some facultative anaerobes can utilize trace amounts of oxygen. The most common genera that include acidogens are *Clostridium*, *Pseudomonas*, *Bacillus*, *Micrococcus*, *Flavobacterium* and *Enterobacterium* (Ziemiński and Frac, 2012).

2.4.1.3 Acetogenesis

During acetogenesis, organic compounds having more than two carbons are degraded to HAc. Acetic acid is not only produced from compounds with multiple carbon atoms, but also from a molecule with a single carbon atom, in which CO$_2$ and H$_2$ produced during acidogenesis are used to form HAc. Acetogenesis by obligate proton-reducing bacteria is thermodynamically favorable under low partial pressure for H$_2$ (pH$_2$) (Khanal, 2011). Formation of H$_2$, CO$_2$ and HAc (although formate is found in a few cases) from the degradation of VFAs containing longer carbon chains lowers the pH levels and enhances the H$_2$ production (Denac et al., 1988). The syntrophic relationship between H$_2$-consuming methanogens, H$_2$-consuming acetogens and H$_2$ producers, assist in maintaining the low pH$_2$ and a balance in the system making thermodynamically favorable conditions for the fermentation reactions to proceed (Schink, 1997). The HAc produced by homoacetogens includes two types: one type grows autotrophically using H$_2$ and CO/CO$_2$ and the other heterotrophically by producing HAc from organic compounds (Ryan et al., 2008). Thus, acetogenesis and acidogenesis are the two steps in anaerobic digestion during which H$_2$ is produced.

2.4.1.4 Methanogenesis

Methanogenesis is the final stage of anaerobic digestion where methane is the end product. Methane is primarily produced from H$_2$ and CO$_2$ (hydrogenotrophic
methanogens) or from HAc (acetoclastic methanogens). The methane-producing bacteria belong to the Archaea class of microorganisms that are sensitive to oxygen and live in a syntrophic relationship with acetogens. Methanogens are affected by pH, high levels of VFAs produced during acidogenesis and acetogenesis, and the amount of H\textsubscript{2} produced (Zeikus, 1977).

### 2.4.2 Electron flow in the metabolic pathway for hydrogen production through dark fermentation

The metabolic mechanisms for dark fermentation are derived from anaerobic digestion. Hydrogen production arising from dark fermentation takes place if organic carbon is available as an energy source for the microflora. Dark fermentative H\textsubscript{2} production is preferred for bio-fuel production because of its high HPR (Levin et al., 2004). The stoichiometric reaction **Equation 2.1** explains how H\textsubscript{2} is produced from glucose metabolism when HAc is the end product. The maximum possible H\textsubscript{2} yield per mole of glucose is 4 mol corresponding to only 33\% of the substrate conversion. However, in practice, attaining this theoretical maximum yield is not possible.

\[
C\textsubscript{6}H\textsubscript{12}O\textsubscript{6} + 2H\textsubscript{2}O \rightarrow 2CH\textsubscript{3}COOH + 4H\textsubscript{2} + 2CO\textsubscript{2} \quad (2.1)
\]

When the end product is HBu (**Equation 2.2**), 2 mol H\textsubscript{2} is produced:

\[
C\textsubscript{6}H\textsubscript{12}O\textsubscript{6} + 2H\textsubscript{2}O \rightarrow CH\textsubscript{3}CH\textsubscript{2}CH\textsubscript{2}COOH + 2H\textsubscript{2} + 2CO\textsubscript{2} \quad (2.2)
\]

These stochiometric equations reveal that the HAc/HBu ratio controls the maximum H\textsubscript{2} yield possible, and that acetogenic fermentation is preferred over HBu fermentation. Furthermore, low yields are characteristic of HPr, HLa or EtOH fermentation (Azbar and Levin, 2012; Levin et al., 2004). A description of glucose metabolism in the following sections describes the different stochiometric reactions.

The metabolic pathway for glucose degradation via anaerobic fermentation is described in **Figure 2.5**. The pathway integrates the formation of an intermediate, pyruvate, by glycolysis during the breakdown of complex sugars. The pathway shows that H\textsubscript{2} can be produced from pyruvate decarboxylation where electrons are transferred to ferrodoxin (Fd). In subsequent a reaction, the reduction of protons (hydrogen ions, H\textsuperscript{+}) takes place resulting in the release of H\textsubscript{2} gas (Jungermann et al., 1973; Saint-Amans et
In this pathway, the NADH formed from glycolysis is used in the formation of H₂, releasing the oxidized form of NAD⁺. **Equation 2.3 and 2.4** represent the formation of pyruvate and NADH through glycolysis, following which evolution of H₂ via oxidation occurs.

\[
C_6H_{12}O_6 + 2\text{NAD}^+ \rightarrow 2\text{CH}_3\text{COCOOH} + 2\text{NADH} + 2\text{H}^+ \tag{2.3}
\]

\[
\text{NADH} + \text{H}^+ \rightarrow \text{H}_2 + \text{NAD}^+ \tag{2.4}
\]

---

**Notes:**

*Enzymes are indicated as follows: (A) hydrogenase; (B) pyruvate-ferredoxin oxidoreductase; (C) NADH-ferredoxin oxidoreductase; (D) phosphate acetyltransferase; (E) acetate kinase; (F) acetaldehyde dehydrogenase; (G) ethanol dehydrogenase; (H) thiolase; (I) acetoacetate decarboxylase; (J) isopropanol dehydrogenase; (K) 3-hydroxybutyryl-CoA dehydrogenase; (L) butyryl-CoA dehydrogenase; (M) phosphate butyryltransferase; (N) butyrate kinase; (O) butyraldehyde dehydrogenase; (P) butanol dehydrogenase; (Q) lactic dehydrogenase; (R) Propionate dehydrogenase; and (S) Pyruvate formate lyase.*
The metabolic pathway is based on the intermediate, pyruvate, from which the pathway branches to different intermediates in anaerobic fermentation. The major fermentation products in the gaseous phase includes H₂, CO₂ and CH₄ while soluble metabolites in the liquid includes HAc, HBu, HLa, EtOH and butanol (Hawkes et al., 2002; Zhou et al., 2007). Figure 2.5 shows the metabolites formed and the enzymes involved at each step of the metabolic pathway. Electron/carbon flow from glucose to other metabolites (e.g. H₂, HAc, HBu, HPr, etc.) occurs and the NADH₂ produced is balanced with the NADH₂ consumed in this pathway.

From pyruvate, the metabolic pathway proceeds into two different branches which are distinguished by the nature of the associated bacterial system (i.e., enteric or Clostridial). In the enteric bacterial system, pyruvate is broken down to acetyl-CoA and formate by pyruvate formate lyase (S in Figure 2.5). The latter metabolite (formate) is then converted to H₂ and CO₂ by formate hydrogenase. The former metabolite (acetyl-CoA) is used for acetic acid production via substrate level phosphorylation (D and E in Figure 2.5) and regeneration of NAD⁺ to maintain glycolysis. However, the NAD⁺ regeneration directly from pyruvate which is also possible under acidic conditions by lactate dehydrogenase results in low H₂ yields. The regeneration of NAD⁺ via non-H₂ producing reactions (such as HLa, EtOH, and butanol formation as shown in Figure 2.5 and described by equations 2.5, 2.6 and 2.7) in enteric bacterial systems results in H₂ yield less than 2 mol H₂ mol⁻¹ glucose, which is only 50% of the theoretical maximum (Hallenbeck, 2005).

\[
\text{CH}_3\text{COCOOH} + \text{NADH} + \text{H}^+ \rightarrow \text{CH}_3\text{CHOHCOOH} + \text{NAD}^+ \tag{2.5}
\]

\[
\text{CH}_3\text{COCOOH} + \text{NADH} + \text{H}^+ \rightarrow \text{CH}_3\text{CH}_2\text{OH} + \text{CO}_2 + \text{NAD}^+ \tag{2.6}
\]

\[
2\text{CH}_3\text{COCOOH} + 2\text{NADH} + 2\text{H}^+ \rightarrow \text{CH}_3(\text{CH}_2)\text{CH}_2\text{OH} + 2\text{CO}_2 + \text{H}_2\text{O} + 2\text{NAD}^+ \tag{2.7}
\]

In Clostridial bacterial systems, pyruvate is broken down into acetyl-CoA and reduced ferredoxin (Fd⁺) by a pyruvate ferredoxin oxidoreductase (B in Figure 2.5) (Hallenbeck, 2005; Zajic et al., 1978). The Fd⁺ is then oxidized to ferrodoxin (Fd²⁺) and the associated electron transfer through hydrogenase activity (A in Figure 2.5) results in evolution of H₂ from the electron acceptance of a proton (hydrogen ion, H⁺). Acetyl-CoA
produced by the Clostridial system is further degraded to HAc and HBu via ATP generation. The maximum H₂ production that is accompanied by HAc and HBu formation is described by the stochiometric reactions (Equations 2.1 and 2.2). The production of other reduced metabolites (such as HLa, HPr, butanol and EtOH) is also important in this pathway for maintaining the balance through which NAD⁺ regeneration occurs (Nandi and Sengupta, 1998).

The NADH consumption is marked by HPr and HLa formation. The formation of these by-products is essential for balancing the NADH produced during glycolysis, since the acceptance of electrons by protons (H⁺ ions) is affected by the corresponding levels of acetyl-CoA and NADH (Lee et al., 2011). In glycolysis, 2 mol of NADH are produced for every mole of glucose consumed and 2 mol of Fd⁺ is produced during pyruvate decarboxylation. Maximum H₂ production is determined by the mechanism in which NADH is recycled through the conversion of pyruvate to fermentation products (Manish et al., 2007). In theory, a maximum of 4 mol of H₂ can be produced if HAc is the end product, but in actuality such an ideal state cannot be achieved because of the fact that the accumulation of H₂ affects the activity of the hydrogenase enzyme and the types of electron carriers present in the metabolic pathway.

As shown in Figure 2.5, the electron flow from acetyl co-A is diverted to HAc and EtOH, and then to HBu and butanol through butyryl Co-A. Note, the electron source is NADH in the case of EtOH and butanol. The depiction of the metabolic pathway shows that the H₂ is produced via the Fd:hydrogenase system, implying that the reduction of ferrodoxin (Fd²⁺ to Fd⁺) is the sole electron source for proton reduction and the release of H₂. The presence of reduced ferrodoxin (Fd⁺) is based on electron flow from the pyruvate node. The electron equivalent (e⁻ eq) of H₂ measured and its e⁻ eq relative to the e⁻ eq of reduced ferrodoxin determines the direction of electron flow between NAD⁺/NADH and the Fd²⁺(oxidized)/Fd⁺(reduced) pools (Lee et al., 2009a).

2.5 Inocula source

2.5.1 Hydrogen producing cultures

A variety of microbial cultures including many types of bacteria belonging to obligate anaerobe or facultative anaerobe genera could be used for microbial H₂ production. The microflora are primarily classified according to the optimal/operating temperature
conditions of their growth environment, such as ambient (20-25 °C), mesophilic (32-42 °C), thermophilic (49-60 °C) and hyper-thermophilic (65-78 °C) conditions. Hydrogen production under ambient temperature conditions is not noteworthy in comparison to H₂ production by mesophiles and thermophiles because of their lag time and/or low H₂ yields (Wang and Wan, 2008). High H₂ yields have been reported at thermophilic and hyper-thermophilic temperatures (O-Thong et al., 2008; Rittmann and Herwig, 2012). However, other authors have reported that the H₂ production rates were lower at thermophilic and extreme thermophilic ranges, which limited the use of these organisms on a larger scale (Hallenbeck, 2005; Rittmann and Herwig, 2012). Most published research was conducted in the mesophilic temperature range. The major H₂ producing bacteria studied in the mesophilic range includes *Clostridium* (Ren et al., 2007), *Enterobacter* (Das and Sen, 2005) and *Bacillus* (Das and Kotay, 2007). Among the H₂ producers reported, *Clostridium* belonging to *Clostridiaceae* are preferred not only for the potential of *Clostridium* to produce high rates of H₂ but also due to the industrial application of these microorganisms in solvent production from different carbon sources (Lee et al., 2011). *Clostridiaceae* are generally rod-shaped bacteria that form endospores at extreme conditions and which are mostly gram positive (Madigan et al., 2012). These bacteria also have the ability to feed on a variety of substrates, and even degrade cellulosic substrates to produce H₂ (Ren et al., 2007; Sparling et al., 2006). An additional advantage is that they have the capability of forming spores, which enable them to survive and adapt to the surrounding environment under various conditions such as higher temperatures or acidic conditions (Hawkes et al., 2002).

Hydrogen producing bacteria have also been reported to include facultative anaerobes such as *Enterobacter*, *Citrobacter*, and *Escherichia coli* (Fan et al., 2009; Kumar and Vatsala, 1989; Palazzi et al., 2000) and aerobic organisms such as *Alcaligenes* and *Ralstonia* (Armstrong et al., 2008; Zorin et al., 1979). The facultative anaerobes are mostly gram-negative, rod-shaped bacteria that produce more reducing equivalents such as EtOH or HPr; however, when exposed to low oxygen levels for a shorter time, they can recover to produce H₂ after depleting the oxygen levels (Das and Nath, 2004). *Escherichia* species studied for H₂ production from glucose and starch hydrolysate has shown a H₂ yield of 0.5 mol⁻¹ hexose (approximate), which is equivalent to 12.5% of the
theoretical maximum (Perego et al., 1998), while, Kumar and Das (2000) reported 20-80% of the theoretical maximum H\textsubscript{2} yield was obtained with the \textit{Enterobacter} species.

Many studies have suggested that \textit{Clostridiaceae} are the most preferred species for bio-H\textsubscript{2} production, having H\textsubscript{2} yields ranging from 1.1 to 2.6 mol mol\textsuperscript{-1} hexose depending on the operating conditions (Lee et al., 2011). As \textit{Clostridiaceae} are more sensitive to oxygen, they could be cultivated along with facultative anaerobes, such as \textit{Enterobacter}. Use of this mixed culture might decrease the length of the lag phase in H\textsubscript{2} production by eliminating the toxic effect of oxygen observed for pure cultures of \textit{Clostridiaceae} in both batch and continuous systems. Hence, a proper understanding of microbial cultures selected for microbial H\textsubscript{2} production is essential.

\subsection*{2.5.2 Pure vs mixed cultures}

Studies that evaluate pure and mixed cultures in bio-H\textsubscript{2} production are limited (Lee et al., 2011). Studies conducted with pure cultures have reported to metabolize complex substrates; however, their H\textsubscript{2} yields are variable from low to high (Evvyernie et al., 2001; Fabiano and Perego, 2002; Kamalaskar et al., 2010). Hydrogen yield and metabolite levels from mixed culture are similar to those of pure cultures at pH levels ranging from 5.0-5.5. Hydrogen production studies carried out using mixed cultures (i.e., combination of 2 or more pure cultures) have shown higher H\textsubscript{2} yields when compared to the results reported for pure cultures (Qian et al., 2011). This was accomplished by using mixtures of facultative anaerobes with strict anaerobes, so that dissolved oxygen could be consumed by the facultative anaerobes, thereby favoring a higher H\textsubscript{2} yield. For example, work of this type was conducted by Yokoi et al. (1998) using a mixture of \textit{Clostridium butyricum} and \textit{Enterobacter aerogenes}. However, a major disadvantage of using pure cultures is that maintaining sterile conditions for the feed and medium on a larger scale poses more practical difficulties (Antonopoulou et al., 2007). Achieving high biomass concentration to feed complex substrates is difficult on a larger scale, furthermore, contamination leading to failure of reactor could incur huge economic losses.

The disadvantages associated with operation of pure culture makes naturally occurring mixed cultures, a preferred source of inoculum because they are able to feed on a variety of substrates and could be cultivated under non-sterile conditions. Furthermore, there are reports demonstrating using wastewater as a source of substrate for H\textsubscript{2}
production (Ke et al., 2005; Li and Fang, 2007). Similarly, using solid and food wastes as substrates has been reported by many researchers (Valdez-Vazquez et al., 2006; Youn and Shin, 2005). The disadvantage of using mixed cultures is the presence of \( \text{H}_2 \) consumers, such as methanogens, homoacetogens and sulfate reducers amongst others (Dinamarca and Bakke, 2009; Zoetemeyer et al., 1982). For this reason, enrichment of culture is essential (see section 2.6). In addition to \( \text{H}_2 \) consumer, there co-exist non-\( \text{H}_2 \) producers such as HPr producing or HL\( \alpha \) producing bacteria that lowers the \( \text{H}_2 \) yield. However, studies by Zhang et al. (2006) revealed that altering environmental parameters, such as hydraulic retention time (HRT), will reduce diversity in the microflora by eliminating non-\( \text{H}_2 \) producers and thereby establish a microbial community that can produce high \( \text{H}_2 \) yields.

### 2.6 Enrichment of the culture

In order to increase \( \text{H}_2 \) production, mixed cultures containing \( \text{H}_2 \) consumers are treated using methods such as heat, chemicals, load-shock, and aeration. Pretreatment of the culture can delay \( \text{H}_2 \) production and may consequently reduce the overall yield or the stability of the system (Hawkes et al., 2002; Minoda et al., 1983). A proper pretreatment method must be selected based on the treatment’s efficiency, the possibility of its application on a larger scale, its effect on the environment and cost-efficiency. Different pretreatment methods are described in the following sections.

#### 2.6.1 Heat treatment

Among the available pretreatment methods, one of the most widely used for enrichment is heat treatment. This method destroy non-spore forming bacteria and enrich the acidogenic spore formers that produce \( \text{H}_2 \) (Lay et al., 1999). During heat treatment, major non-spore forming organisms such as methanogens are destroyed and only the spore forming bacteria survive (Oh et al., 2003; Van Ginkel et al., 2001). However, not all \( \text{H}_2 \) consumers belong to the non-spore forming group. For example, homo-acetogens (such as *Clostridium aceticum*, which are \( \text{H}_2 \) consumers) are spore formers that can survive heat treatment (Oh et al., 2003; Ohwaki and Hungate, 1977). Hussy et al. (2003) reported that heat-treatment did not eliminate \( \text{H}_2 \) consumers such as homoacetogens and HPr producing bacteria.
Conditions for heat treatment can vary, i.e., the incubation temperature and residence time. The temperature range that is normally used in heat shock treatment is 80 to 105 °C and the retention time is 15 to 120 min (Chang and Lin, 2004; Lay et al., 1999; Zhu and Beland, 2006). However, Alibardi et al. (2012) and Lay et al. (2011) reported that optimum temperature and retention time for high H₂ yield would be 100 °C for 4 h or 60 °C for 40 minutes. Note, Lay et al. (2011) observed CH₄ in addition to H₂ at these conditions for the reactors operated at 55 °C. Ren et al. (2008) studied the effect of heat shock using a sterilization temperature of 121 °C for 20 min, and achieved a maximum H₂ yield of 190 mL, corresponding to 1.65 mol mol⁻¹ glucose.

Although these studies reported high H₂ yields following applications of heat treatment, there are drawbacks to their use as a selective means for the enrichment of microorganisms. The use of heat shock may not only kill the H₂ consuming methanogens, but also inactivate some of the H₂ producing non-spore forming vegetative cells and also in addition, spore forming acetogens are not killed (Kraemer and Bagley, 2007). A lag in the initiation of H₂ production was observed for the heat treated cultures in both batch and continuous systems (Duangmanee et al., 2007; Hawkes et al., 2002). Duangmanee et al. (2007) reported that repeated heat treatment was required to maintain H₂ production in continuous systems. However, repeated heat treatment or prolonged heat treatment may affect the microbial granular structure in high rate systems such as an upflow anaerobic sludge blanket reactor (UASBR). In comparison, Liang et al. (2010) reported that heating followed by acid treatment of a granular culture resulted in low HPR and partial granulation; however, with subsequent heat shock treatment, the granulation and HPR improved. Heat treatment at a large scale is not economically viable as in case of reactor failure or revival of methanogens providing repeated heat treatment to the inoculum becomes inevitable.

2.6.2 Acid and alkali treatment

The most widely used pretreatment apart from heat is acid treatment. This is because the main H₂ consuming organisms (methanogens) are active at pH ranging from 6.5 to 7.5 and most methanogens are inhibited at a lower pH (<5.5) (Fang and Liu, 2002; Fang et al., 2002b). Acid treatment is employed by adjusting the pH of the culture to 2.0 - 3.0 for an incubation period of 24 h, during which only the spore forming bacteria survive.
Elimination of the non-spore forming methanogens thereby represses methanogenic activity (Chang et al., 2002; Lee et al., 2009b). However, the acid pretreatment may not be effective over sustained long periods of operation. Studies by Luo et al. (2011) suggest that the H₂ production potential decreased over repeated batches. These authors reported that approximately 80% of the H₂ was consumed by fifth generation acid pretreated inoculum compared to 10% in a freshly pretreated inoculum. Demirel et al. (2010) reported that H₂ production increased by 80% with alkaline treatment (pH 11.0 for 24 h). However, Ren et al. (2008) reported low H₂ yield and increased methane yield with alkali treatment of cultures with these parameters (incubation of culture at pH 11.0 for 24 h). Mu et al. (2007) studied both acid and alkali treatments for enriching the microflora to enhance bio-H₂ production and suppress methanogens. Variability in this process is a major concern and this has caused concerns related to implementing this technology in full-scale systems.

2.6.3 Chemical treatment

Chemical inhibitors include both synthetic and biochemicals. Some of the common synthetic chemical treatments include 2-bromoethanesulfonate (BES), iodopropane and acetylene (Sparling et al., 1997; Zhu and Beland, 2006). Among these, BES is a well known inhibitor for suppressing methanogenesis. The inhibitor binds to the co-enzyme M reductase complex, a prime component of the methanogenesis present in methanogens (Zhu and Beland, 2006). For example, in *Methanobacterium thermoautotrophicium*, when BES (an analog of co-enzyme M) was applied, reduction of methyl co-enzyme to CH₄ was inhibited (Gunsalus et al., 1978). Sparling et al. (1997) reported that 25 mM of BES was effective in inhibiting methanogens and increasing the H₂ production. However, studies by Cheong and Hansen (2006) have shown that the COD of a BES treated culture would be higher and may reduce the degrading efficiency of the feed or waste stream and hence cause environmental pollution problems at discharge. Kotsopoulos et al. (2006) reported that BES is not an efficient pretreatment method for long-term operation and may be toxic to H₂ producers.

Sparling et al. (1997) reported that 1% (v/v) acetylene could be used for inhibiting methanogens and enhancing H₂ production. Exposing methanogens to acetylene causes them lose the ability to maintain their transmembrane pH thus, resulting in reduced
methanogenic activity. Ethylene, a similar compound to acetylene, can also inhibit methanogens. The disadvantage of using ethylene is that, the amount of ethylene required for the pretreatment is larger than that of acetylene due to their difference in solubility by a factor of 400 (Gordon and Ford, 1972; Sprott et al., 1982). Chloroform has been used to inhibit methanogens. Studies by Xu et al. (2010) have shown that inhibiting methanogens with chloroform increased $H_2$ production. Chloroform or any methyl chlorinated compound are able to block corrinoid enzymes which leads to inhibition of methyl Co-enzyme A in methanogens (Oremland and Capone, 1988). However, the use of these chemicals could pose a threat to the environment if they are discharged in effluents from a bioreactor (Valdez-Vazquez and Poggi-Varaldo, 2009).

Another type of inhibitors used include biodegradable chemical that are able to inhibit methanogens. Long chain fatty acids (LCFAs) are a group of chemical inhibitor which can act on both aceticlastic methanogens and hydrogenotrophic methanogens (Koster and Cramer, 1987; Lalman and Bagley, 2002). However, LCFAs may not inhibit $H_2$ consuming-spore forming *Clostridium aceticum* and *Desulfotomaculum geothermicum* (Park et al., 2004b). The advantage of using LCFAs for pretreatment is that they are biodegradable compared to other synthetic chemicals and degrade to shorter chain fatty acids and HAc plus $H_2$ (Weng and Jeris, 1976). The quantity of $H_2$ produced from LCFAs is much lower than that of sugars such as glucose and xylose because they degrade very slowly (Chaganti et al., 2012a; Saady et al., 2012b).

### 2.6.4 Other treatment methods

Load shock is another form of pretreatment, in which no chemical treatment is involved. High loading of substrate is applied to the system, which makes the environment unsuitable for many microorganisms. Van Ginkel et al. (2001) reported that with high substrate loadings, the higher levels of volatile fatty acids produced reduced the survival of methanogens under acidic pH levels of 5.0-4.5. In continuous systems, increase in loading is an effective mechanism to eliminate a larger percent of methanogens and ultimately enhancing the $H_2$ produced (Prasertsan et al., 2009).

Since methanogens are sensitive to oxygen, aeration could be used to inhibit methanogens. Ueno et al. (1996) reported complete inhibition of methanogenesis with no methane detected while achieving 65-70% of the theoretical maximum $H_2$ yield in
chemostat studies conducted with industrial wastewater. In comparison, Zhu and Beland (2006) reported no significant impact on the H₂ yield with aeration. They observed similar levels of CH₄ in aerated cultures as and control cultures.

Guo et al. (2008a), reported a shorter lag phase in culture treated with ultrasonication and microwaves. Studies conducted by Thungklin et al. (2011) revealed that microwave irradiation inhibited the H₂-consuming activity and greater than 23 mL H₂ L⁻¹ culture was obtained from microwave inhibited culture containing slaughterhouse waste.

2.6.5 Summary

Although various pretreatment strategies have been employed for culture enrichment, there is no study demonstrating which of these methods is the most effective in a full-scale application. For example, Ren et al. (2008) and Luo et al. (2010) evaluated different pretreatment strategies for suppressing H₂ consumption and enhancing H₂ yield. Ren et al. (2008) concluded that the maximum H₂ yield was obtained by repeated aeration and the lowest H₂ yield was obtained with acidified culture. In comparison, Luo et al. (2010) reported that an untreated culture and a culture treated with load-shock performed the same and that the lowest yield was obtained with chloroform pretreated culture. Evaluation of acid, alkali and heat treatments by Mu et al. (2007) revealed that heat treatment may be considered as a potential treatment method for bio-H₂ production. Recently, Pendyala et al. (2012) reported that the conflicting data was due to variation in the fermentation conditions and that the conclusions about the optimum pretreatment strategy had no statistical basis. The study by Pendyala et al. (2012) revealed that the treatment methods employed did not reveal any statistical difference between flocculated cultures whereas for granulated culture the effect of linoleic acid (LA, an unsaturated LCFA) and BES treatment was statistically the same, while other pretreatment methods showed lower yields. Based on the above discussion, the criteria for selecting the appropriate pretreatment technology involves not only efficiency in suppressing H₂ consumption and enhancing H₂ production but also in establishing diverse H₂ producing microflora for long-term operation. Optimal pretreatment must result in efficient biological H₂ production from a mixed anaerobic community. The selection of an appropriate technology should also consider the cost of implementation and addressing environmental concerns associated with them.
2.7 Long chain fatty acids (LCFAs) and their role in hydrogen production

2.7.1 LCFA characteristics, degradation and mechanism of their action

Long chain fatty acids are characterized by functional hydrophilic and hydrophobic groups. Normally, in naturally available lipid containing wastewater streams, LCFAs occur in the form of triglycerides (a LCFA linked to glycerol by ester bonds). The LCFAs are categorized based on the length of the carbon chain and the number of double bonds present. LCFAs without double bonds are saturated fatty acids while those with a single double bond are mono-unsaturated fatty acids and those with two or more double bonds are designated as poly-unsaturated fatty acids.

The LCFAs that are commonly available in wastewater include myristic (MA, C14:0), palmitic (PA, C16:0), oleic (OA, C18:1) and LA (C18:2) acids. The occurrence of these fatty acids in animal and plant lipids and some of the vegetable or seed oils is very common.

Bio-degradation of LCFAs has been reported for ambient, mesophilic and thermophilic temperatures (Angelidaki and Ahring, 1992; Lalman and Bagley, 2000; Saady et al., 2012b). LCFAs impose a bacteriostatic effect and no adaptation of the culture to the presence of LCFA concentrations at low levels were observed in methanogens (Angelidaki and Ahring, 1992). LCFA degradation involves the metabolism of fatty acids by syntrophic bacteria present in mixed anaerobic community through β-oxidation (Weng and Jeris, 1976). According to the model proposed by Batstone (1999), LCFA degradation takes place under low partial pressure of $H_2$ (pH$_2$) with four intermediate steps in the transformation: 1) assimilation and transport; 2) activation; 3) acetyl Co-A intermediate formation; and 4) β-oxidation. Absorbed LCFAs are transported to mitochondria after activation in the cytoplasm by ATP. β-oxidation takes place inside the mitochondria, during which the fatty acids containing carbon atoms are oxidized to form HAc along with the creation and release of $H_2$ (Mackie et al., 1991). The following figure illustrates the LCFA degradation pathway involving a β-oxidation mechanism.
The generation of $\text{H}_2$ via $\beta$-oxidation is due to the presence of $\text{H}^+$ ions which serve as electron acceptors during the reaction. Experimental results from studies conducted by Hanaki et al. (1981) revealed that higher levels of LCFA can inhibit the $\beta$-oxidation pathway. According to Shin et al. (2002), higher loading of LCFA in an UASBR resulted in accumulation of LCFA in the reactor. Although LCFA degradation rates are slow, degradation depends on the culture type and the composition of the inoculum containing the microflora required for bio-degradation. For example, Saady et al. (2012b) reported degradation rates of 29 and 21 $\mu$g LA mg$^{-1}$ VSS day$^{-1}$ for granulated and flocculated cultures, respectively. Kim et al. (2004) reported a degradation rate of 58.8 $\mu$g LA mg$^{-1}$ VSS day$^{-1}$ for linoleate and 23.04 $\mu$g PA mg$^{-1}$ VSS day$^{-1}$ for palmitate-fed reactors. The study also revealed that the lag phase of the $\beta$-oxidation rate is dependent on the LCFA concentration employed in the system.

### 2.7.2 LCFA toxicity

The mechanism for LCFA toxicity is dependent on the absorption of the LCFA onto the surface of the microbial cells. Many studies have suggested that LCFA toxicity is dependent on the ratio of LCFA concentration to that of biomass concentration and the surface area available for LCFA to attach onto cellular membranes (Demeyer and...
Henderickx, 1967; Rinzema, 1988; Saady et al., 2012b). However, Koster and Cramer (1987) reported that LCFA inhibition is dependent only on the initial concentration of LCFAs and not on the characteristics of the biomass. The degree of toxicity imposed is greater for a mixture of LCFAs than for a single LCFAs. For example, Koster and Cramer (1987) observed increased toxicity for MA and capric (CA) acid in the presence of lauric acid (LAU) compared to the toxicity for individual acids. LCFA inhibition is dependent on the number of carbon atoms present and their degree of unsaturation (Galbraith et al., 1971). Lower levels of LCFAs could be toxic to gram positive organisms but not gram negative strains. It is for this reason that methanogens are inhibited by LCFAs because their cell walls resemble those of gram positive strains (Kabara et al., 1977; Zeikus, 1977).

Although LCFA treatment offers the advantage of inhibition of H$_2$-consuming methanogens, there are two problems associated with the use of LCFAs: 1) delay of substrate degradation; and 2) increased buoyancy of the biomass. In the first case, LCFA delay substrate degradation in batch anaerobic degradation systems. Angelidaki and Ahring (1992) studied the degradation of HAc, HBu and HPr, the major VFAs produced by dark fermentation in the presence of LCFAs (OA and stearic acid (SA)). They observed a lag phase in the degradation of these VFAs in the presence of the LCFAs. Lalman and Bagley (2002) reported that inhibition of the degradation of glucose, HBu and H$_2$ by LA was greater than that imposed by SA and OA. Alosta et al. (2004), however, noted that only at concentrations greater than 300 mg L$^{-1}$ LA, the degradation of glucose and the production of byproducts was considerably decreased. These authors observed a change in the pattern of VFA production and degradation in LCFA inhibited cultures fed with glucose. For example, HBu was detected only in cultures receiving more than 300 mg L$^{-1}$ of LA and HAc increased even after 20 days of incubation at LA concentration $> 500$ mg L$^{-1}$, whereas HBu was absent in both OA and SA fed cultures. LCFAs adsorbed on the surface of the microbial biomass caused flotation of sludge in granular systems, leading to washout of the biomass in continuous systems (Rinzema et al., 1989). Although the addition of calcium would reduce the toxicity imposed on the culture by LCFA treatment, prevention of sludge flotation is not possible (Alves et al., 2001).
Hwu and Lettinga (1997) studied the effect of OA on aceticlastic methanogens to find the levels of OA required for 50% reduction in methanogenic activity ($I_{50}$ value for this LCFA). They reported that the $I_{50}$ value was dependent on the culture source and operating temperature. For example, the $I_{50}$ values of OA were 0.79 and 0.39 mM for cultures from two different sources operating in the thermophilic range and was 4.35 mM for tolerant culture (LCFA adapted culture) at mesophilic temperature. Studies using high rate systems (e.g. UASBR, expanded granular sludge bed (EGSB), and down-flow anaerobic expanded bed (DAEB) systems) reported that the methanogenic activity decreased with increasing LCFA concentration (Hwu et al., 1998b; Miranda et al., 2006). These studies also confirmed that the culture washout contained an appreciable amount of the LCFAs, which was present in the flotation matter. Batch studies conducted by Kim et al. (2004) at mesophilic conditions using granular sludge showed $I_{50}$ values of 5.71 mM for PA, 5.37 mM for SA, 3.1 mM for OA and 0.73 mM for LA, which suggest that an increasing degree of unsaturation corresponds to high levels of inhibition by the LCFA.

Hwu et al. (1998a) proposed a hypothesis of bio-adsorption and bio-degradation, according to which the LCFA is adsorbed to the granular surface and no degradation of is observed initially. Adsorption of LCFA to the granular surface causes inhibition or the lag phase of the methanation period. However, over a specific time LCFA disappears from the aqueous phase and degradation of the LCFA that was initially adsorbed takes place. This hypothesis suggests that $IC_{50}$ values for granular cultures would be greater than those of the flocculated cultures because more surface area is available for LCFA adsorption in flocculated cultures. For example, the $IC_{50}$ determined for granular cultures ranged from 1.75 to 3.34 mM, but values for flocculated cultures ranged from 0.26 to 0.53 mM in (Hwu et al., 1996).

### 2.7.3 Synergistic effect of pH and LCFAs

Both degradation and toxicity effects of LCFA treatment is dependent on pH (Rollón, 1999). At higher LCFA levels, the inhibition of methanogenesis at lower pH is enhanced (McCarty, 1964). Likewise, Komatsu et al. (1991) reported that the increased toxicity to methanogens was observed at a lower pH for HAc as substrate in the presence of oleic acid. Alves et al. (2009) reported that, at neutral pH, LCFA acts as a surfactant, causing disintegration of cells by lowering the surface tension over the cell surface. Lindblad et
al. (2002) observed that the toxicity imposed by LAU treatment was greater at pH 6.0 than at pH 8.0. This suggest that at higher pH, LAU was less inhibitory to methanogens. However, the degradation of LCFAs by the species associated with Syntrophomonas took place within the neutral pH range (Sousa et al., 2007). The outcome of these studies suggest that a pH range 5-6 is effective in suppression of methanogens and LCFA degradation.

2.7.4 Use of LCFAs in bio-H\textsubscript{2} production

Use of LCFAs as inhibitors of methanogens has been studied widely as described in previous sections. LCFAs are readily available from inexpensive sources, such as animal fats, agricultural crops, fish oils, vegetable oils and oils from seed (See Table 2.4 for composition of LCFAs available from different sources). This makes LCFAs suitable for use as a methanogenic inhibitor in full scale systems. LCFAs have been examined for use in H\textsubscript{2} production because of its biodegradable nature that poses less threat to the environment while being toxic to methanogens under the specific operating conditions of a biofuel production system. Various studies have been carried out in LCFA-treated batch reactors demonstrating the impact of LCFA on bio-H\textsubscript{2} production (Ray et al., 2008; Saady et al., 2012a). Ray et al. (2010) employed LA as a methanogenic inhibitor for diverting electron fluxes from methane to H\textsubscript{2}. The effects of treating mixed anaerobic cultures with a mixture of LCFAs on bio-H\textsubscript{2} production has also been studied by Saady et al. (2012a) using MA, PA and LAU, which have a strong presence in lipid-containing wastewater. However, LA, a polyunsaturated fatty acid, has been used widely in H\textsubscript{2} production for inhibition of H\textsubscript{2}-consuming methanogens. Studies using LA as a methanogenic inhibitor for bio-H\textsubscript{2} production were conducted using anaerobic sequential batch reactors with a working volume of 6 L and fed lignocellulosic pure sugars (Chaganti et al., 2013). This study reported a maximum yield of 2.89 mol mol\textsuperscript{-1} hexose which is equivalent to 73% of the theoretical maximum H\textsubscript{2} yield from dark fermentation.
Table 2.4 Table showing the major fatty acid composition of common oil, seed, and other waste sources (Rinzema, 1988; Van Gerpen et al., 2004)

<table>
<thead>
<tr>
<th>Raw material</th>
<th>Lauric</th>
<th>Myristic</th>
<th>Palmitic</th>
<th>Stearic</th>
<th>Palmitoleic</th>
<th>Oleic</th>
<th>Linoleic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linseed oil</td>
<td>3.3</td>
<td>7.1</td>
<td></td>
<td>11.4</td>
<td>57.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cotton seed oil</td>
<td>1.4</td>
<td>25.7</td>
<td>2.9</td>
<td>15.2</td>
<td>51.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chicken Fat</td>
<td>1.4</td>
<td>21.0</td>
<td>4.3</td>
<td>0.5</td>
<td>42.4</td>
<td>20.4</td>
<td>1.0</td>
</tr>
<tr>
<td>Raw Sewage</td>
<td></td>
<td></td>
<td>27.6</td>
<td>16.7</td>
<td>48.3</td>
<td>5.1</td>
<td></td>
</tr>
<tr>
<td>Domestic</td>
<td>2.2</td>
<td>16.4</td>
<td>8.1</td>
<td>0.7</td>
<td>30.5</td>
<td>29.2</td>
<td>1.0</td>
</tr>
<tr>
<td>Safflower oil</td>
<td>5.9</td>
<td>1.5</td>
<td></td>
<td></td>
<td>8.8</td>
<td>83.8</td>
<td></td>
</tr>
<tr>
<td>Corn oil</td>
<td>1-2</td>
<td>8-12</td>
<td>2-5</td>
<td></td>
<td>19-49</td>
<td>34-52</td>
<td></td>
</tr>
</tbody>
</table>

2.8 Factors affecting fermentative bio-hydrogen production

The \( \text{H}_2 \) production from fermentation is highly dependent on the environmental conditions of the process. Parameters influencing yield include both physical and chemical conditions. To maximize the \( \text{H}_2 \) yield and HPR, optimal conditions for fermentation need to be selected. In order to accomplish this, a better understanding of the factors influencing the \( \text{H}_2 \)-producing fermentation process is required. The following sections describes the effects of various parameters on bio-\( \text{H}_2 \) production.

2.8.1 Nutrients

The proper balance of nutrient sources containing both organic and inorganic materials is required for bacterial growth and fermentation to complement the carbon sources used as the electron donor. Substrate feedstocks, such as lignocellulosic sugars, carbohydrate-rich starchy material and woody biomass are rich sources of carbon. However, these feedstocks are deficient in nitrogen, phosphorous and other minerals which are essential for microbial metabolism and bio-\( \text{H}_2 \) production. Lacking these nutrients could depress bacterial growth during fermentation (Lettinga, 1995). The major nutrients required for bacterial growth and metabolism are carbon (C), nitrogen (N) and phosphorous (P). The advantage of producing \( \text{H}_2 \) via dark fermentation is that since the process is anaerobic, the biomass production or growth is limited in comparison to
aerobic processes, which suggests low-nutritional requirements for the support of bacterial growth (Cheong, 2005). Apart from the C source required for energy metabolism, N and P are constituents of the amino acids, nucleic acids and nucleotides that are involved in protein and DNA synthesis. A proper balanced ratio of these nutrients is required for bacterial function and high product yield. For example, several authors have reported that the C:N and C:P ratios affect fermentative H$_2$ production using mixed culture (Argun et al., 2008; Lin and Lay, 2004). These authors reported that the optimal ratio for C:N:P is 100:0.5:0.1 (on a weight by weight basis), while, Sreethawong et al. (2010a) reported COD:N of 100:2.2 as the optimal ratio for bio-H$_2$ production. This is similar to the COD:N:P (100:2:0.5) ratio used by Intanoo et al. (2012). The other essential inorganic nutrient in bio-H$_2$ production is iron (Fe$^{2+}$). During dark fermentation (as described in section 2.4.3), oxidation of reduced ferrodoxin takes place through the actions of the hydrogenases (Adams et al., 1980). Hydrogenase, an iron-containing enzyme (12 atoms of iron and 12 atoms of sulfur groups per molecule), is responsible for H$_2$ evolution (Chen and Mortenson, 1974). Studies showing the effect of external iron concentration on H$_2$ production, and how this affects the in vivo processes of hydrogenase activity, were carried out in anaerobic bacterial systems (Dabrock et al., 1992; Lee et al., 2001). Oztekin et al. (2008) reported a maximum H$_2$ yield of 2.89 mol mol$^{-1}$ hexose at optimum ratios of N:C, P:C and Fe(II):C equals 0.02, 0.008 and 0.015, respectively. In addition to the source of iron, Fe$^{2+}$, a sulfide source is required for anaerobic bacterial systems. Speece (1983) reported that sulfide in its un-ionized form is essential for anaerobic bacterial growth. Other sources of nutrients includes yeast extract (peptone), minerals such as potassium, magnesium, and ammonium, buffering agents such as bicarbonate and trace amounts of metals and vitamins are essential for fermentation and bacterial growth. Many studies have demonstrated optimizing these components in media that are suitable for bacterial growth (Azbar et al., 2009; Liu and Shen, 2004; Zhao et al., 2009).

2.8.2 Temperature

Various external factors such as temperature, pH and substrate concentration which contribute to the bio-H$_2$ fermentation process can play an important role in optimizing H$_2$ production. Temperature plays a vital role on H$_2$ production because enzymatic activity
(e.g. hydrogenase activity) is influenced by the temperature. According to Dinamarca and Bakke (2011), in biochemical systems, the enzymatic activity doubles for every 10 °C rise in temperature. In comparison, in H₂ producing systems, hydrogenase activity have been reported to increase to optimum temperature and decreased thereafter (Upadhyay and Stokes, 1963). The optimum temperature for H₂ production is variable from 37 to 60 °C and even up to 83 °C, depending on the type of organism present in the inoculum source (mesophilic, thermophilic or hyperthermophilic) (Abreu et al., 2007). Most of the fermentation studies have been carried out in the mesophilic range (25- 40 °C) (Dinamarca and Bakke, 2011; Rittmann and Herwig, 2012). Higher temperatures are advantageous for achieving higher H₂ yields. However, achieving high cell densities have proven challenging at high temperature ranges, and low volumetric H₂ production rates were observed (Hallenbeck, 2005).

A change in temperature not only affects HPR but it also affects substrate utilization and changes the metabolic pathway of the microbial population leading to variation in the liquid metabolite distribution and microbial communities (Luo et al., 2011). Although several studies have examined the effect of temperature on bio-H₂ production, the results obtained are not uniform. For example, Wang and Wan (2008) reported that the optimum temperature for mixed culture fermentation for H₂ production was 40 °C; however, Valdez-Vazquez et al. (2005a) reported that the optimal temperature was 55 °C and Danko et al. (2008) reported that the maximum H₂ production was observed at 60 °C. These inconsistent findings for the determination of the optimal temperature required for H₂ fermentation may be due to variations in the methodology, including the use of different sources of culture and different carbon sources used for the fermentation experiments conducted by these researchers. Note complex substrates such as foodwaste and starch require high temperatures for promoting high hydrolysis rates while simple substrates such as glucose, xylose and sucrose are easily fermentable substrates at low temperatures (25-35 °C).

2.8.3 pH

pH is another important parameter affecting H₂ fermentation. The quantity of H₂ produced is based on the enzymatic activities in the substrate to H₂ metabolic pathway. Each enzyme has a specific pH at which they operate at optimum. Changes in pH affect
the hydrogenase activity causing alteration in the metabolic pathway of the microflora and hence, product distribution in the fermentation process (Dabrock et al., 1992). The optimum pH range lies between 4.5 to 6.0 for dark fermentation (Fang and Liu, 2002); however, the optimal pH conditions may vary depending on the nature of the operation in which microbial fermentation is carried out. For example, in batch studies where pH generally cannot be controlled, the initial pH is important while in continuous systems (e.g. CSTR), pH can be controlled and an optimal pH can be attained. Various studies have reported using different initial pH conditions for bio-H₂ production. Ferchichi et al. (2005a) reported that an initial pH of 6.0 yielded the maximal HPR with cheese whey as the substrate. O-Thong et al. (2011) reported an initial pH of 5.5 favored H₂ production from POME with a HPR equal to 4.8 L L⁻¹ POME. However, Lee et al. (2002) reported contradictory findings and found pH 9.0 was optimum for maximal HPR from sucrose and that no methane was detected in the range of pH 6.0-9.0. Note, the pH range at the conclusion of most of these studies decreased to the acidic pH range due to the VFA production. Therefore, usage of controlled pH system would be best to study the effect of initial pH on batch fermentation. Kim et al. (2011) studied the effect of initial pH decoupled from the operating pH and reported that an initial pH of 8.0 resulted in maximal H₂ production using food waste as the substrate. However, this may vary depending on the substrate used and other factors controlling the net H₂ production such as H₂ consumption and soluble metabolite productions.

The production of metabolites is also dependent on pH condition. Depending on the pH conditions, the production of alcohols and/or volatile fatty acids can be expressed (Temudo et al., 2007). The pH can also control the substrate degradation rate (Cheng et al., 2002). Studies by Han and Shin (2004) have shown that a sudden change in pH caused the pathway to shift towards HLa production. Masset et al. (2010) reported that maximum HBu levels were produced at low pH conditions (4.85), whereas HAc and EtOH production was favorable at pH 7.3. Similarly, Lay et al. (2010) reported that maximum HAc levels ranging from 1700 to 1988 mg L⁻¹ occurred at pH 6 to 7 while the maximum HBu concentration of 1512 mg L⁻¹ was detected at pH 5.0 and EtOH concentrations of 1951 mg L⁻¹ and 1632 mg L⁻¹ were observed at pH 8 and pH 7, respectively.
The pH level is an important factor for containing H\textsubscript{2} consuming reactions. For example, suppression of methanogenesis begins when the pH is lower than 6.0 (Liu et al., 2008a). In addition to suppression of methanogenesis, lower pH conditions are able to suppress homoacetogenesis (Hwang et al., 2004). The preferred pH for higher H\textsubscript{2} yield is acidic, in the range of 5.0-5.5. Fang and Liu (2002) reported that methanogenic activity was not detected with pH below 5.5 and that methane production increased with increasing pH (from 6 to 7.5). Park et al. (2005) observed suppression of Archeae populations (both aceticlastic and hydrogenotrophic methanogens) at low pH conditions. Calli et al. (2008) reported increased acetogenic H\textsubscript{2} consumption above pH 5.5, suggesting that pH lower than 5.5 is favorable for H\textsubscript{2} production.

Changes in external pH could also induce changes in the internal pH of the microorganisms as well, altering membrane potential and proton motive force (Kaback, 1986). Gottwald and Gottschalk (1985) reported that cells try to maintain a small pH gradient between internal and external pH, and noticed that at internal pH less than 5.7 a shift to solvent production was observed in the metabolic pathway. Hwang et al. (2004) and Ren et al. (1997) reported observations of EtOH-HAc products for H\textsubscript{2} fermentation at low pH which contradicts the findings reported by Masset et al. (2010) and Lay et al. (2010) where HAc and EtOH production were observed in dominance at higher pH levels. The formation of these reduced end products such as EtoH and HPr is to provide balance of the oxidized and reduced end products, where at low pH the pathway shifts to EtOH-acetone-butanol fermentation type, with acetone further reducing to i-propanol (i-PrOH) (Moat et al., 2002).

### 2.8.4 Hydraulic retention time and organic loading rate

Bioreactor design factors such as HRT and OLR can impact H\textsubscript{2} production (Li and Fang, 2007; Show et al., 2007; Wang and Wan, 2009a). HRT is defined as the time required for a volume element to transiton from the inlet to the outlet of the reactor system. HRT is calculated from the ratio of volume of fluid present inside the reactor system to the volumetric flow rate applied to the system. HRT is an important parameter from the perspective of engineering design, as HRT is used in the determination of reactor capacity (Karia and Christian, 2006). OLR is defined as the mass rate at which the organic material is applied to a volume element of the reactor and is normally expressed
as kg COD applied for a unit volume per day (kg COD m\(^{-3}\) d\(^{-1}\)) (Bitton, 1997). HRT and OLR do not apply to batch systems as these parameters are relevant to continuous, semi-continuous and sequential batch systems where both influent and effluent streams are present. HRT and OLR are the main parameters that need to be optimized for H\(_2\) producing continuous systems using different sources of substrate because HRT and OLR not only affect the H\(_2\) yield and HPR, but also the metabolic stability of the H\(_2\) fermentation system (Hawkes et al., 2002; Ueno et al., 1996). In addition to these parameters, sludge (culture)/solid retention time (SRT), which is referred to as the mean residence time of the biomass present in the bio-reactor (Bitton, 1997), plays influential role in the anaerobic fermentation. SRT is also an operational parameter in continuous systems that needs to be regulated to ensure that enough microorganisms are available to maintain the loading concentration at levels required to maintain a specific food to microorganism ratio (F/M ratio) for better performance of the reactor (Lee et al., 2006) (see section 2.9.1). In batch systems, substrate concentration and reaction time play as important a role as pH and temperature.

HRT influences the time that the feed substrate is available for the microorganisms to act upon them. Hence, a high HRT should improve the substrate utilization and hence, substrate conversion efficiency. However, a retention time that is too long may be unfavorable because the H\(_2\) produced may be consumed by methanogens. Hydrogen producers reportedly grow at faster rates than methanogens, which means that lower HRTs (i.e., a high dilution rate) would favor the H\(_2\) producers (Hawkes et al., 2002; Valdez-Vazquez and Poggi-Varaldo, 2009). Thus, in continuous operating bio-reactor systems, the slow growing methanogens (major H\(_2\) consumers) might washout at high dilution rates (Chang et al., 2001). With shorter HRTs, the electron flow shifts towards the production of metabolic products rather than microbial growth or cell maintenance this results in high HPR, which might cause an operation failure for HRTs below a critical biomass washout condition (Jo et al., 2008b; Mu and Yu, 2007; Zhang et al., 2007b). Unlike pH, there is no particular range of HRT that are optimal for H\(_2\) production. For example, Kim et al. (2008b) reported that HRT ranging from 30-33 h was optimal for high HPR, whereas Show et al. (2007) reported that 0.5 h was the optimum HRT. The extreme deviation of optimal values for HRT may be due to variations in
methodology, which includes the range of HRT selected for the experimental design, the inoculum source, substrate type, reactor configuration and operating conditions. The pH level and HRT are the most important parameters for continuous systems such as CSTRs and UASBRs. In these reactor configurations, activities of H$_2$ producing bacteria are uncoupled from the non-H$_2$ producing Archaea (Oh et al., 2004). Shorter HRTs not only eliminate the H$_2$ consuming methanogens, but also reduce the numbers of HPr producers as well without jeopardizing the dominant H$_2$ producing species (Zhang et al., 2006). Hawkes et al. (2007) suggested that continuous fermentation with low HRTs will not only improve the H$_2$ production process but also benefit the engineering and economic significance of designing small reactors for a lower cost.

The OLR is directly related to HRT. The HRT and organic substrate concentration (i.e., the feed concentration) are used to determine the OLR. Operating conditions of either high OLR with high HRT or low HRT and low OLR would be suitable for H$_2$ production. For example, Lin et al. (2009) reported increased H$_2$ yield when shifting the system to higher HRT and increasing OLR by varying the substrate concentration. However, higher OLR with longer HRT does not result in a high HPR. Van Ginkel and Logan (2005) reported decoupling the HRT and OLR and they studied the effect on H$_2$ production by varying the feed concentration at different HRT. The study revealed that reduced OLR improved H$_2$ yield, but not the HPR. According to Van Ginkel and Logan (2005), the HPR increased by lowering the HRT while maintaining a high OLR, the maximum HPR observed was 4.33 L h$^{-1}$ at a 1 h HRT and corresponding to an OLR of 240 g COD L$^{-1}$ d$^{-1}$ (H$_2$ yield observed under this condition was 1.7 mol mol$^{-1}$ hexose). The operating conditions under which the maximum H$_2$ yield of 2.8 mol mol$^{-1}$ hexose was obtained by these authors correspond to a HRT of 10 h and an OLR of 6 g COD L$^{-1}$ d$^{-1}$ (the corresponding HPR obtained in this condition was 0.16 L h$^{-1}$). In summary, low OLR and low HRT favours optimizing the HPR and H$_2$ yield. Sufficient time is required for the microorganisms to acclimatize to an OLR in increments over a specific period. Several studies revealed that sudden changes in OLR or operating with a high OLR leads to changes in the metabolic pathways. (Abreu et al., 2012; Jo et al., 2008b), these sudden changes can influence the H$_2$ yield and HPR.
An important aspect of operating under high OLR which needs to be considered is the substrate removal efficiency. Abreu et al. (2012) observed decreased COD removal efficiency with increased OLR. Balancing the substrate removal efficiency at high OLR and achieving higher HPR with low HRT is a major challenge for operating pilot and full-scale facilities. Hence, optimizing the the H_{2} yield and HPR by adjusting the pH, HRT and OLR is important. Valdez-Vazquez and Poggi-Varaldo (2009) reported that the easiest method for optimizing a bio-H_{2} producing system without the addition of an inhibitor to increase the HPR would be to operate the system at high OLR, low pH (5-6) and low HRT.

### 2.8.5 Hydrogen partial pressure

The accumulation of H_{2} reduces H_{2} production due to high H_{2} partial pressures (pH_{2}). Change in the pH_{2} leads to a shift in the metabolic pathway to produce end products (such as volatile fatty acids and alcohols) such that the system is thermodynamically stable (van Niel et al., 2003). For example, the pH_{2} is able to influence the HPr to HAc ratio with low pH_{2} favoring HPr formation, whereas high pH_{2} is characterized by HAc formation (Schink and Stams, 2006; Stams and Hansen, 1984).

The pH_{2} is dependent on the amount of H_{2} dissolved in the liquid phase (Levin et al., 2004). Work by Stams (1994) has shown the effect of pH_{2} on H_{2} formation. Their study revealed that oxidation of Fd^{+} including proton reduction requires low pH_{2} compared to the oxidation of NADH with the formation of H_{2}, which is feasible at pH_{2} of 10 Pa. Hence, with increasing H_{2} levels in the liquid phase, the oxidation of Fd^{+} mediating the activity of H_{2} forming co-enzymes is less feasible.

In natural processes, reducing the pH_{2} by syntrophic H_{2} consuming microorganisms leads to thermodynamic stability. Several studies have shown increasing the H_{2} yield by using different methods to remove H_{2} from the bioreactor. Sparging with an inert gas such as nitrogen or argon is the most widely used method for displacing H_{2} from the fermentation broth. Mizuno et al. (2000) noticed increasing H_{2} yield from 0.85 to 1.45 mol mol^{-1} glucose with N_{2} sparging at a flow rate 15 times greater than that of the HPR. According to Tanisho et al. (1998), sparging with argon was responsible for increasing the NADH residual content and removing of CO_{2} from the liquid phase.
Since \( \text{H}_2 \) and \( \text{CO}_2 \) are the primary substrates for methanogens and acetogens, decreasing the pH reduces the \( \text{H}_2 \) consuming activity. Kraemer and Bagley (2006) reported that sparging \( \text{N}_2 \) at a low flow rate assisted in reducing the dissolved \( \text{CO}_2 \) level in the fermentation medium to \( \leq 10.1 \text{kPa} \) and thereby increased the \( \text{H}_2 \) yield by 38%.

Sparging can involve not only inert gases, but can also include one or two mixtures of biogas. Sparging with biogas (composed of \( \text{H}_2, \text{CH}_4 \) and \( \text{CO}_2 \)) has been employed for the removing dissolved gases from fermentation broth. Kim et al. (2006) studied the effect of sparging with \( \text{CO}_2 \) at different rates on improving \( \text{H}_2 \) yield in continuous fermentation and observed an increase in \( \text{H}_2 \) yield ranging from 82-118%. Sparging with \( \text{CH}_4 \) or biogas was also applied to continuous fermenting systems by Kim et al. (2006) and Liu et al. (2006a) to improve the \( \text{H}_2 \) yield. These authors concluded that increase in \( \text{H}_2 \) yield after sparging with \( \text{CH}_4 \) was less than that from inert gas or \( \text{CO}_2 \).

Another method which can be used to remove \( \text{H}_2 \) is membrane separation technology to selectively remove \( \text{H}_2 \) (Lee et al., 2007). The smaller molecular size of \( \text{H}_2 \) is an advantage that allows \( \text{H}_2 \) to be easily separated from the other larger biogas molecules (Liang, 2003). However, this method is not cost-effective because the pore size needed to remove \( \text{H}_2 \) is expensive. Liang et al. (2002) studied the efficiency of separating biogas from liquid broth with a silicone rubber membrane, which reduced the partial pressure inside the bio-reactor; however, \( \text{H}_2 \) formation was enhanced by only 10%.

Kraemer and Bagley (2007) reviewed other non-sparging techniques employed in \( \text{H}_2 \) fermentation systems, such as applying a vacuum to the reactor head space and vigorous stirring to discharge the dissolved \( \text{H}_2 \) from the fermentation broth to the head space. However, all of these methods for improving \( \text{H}_2 \) yield depend on the inoculum source and the reactor configuration that is used for \( \text{H}_2 \) fermentation.

**2.8.6 Other factors**

Other factors influencing \( \text{H}_2 \) fermentation include byproducts formed during fermentation. End-product inhibition occurs at high \( \text{pH}_2 \) levels and high VFAs concentrations leading to the development of a pH gradient across microbial membranes causes inhibition of many populations (Hegarty and Gerd, 1999). The \( \text{H}_2 \) fermentation pathway is affected by the presence of end-products and by pH (as discussed in sections
For example, pH affects the dissociation of organic acids and undissociated HBu affect the H₂ yield more than HAc (Chin et al., 2003).

The substrate concentration and substrate type can affect H₂ fermentation. An optimal F/M ratio is essential for achieving stable H₂ yields in continuous operation systems. Van Ginkel et al. (2001) reported that increasing substrate concentration results in a high F/M ratio that is not in the optimal range for operation. The inhibitory effect of a high F/M ratio is due to substrate inhibition leading to elevated acid levels which in turn reduces the pH.

The choice of substrate source for H₂ fermentation plays a significant role in the economic feasibility of bio-H₂ production. Cheng et al. (2011) reviewed a variety of carbonaceous feedstock material for H₂ production and suggested that lignocellulosic feedstock has the potential for producing H₂ in spite of high pretreatment costs. Using pure sugars (e.g. glucose, xylose, sucrose, etc.) has been widely studied; however, the use of these readily degradable sugars on a larger scale is less feasible because of their cost (Kapdan and Kargi, 2006) and more importantly these chemicals are used in the production of various food products. Micro-algae, a third generation feedstock, is considered a promising source (after lignocellulosic biomass) because of their high carbohydrate and lipid content (Wijffels and Barbosa, 2010). Cultivation of this biomass source in large quantities for large-scale use is a major challenge; hence, further research development is required to cultivate algal biomass for biofuel production.

### 2.9 Bioreactor configuration

Bioreactors are classified primarily based on the movement of fluid in and out, mixing the contents and solids/liquid separation. Biological H₂ fermentation studies have been performed using batch, continuous or semi-continuous systems. Continuous and semicontinuous systems for H₂ production are also broadly classified based on the physical characteristics of the culture. The physical characteristics includes suspended, immobilized or fixed and granulated.

#### 2.9.1 Batch and semi-continuous reactors

Hydrogen production from batch reactors has been widely studied because of their ease of operation and low cost. These reactors include serum bottles and bench scale
reactors constructed from glass or Plexiglas and configured with systems for pH and temperature control. For example, Chen et al. (2006) examined H₂ production kinetics from food waste material in serum bottles, whereas Datar et al. (2007) conducted studies of H₂ production from steam-pretreated CS in a 2.5-L glass reactor equipped with pH and temperature controls. Though high yields of H₂ were obtained in batch operating systems, the HPR in these systems was low in comparison with continuous systems. For example, the maximum H₂ yield obtained by Datar et al. (2007) was 3.21 mol mol⁻¹ glucose, which corresponds to an HPR of 0.26 L L⁻¹ h⁻¹. The other advantage in using lab-scale batch reactors; however, is to study the characteristics of the microbial population and to optimize the operating conditions for H₂ production on a small scale before scaling the process up to pilot or industrial scale. Hydrogen production using pilot-scale, semi-continuous or continuous processes have been reported to develop operational strategies and examine the impact of varying design factors. Studies using serum bottles on the other hand are used as a screening tool to examine the impact of chemical and environmental factors (Hallenbeck and Ghosh, 2009).

Gomez et al. (2006) studied H₂ production by dark fermentation of slaughterhouse waste using a semi-continuous operating reactor and was able to obtain stable operation with a H₂ yield in the range of 52.5-71.3 L Kg⁻¹ VS (calculated under normal conditions). Hwang et al. (2004) reported operating a semi-continuous reactor for 100 days using glucose and observed H₂ yields ranging from 100-200 mL g⁻¹ glucose. Valdez-Vazquez et al. (2005a) evaluated H₂ production from municipal solid waste at both mesophilic and thermophilic conditions. They reported that the semi-continuous process was efficient as the continuous process and approximately 80% of the theoretical H₂ maximum yield was observed under thermophilic conditions.

Anaerobic sequencing batch reactors (AnSBRs) are an alternative configuration to continuous reactors. AnSBR have the following advantages over continuous systems: retention of high biomass content, ease of operation on par with a batch process, and no requirement for a clarifier to separate the biomass in liquid phase from the effluent (Dague et al., 1992). Thus, dissociation of the solid retention time (SRT) from the HRT will facilitate high OLR and thus, achieve high levels of H₂ production. Operating AnSBR is based on the following phases: fill, reaction, settling, and decant time. Shizas
and Bagley (2002) studied the effect of these cycle time components on H$_2$ performance. These authors found that a larger fill time to cycle time ratio improved H$_2$ production by decreasing the initial substrate concentration.

AnSBRs are similar to the high rate digester used for methane production, but unlike the methane systems, H$_2$ producing AnSBRs need to be configured according to the requirements of their operating conditions. For example, AnSBR used for H$_2$ production requires a short HRT with a long SRT because of the growth rates of H$_2$ consumers and non-H$_2$ producers (Chang et al., 2002).

Another advantage of AnSBR is that this system can also be used in wastewater systems and can treat high volumes of wastewater compared to conventional systems (Chiang and Dague, 1992). Furthermore, the use of a high F/M ratio at the start of the cycle allows high substrate degradation rates, which in turn results in higher production rates. The depletion of substrate at the end of the cycle results in lower production rates of bio-gas, facilitating the settling of biomass (Dague et al., 1992; Zhang et al., 1997).

Several studies have demonstrated the use of AnSBR for H$_2$ production from different sources of substrate, ranging from simple to complex substrates including glucose, sucrose, dairy wastewater and palm oil mill effluent (POME) (Lin and Jo, 2003; Mohan et al., 2007; O-Thong et al., 2007; Srithawong et al., 2010b). Cheong et al. (2007) reported high HPR of 4.6-5.5 L L$^{-1}$ d$^{-1}$ compared to 3.2 L L$^{-1}$ h$^{-1}$ at 0.5 h HRT in a CSTR system (described in Section 2.9.2) for a feed containing glucose. The Badiei et al. (2011) were able to achieve a higher HPR of 6.7 L L$^{-1}$ d$^{-1}$ at a 3 d HRT and a SRT of 11 day on average for reactors fed with POME.

### 2.9.2 Continuous system with suspended sludge: Continuous stirred tank reactor

Continuous stirred tank reactors (CSTRs) are the most widely used laboratory scale systems for bioH$_2$ production (Li and Fang, 2007). CSTRs are useful in operating at low HRTs and the relatively good mixing employed in these systems assist in reducing the mass transfer limitation between the substrate and the biomass (Majizat et al., 1997). HRTs equal to the critical washout condition are essential to achieve high HPR in reactors containing flocculated cultures (Show et al., 2010). For example, Li and Fang (2007) and Show et al. (2010) achieved HRT ranging from 3 to 8 h with high HPRs. However, at low HRTs i.e., HRT is equal to the SRT, rigorous washout of the biomass
was observed (Show et al., 2010; Wu et al., 2008). Note a dilution rate greater than the critical washout condition leads to low substrate conversion efficiency and eventual failure of the reactor (Chen et al., 2001).

The critical HRT value is dependent on substrate loading as well as the carbon source substrate type. For example, use of simple sugars such as glucose and sucrose allow operation with low HRT ranges from 6 to 8 h (Chen and Lin, 2003; Fang et al., 2002a) while for complex organic sources, such as cheese whey higher HRTs (> 24 h) are required (Venetsaneas et al., 2009).

Applying low HRT seems to be economical from the aspect of design (note HRT is indirectly proportional to the volume of reactor), as a smaller reactor is adequate for operation compared to the size required for anaerobic digestion where longer HRTs are applied (Jung et al., 2011a). Operating bioreactors at low HRTs with high HPRs could be achieved dense and compact microbial cultures. Many studies have reported H$_2$ producing granules (HPG) formation in CSTRs. In CSTRs, culture granulation has lead to enhanced high biomass retention and increasing HPRs. Zhang et al. (2007a) reported granule formation induced by 120 h of acid incubation, achieving HPR up to 3.2 L L$^{-1}$ h$^{-1}$ at 0.5 h HRT. Similarly, Show et al. (2007) attained a HPR of 3.26 L L$^{-1}$ h$^{-1}$ at a HRT of 0.5 h with an OLR of 20 g L$^{-1}$ h$^{-1}$ with glucose as the substrate.

Apart from culture granulation in CSTR, studies with self-flocculation of seed cultures and cell immobilization have been reported by different researchers (Han et al., 2010; Wu et al., 2006). These studies revealed that high HPR (up to 15 L L$^{-1}$ h$^{-1}$) and H$_2$ yield of 0.279 L g$^{-1}$ COD could be achieved with short HRT of 0.5 h and biomass concentrations of 35.4 g VSS L$^{-1}$ for self flocculated cells.

### 2.9.3 Upflow anaerobic sludge blanket reactor

Upflow anaerobic sludge blanket reactor (UASBR) have been widely used in CH$_4$ production for the treatment of high COD containing wastewater (Lettinga et al., 1980). As the name suggests, the presence of a blanket separating the gas, liquid and solid phases makes the system suitable for bio-H$_2$ production allowing granule formation with high settling velocity (Jung et al., 2010). UASBR are primarily studied for H$_2$ production using wastewater or synthetic wastewater as substrate (Yu et al., 2002a).
The main design parameter in a UASBR reactor is the height to diameter (H/D) ratio. Chang and Lin (2007) reported that a high H/D ratio of 7.4 favored H\textsubscript{2} production from sucrose with good settling characteristics and little variation in biomass with decreasing HRT. The maximum H\textsubscript{2} yield obtained was 2.9 mol mol\textsuperscript{-1} sucrose at an HRT of 8 h, which was high compared to the amount of H\textsubscript{2} produced from a reactor with a low H/D ratio.

Operation under optimum conditions (HRT and OLR) could yield high production rates or higher H\textsubscript{2} yield. For example, Chang and Lin (2004) reported stable operation of long duration with a maximum H\textsubscript{2} yield of 53.2 mmol d\textsuperscript{-1} g\textsuperscript{-1} biomass at an HRT of 8 h from sucrose fed culture. These authors reported operation below optimal conditions (HRT) led to washout of the biomass and decreased performance of the reactor. Similarly, Tawfik and Salem (2012) studied the effect of OLR on H\textsubscript{2} production using UASBR and found that at OLRs ranging from 7.1 to 21.4 g COD L\textsuperscript{-1} d\textsuperscript{-1}, the HPR increased up to 2.6 L d\textsuperscript{-1}, but then decreased with increasing the OLR. However, these results depend on the type of operation and other working parameters used for the bioreactor’s function. Studying the individual effects of each parameter give a better understanding of the reactor’s performance. Supportingly, Yu et al. (2002b) studied the impact of temperature, pH, HRT and OLR on H\textsubscript{2} production from rice winery wastewater and achieved a H\textsubscript{2} yield in the range of 1.3-2.1 mol mol\textsuperscript{-1} glucose.

The performance of the UASBR H\textsubscript{2} production system increased with granular biomass formation in the reactor (Abbasi and Abbasi, 2012). McHugh et al. (2003) proposed that the layer structure of granules was beneficial and noted their advantages in methane producing bioreactors. Jung et al. (2011b) suggested that the H\textsubscript{2} producing granules was different from methanogenic granules which have layers of H\textsubscript{2} producing and H\textsubscript{2} consuming groups. The presence of fast growing acidogens enables quicker formation of HPG. However, many studies have reported a very long lag phase in the H\textsubscript{2} production using UASBR compared with that of other reactor configurations. Strategies to overcome this problem have been proposed and they include using flocculants or application of immobilization techniques (Boonsawang et al., 2008; Wenjie et al., 2008).

According to Lee et al. (2004), using a carrier based granular sludge blanket reactor (CGSB) resulted in increased HPR ranging from 3.4-7.1 L L\textsuperscript{-1} h\textsuperscript{-1} with HRT up to 0.5 h.
This study showed that, of the four carriers tested, spherical activated carbon proved to be more efficient with granular formation occurring within 100 hours. However, the drawback in using CGSB is a decrease in the mass transfer efficiency of the substrate to biomass. In order to overcome this limitation an UASBR with a high H/D ratio was used to increase the upflow velocity or extended circulation of the liquid can be employed to enhance mixing with a high upflow velocity (Hwu et al., 1998b; Seghezzo et al., 1998).

Since a methanogenic granule is used as the seed sludge for H\textsubscript{2} production, treatment of the microbial consortia to suppress the methanogens is inevitable. Care should be taken in selecting the treatment process for start-up of the reactor. Hu and Chen (2007) tested various pretreatment methods on methanogenic granules for H\textsubscript{2} production in UASBR and found that chemical pretreatment was able to maintain the granular structure yielding high production rates of up to 11.6 L H\textsubscript{2} L\textsuperscript{-1} d\textsuperscript{-1}.

In addition to factors affecting the H\textsubscript{2} production, it is also noteworthy studies have reported that adaptation to a particular substrate can also reduce the lag phase (Liu et al., 2008b). Yusoff et al. (2009) reported that the long lag phase of reactor operation was due to non-adaptation of the culture to the feed containing low degrading substrates such as POME. However, after 4 days of adaptation to the POME containing wastewater, an increase in H\textsubscript{2} content was detected. Kim et al. (2008a) suggested that an early switch over from batch to continuous mode of operation could reduce the startup period in H\textsubscript{2} producing culture. The disadvantage associated with early startup with continuous operation would be stimulation of the growth of other non-H\textsubscript{2} producers, such as HLa and HPr producers.

### 2.9.4 Other reactor configurations

Membrane bioreactors (MBR) are the next most widely used reactor configuration in bio-H\textsubscript{2} studies due to increased retention of the biomass (Lee et al., 2010). An external cross flow type is preferred over a submerged type of MBR due to ease of operation and the ability to operate as a CSTR, but the disadvantage of using these cross flow reactors is the observed deficit in membrane permeability, which causes membrane fouling (Al-Halbouni et al., 2008). The membrane fouling mechanism proposed by Lee et al. (2008) suggests that colloidal adhesion of the membrane due to extracellular polymeric substance (EPS) production leads to a reduction in permeability. It is for this reason that
using MBR for commercial scale applications remains limited worldwide (Yang et al., 2006).

Expanded granular sludge bed reactors are a modified form of UASBR, where the limitations of the UASBR system (such as dead zones and low up-flow velocities) are overcome in the EGSBR design (Jeison and Chamy, 1999). In order to overcome these problems and increase the mass transfer efficiency between the feed and the culture as discussed earlier, a high H/D ratio with extended circulation is preferred (Lettinga, 1996). An EGSB reactor with attached growth system (granular activated carbon) showed an increased HPR of 0.71 L L\(^{-1}\) h\(^{-1}\) with EtOH type fermentation for a molasses fed reactor operating at a 2 h HRT (Guo et al., 2008b). Bio-H\(_2\) production from arabinose and glucose showed HPR ranges from 2 to 3.2 L L\(^{-1}\) d\(^{-1}\) in the EGSBR with an HRT of 24 h.

The other reactor configuration studied for bio-H\(_2\) production includes fluidized and packed bed reactors. These reactors are mostly applied to wastewater treatment facilities because of their high efficiency with high biomass content (Hickey and Owens, 1981). Attempts to study bio-H\(_2\) production in these configurations has been made but the usage of these reactor systems on a wide scale is relatively limited due to lower production rates (Barros et al., 2010; Wu et al., 2003).

### 2.9.5 Conclusion

Various studies have examined H\(_2\) production by comparing different reactor design and operation configurations. Kongjan and Angelidaki (2010) studied H\(_2\) production using different reactor configurations and achieved a maximum H\(_2\) yield of 212 ml g\(^{-1}\) COD corresponding to a HPR of 0.82 L L\(^{-1}\) d\(^{-1}\) in UASBR. Similarly, Gavala et al. (2006) studied bio-H\(_2\) production in suspended and granular systems and reported that the UASBR system was more stable with significantly increased HPR compared to that of CSTR. Many studies of reactor configuration for bio-H\(_2\) production has recommended granular or immobilized systems because they offer better HPR which can handle feeds containing elevated substrate levels (Jung et al., 2011a; Show et al., 2008). Achieving high HPR from these reactor systems will be a challenge when using different sources of waste material.
2.10 Microbial techniques for characterizing the mixed cultures

Hydrogen production is characterized by the presence of the H$_2$ forming micro-organisms present in the microflora (Wirth et al., 2012). Therefore, establishing an enriched H$_2$ producing microflora for sustainable bio-H$_2$ production is of great importance (Koskinen et al., 2007). An understanding of the microbial composition of the heterogenous microflora present in mixed anaerobic communities will assist in optimizing the parameters for stable operation of the bioreactor.

Numerous methods based on molecular biology have been employed in characterizing these microorganisms. Most of the molecular biology methods are based on nucleic acid based assays, which are employed to examine the diversity of the microbial community (Zoetendal et al., 2004). The identification of species present in the microflora will assist us in understanding the metabolic activities associated with the microflora, including the characteristics of the identified microflora under different operational conditions and interactions between different groups of micro-organisms in mixed communities.

All molecular biology methods begin with extraction nucleic acid from microbial samples. These molecular techniques involve cell lysis, contaminant removal, solvent extraction, precipitation and purification (Miller et al., 1999). The extracted DNA is subjected to polymerase chain reaction (PCR) amplification using primers which are designed based on either the relative DNA sequences or adapted from published findings according to the source of culture been used and target of interest (i.e., targeting specific group of microbial population). The amplified DNA is then cloned and sequenced to identify the species present in the microflora. The most commonly used gene sequence is the 16S rRNA gene (16S RDNA). The 16S rRNA gene has a huge database of over 3 million sequences available at GenBank (RDP, http://rdp.cme.msu.edu/). For this reason, the 16S rRNA gene-based technique is widely used for monitoring changes in microbial communities under different conditions.

Several studies have described molecular techniques used for characterizing H$_2$ producing cultures and presented their advantages and disadvantages (Li et al., 2011; Nocker et al., 2007). The most widely used fingerprint techniques for identifying the diversity profiles of the microflora are denaturing gradient gel electrophoresis (DGGE)
and terminal restriction fragment length polymorphism (T-RFLP). T-RFLP is preferred for comparing complex communities when high throughput and high sensitivity are required. In comparison, the DGGE method is widely used because of visualization, ease of sequencing of DGGE bands and its affordability. In addition, the separate bands may be isolated and sequenced to identify a specific species. The disadvantages of DGGE includes less sensitivity, long time to conduct the analysis (involving many intermediate steps), highly diversified communities are not easily identified and the method produces less resolved bands in samples containing small quantities of biomass. Therefore, T-RFLP is preferred for characterization of complex mixed microbial system.

The T-RFLP method is a high throughput community profiling technique with high reproducibility in terms of both qualitative and quantitative analysis of the microbial genome. The other main advantage of the T-RFLP method is that it can be standardized and used to compare data published by other researchers. The phylogenetic information (i.e. taxonomic identification) can be inferred from the T-RFs sizes by comparing them with sequences of known bacteria from standard databases available such as T-Align, PAT, MiCA, TRFMA etc. A background of T-RFLP used in the current research work is described in section 2.10.1.

2.10.1 Terminal restriction fragment length polymorphism

Terminal restriction fragment length polymorphism (T-RFLP) is a fingerprint technique used to identify the composition of bacterial communities through the use of restriction enzymes. Moeseneder et al. (1999) studied optimization of the T-RFLP method and compared that to DGGE. These authors observed that results obtained from T-RFLP had better or similar outcomes in comparison to the DGGE. In T-RFLP, the PCR amplification is carried out by labeling one end (5’end) of the primer with fluorescence to amplify the targeted region of the 16S rRNA gene. The PCR amplified product is then treated with restriction endonuclease which generates fragments of different sizes based on the specificity of the restriction enzyme used. The terminal restriction fragments (TRFs) generated by the restriction enzymes are used for both qualitative and quantitative analyses of the microbial diversity of the cultures (Liu et al., 1997). A schematic representation of the T-RFLP technique is shown in Figure 2.7.
Figure 2.7 Schematic representation of the steps involved in T-RFLP analysis

In T-RFLP analysis, only the labeled end fragment is detected and this makes for an easier analysis of a complex microbial communities. Each labeled end fragment refers to a single operational taxonomic unit (OTU) present in mixed microbial cultures with a restriction site at the same location (Avaniss-Aghajani et al., 1994). Thus, the pattern of the fragments depicted in the T-RFLP profile represents the number of taxonomical units present in the microbial population.

Advantages of T-RFLP, which include higher resolution than other molecular techniques involving gel electrophoresis that use capillary electrophoresis has been outlined by Marsh (1999). Marsh (1999) also reported that the output of T-RFLP (a profile comprised of digital data) can be used readily in statistical analyses by converting the information to numerical data based on the size of the fragments obtained from T-RFLP. Other advantages of using T-RFLP in the analysis of mixed microbial communities include the capability to identify rare species within the population and the phylogenetic information that can be obtained from the size of the restriction fragments that were generated. The sizes of the terminal restriction fragments of the known bacteria can be obtained from databases, such as those maintained by T-align, TRFMA, and TAP as discussed previously (Li et al., 2011).
Limitations of using T-RFLP  Include the primers and salts must be removed from the PCR-products using clean up systems prior to analysis because the presence of these charged molecules can be misleading and bias the selective detection of charged molecules (Hoshino et al., 2006; Osborne et al., 2005). Thus, the assessment of phylogenetic information obtained from the T-RFLP profile of the diversity of the microbial community becomes difficult (Nocker et al., 2007). Furthermore, using a single restriction enzyme for the analysis of a complex microbial community may reduce and over-simplify the data set leading to errors, therefore, using more restriction enzymes to obtain a diverse dataset is preferred.

Nevertheless, T-RFLP has been used widely for evaluating and identifying the dynamics and variability of mixed microbial communities present in H₂ producing systems because of the reproducible characterization of the microbial cultures (Castello et al., 2009; Ueno et al., 2006). Chaganti et al. (2012b) conducted analyses of mixed anaerobic communities using clone library sequencing and T-RFLP and found that the T-RFLP technique (applied to three different sources of H₂ producing mixed microbial culture to assess variation in the samples) produced findings that were reproducible. Hartmann et al. (2005) conducted studies using T-RFLP and ribosomal intergenic spacer analysis (RISA) and revealed that although T-RFLP is in principle more demanding, this technique offers the benefit of phylogenetic information about microorganisms detected in the soil sample.

2.1.1 Statistical methods used in analysis of biohydrogen fermentation

Experimental design is important in analyses of fermentative H₂ production from mixed anaerobic communities because of the influence of more than one factor. Both individual parameters and interactions between parameters acting in combination affect H₂ production (Li and Fang, 2007; Nath and Das, 2011). Studying the effect of experimental factors on biological H₂ fermentation one factor at a time or in combination is essential in developing a sustainable H₂ production process.

Examination of one factor at a time assists in understanding the effect of each operational parameter on H₂ production. This method of experimental reduction has been widely applied because this degree of control is relatively easy to implement, analysis of the results is straightforward and does not require complex statistical methods.
Chittibabu et al. (2006) and Ferchichi et al. (2005b) have examined the effects of various operating parameters (such as components of the medium, inoculum size, pH, agitation speed, substrate concentration, FeSO$_4$ and yeast concentration, dilution rate, and temperature) on H$_2$ production by adjusting one factor at a time while maintaining other factors constant. This approach is an important preliminary step, but has the disadvantage of neglecting the effects of interactions between the parameters. For example, Antonopoulou et al. (2011) examined the effect of substrate concentration on H$_2$ production, but ignored the individual effects of HRT and pH and their interaction with substrate concentration on the H$_2$ production. In order to optimize the performance of a H$_2$ production method for broader application, such as large-scale commercial bio-fuel production, the impact of adjusting multiple parameters needs to be analyzed, and this requires more complex statistical procedures.

A factorial design, which includes more than one variable applied at more than two levels, is preferred. The factorial design might be a full factorial analysis, in which each possible combination of factors is tested at each level of every factor. The number of runs in a full factorial design is $a^n$, where ‘a’ is the number of levels tested for each factor and ‘n’ represents the number of factors. A full factorial design is generally avoided because of the large number of experiments required in the design. Increasing the number of factors adjusted and increasing the number of levels tested makes this approach unrealistic and inefficient (in terms of cost, time, labour, and usefulness of all elements in the large dataset) in comparison a fractional factorial design, which comprises only a fraction of the operating conditions tested in a full factorial design (Lazic, 2004). The most widely studied fractional factorial designs include the Plackett-Burman design, central composite design, Box-Behnken design and Taguchi design (Jo et al., 2008a; Pan et al., 2008; Wang and Wan, 2009b; Wang et al., 2013). Among the research designs listed above, the Box-Behnken design (a 3-level fractional factorial design) formulated by Box and Behnken (1960) has been used in H$_2$ production studies because the spherical design involving 3 levels of each factor is rotatable or nearly rotatable.

The experimental data obtained from H$_2$ fermentation studies need to be analyzed carefully for a proper understanding of the outcomes. Since there are several variables studied in each experiment generating huge dataset as an outcome, a statistical approach
to the analysis of the copious dataset is needed. A statistical approach allows inferences to be drawn about the impact of individual parameters (independent variables) as well as their interactions on the experimental outcomes (dependent response variables), which may improve an understanding of the relative importance of these variables in H₂ production and may elucidate how the outcome from adjusting or optimizing various operating parameters relates to the response variable of interest (H₂ production).

Carpi and Egger (2011) outlined the importance of statistical analysis in scientific research and described two types of statistical analyses which includes descriptive statistics and inferential statistics. Descriptive statistics summarize the major attributes of the dataset, such as providing an assessment of the average response (e.g. mean) and an assessment of the amount of variation around the mean response (e.g. standard error of the mean or standard deviation), thus providing a general characterization of the group’s response. For example, HPR at different HRTs can be represented by the calculated average response and standard deviation at each of the tested HRTs. The results of the statistical analysis can be plotted to illustrate not only any trends in the response (HPR) to various HRTs, but also the degree of variation in the responses obtained for each HRT (Thanwised et al., 2012).

The second type of statistical analysis includes inferential statistical methods, which are used to identify the relationships between variables in the dataset in order to make inferences about the representativeness of the small experimental dataset (i.e., whether or not the findings can be generalized beyond the experimental conditions to the world at large). For example, the experimental data from a pilot study may be described in the form of a graph or table (descriptive statistics). Inferential statistics determine whether differences in the responses obtained for different treatments or levels of treatment are meaningful (i.e., statistically significant) or due to chance alone and also facilitate in characterizing the data sets in the groups.

Significance is expressed as the level of confidence, normally specified as a 95% confidence interval. The term ‘statistically significant at a 95% confidence interval’ refers to the probability that the differences observed when comparing the results of different operating conditions or treatments are unlikely due to chance alone (Carpi and Egger, 2011). The test of statistical significance is related to the p-value indicating the
probability that the event in the data set was observed by chance. This means repeating
the same experiment/treatment condition similar difference in the treatment conditions
applied would be observed. For example, Cubillos et al. (2010) studied the effect of
simultaneous adjustments to pH and substrate concentration on H₂ production, and
concluded that there was no significant difference in the degradation rates within the pH
range tested. The observed differences in the H₂ yield and glucose degradation were
statistically insignificant because the probability that apparent differences were due to
chance was greater than 5% (p> 0.05). A p-value greater than 0.05 indicates that there is
no relationship between the initial pH and glucose degradation. This statistical test is
conducted using Tukey’s test used to compare the statistical difference between multiple
means and determine whether they are significant or due to chance (Box et al., 1978). A
variety of inferential statistical methods have been developed for application to a wide
variety of research designs in order to elucidate the differences that exist and to determine
whether apparent differences between datasets are ‘real’ or due to chance.

A multivariate analysis is required to understand the relationships between several
process variables (i.e., external variables such as culture source, pH, inhibitor
concentration, substrate loading and HRT or internal variables such as soluble
metabolites and gas products) and their impact on the response variables. A principal
component analysis (PCA) is one such multivariate statistical tool used in the analysis of
large data sets. PCA presents results in a reduced form, as a visual plot on a two-
dimensional plane that presents the relationships between underlying components and the
response variables analysed In addition, PCA also highlights the differences and
similarities found within the dataset (Wise and Gallagher, 1996).

Abreu et al. (2009) studied the effect of inoculum type at different pH levels on bio-
H₂ production from arabinose. The study used 4 different seed cultures with 8 pH
conditions for each source of inoculum tested, resulting in 32 samples. Each sample
included measures for 13 response variables, such VFAs, gas products and alcohols. A
PCA was used by the authors to visualize the main differences between the 4 culture
sources and how they varied from each other.

Similarly the genomic data obtained from microbial characterization using molecular
biology techniques (such as T-RFLP and DGGE) are analyzed using statistical tools to
identify changes in the structure and composition of the bacterial community due to different operational or treatment conditions employed to enhance the HPR. PCA and multidimensional scaling (MDS) are widely used methods to visualize similarities and differences within complex microbial communities (Schutte et al., 2008). Schutte et al. (2008) also describes how these statistical tools are employed in the analysis of the microbial dataset. PCA transforms the correlated variables to discrete variables designated principal components thus, reducing the dimensions of a complex dataset. The first principal component (PC 1) is an underlying factor that may contribute to the effects of several parameters and accounts for the largest amount of variability in the dataset. The succeeding principle components account for the remaining variability in the data set. PCA uses a linear combinations of variables to form the components which attempt to capture the maximum possible variation found in the original dataset (Johnson, 1998).

Multidimensional scaling methods are classified as metric or non-metric. A metric MDS known as principal coordinate analysis (PCoA) is often preferred because the distinction between the original profiles (i.e., distance) is reflected with maximum accuracy. PCoA assesses the ‘goodness of fit’, which reflects how well the plot represents the actual dataset in that plane, which is more powerful technique than the PCA (Chae and Warde, 1987). An additional advantage of PCoA is that a pairwise distance measure is calculated for all of the profiles, whereas with non-metric MDS, the distance measures are based on the rank ordering of the distance between the profiles.

Another statistical method used widely is multivariate cluster analysis (MCA). MCA aims at minimizing variance within the group and also maximizing differences between the groups. This approach assists in grouping the samples to well defined categories grouped into few rows (clades) (James and McCulloch, 1990). In cluster analysis, the relevant similarity or dissimilarity measure of association is selected first to derive the association coefficient, followed which the calculated association matrix is represented as the horizontal tree (hierarchical clustering) or as objects of distinct groups (k-means clustering).

Further identification of the similarities, grouping pattern and linkage between the samples, correlation of the species abundance at each condition with the environmental factors associated with them would be of good understanding of the data set. A canonical
correspondence analysis (CCA) is used to identify the association of the environmental species along with changes in the environmental gradient. Using CCA is practical and has been a method of choice by ecologists (Terbraak, 1986). For example, CCA has been used for understanding the effect of environmental factors on the bacterial community composition from 30 different lakes in Wisconsin, showing patterns in the distribution of the biological species associated with eleven different factors such as pH, water clarity, regional- and landscape-level factor (Yannarell and Triplett, 2005). A similar analysis in H$_2$ producing environments may assist in understanding species association with the operating conditions. Thus, conditions favouring H$_2$ producing bacteria showing high H$_2$ yields may be used in the reactor operation.

2.12 Current status of biohydrogen production research

2.12.1 Hydrogen production from lignocellulosic biomass via dark fermentation

Various studies have reported the use of lignocellulosic biomasses for bio-H$_2$ production. Kongjan et al. (2010) reported that H$_2$ can be produced as the major product (with respect to gas composition) of fermentation from hemicelluloses rich in hydrolysate in batch and continuous operation systems under thermophilic conditions. de Vrije et al. (2009) studied the effective synchronized utilization of saccharides from the hydrolysate in lignocellulosic feedstock (Miscantahus) for efficient H$_2$ production by thermophilic bacteria.

The processes involved can feature a single stage or involve a two-stage process. In the single stage process, the direct fermentation of cellulosic biomass has been reported with both pure cultures where thermophilic conditions need to be maintained (Carere et al., 2008). In the co-cultivation of pure cultures, the effective degradation of cellulose, H$_2$ production and control of consumption by non-H$_2$ producers was reported as difficult (Saratale et al., 2008). The maximum yield obtained by Liu et al. (2003) was 102 ml g$^{-1}$ cellulose and the maximum specific HPR was 287 ml d$^{-1}$ g$^{-1}$ VSS at an optimum pH of 6.5. Taguchi et al. (1996) worked with a two-stage process, in which the first stage involved hydrolysis of the cellulosic feedstock and the second stage involved formation of H$_2$ from the hydrolyzed sample by Clostridium sp. Bao et al. (2013) reported that the pretreatment of substrate in the first stage improved the H$_2$ production and increased H$_2$ yield by 80% (approximately). The problem in using raw lignocellulosic biomass as a
substrate would be a longer fermentation time and relatively low yields. For example, Monlau et al. (2012) studied H₂ and CH₄ production using 20 different lignocellulosic substrates in batch reactors for a period of 5 and 40 days, respectively. The study concluded that H₂ production was majorily attributed to the soluble sugars and carbohydrates from the substrate and the average H₂ yield obtained by the authors varied from 2 to 120 mL g⁻¹ TVS, while the presence of lignin, crystalline and amorphous cellulose, proteins had negative or no impact on H₂ production. In cases were raw substrates (i.e. without pretreatment) were used but were reported with high H₂ yields, a genetically modified system (microbe) were used for enhancing H₂ production from the lignocellulosic biomass (Cha et al., 2013). However, feasibility of cultivating these genetically engineered microbe at a larger scale is still unknown. In addition, there is ongoing research in the field of metabolic engineering of microorganisms exploring methods for achieving higher H₂ yields from dark fermentation. These studies explore the potential of shunting the metabolic pathway through other products (Rachman et al., 1997; Rachman et al., 1998). Vardar-Schara et al. (2008) suggested that instead of deleting known pathways, elucidation of the unknown metabolic pathways by application of metagenomics and random chemical mutagenesis followed by DNA microarray is of great importance. These authors also stated that application of metabolic engineering is important; however, further research and development in this field is necessary for establishing co-cultivation of genetically engineered strain with the mixed population or achieving high cell densities with defined mixed cultures i.e., combinations of two or more pure strains. In addition, to date there is no published reports in assessing the feasibility of co-cultures with use of metabolic engineering tools on a larger scale for H₂ production.

Hence, using reactor based methods to improve the H₂ production is of great significance. Varying the parameters responsible for H₂ production and pretreatment of the inoculum is one such method to improve the H₂ yield and HPR. Table 2.5 shows the H₂ production performance of different reactor systems under different operational conditions with different substrate sources.
<table>
<thead>
<tr>
<th>Seed Culture*</th>
<th>Substrate</th>
<th>Working volume (L)</th>
<th>pH</th>
<th>HRT (h)</th>
<th>Substrate concentration or OLR</th>
<th>Culture pretreatment</th>
<th>$\text{H}_2$ production rate</th>
<th>Max-$\text{H}_2$ yield</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Batch Studies</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anaerobic digester sludge</td>
<td>Glucose</td>
<td>0.14</td>
<td>7.0</td>
<td>NA</td>
<td>10.0 g L$^{-1}$</td>
<td>Heat</td>
<td>0.06 L L$^{-1}$ h$^{-1}$</td>
<td>2.18 mol mol$^{-1}$ glucose</td>
<td>(Baghchehsaraee et al., 2010)</td>
</tr>
<tr>
<td>Rice-rhizosphere microflora</td>
<td>Apple pomace</td>
<td>0.60</td>
<td>6.0</td>
<td>NA</td>
<td>73.4 g L$^{-1}$</td>
<td>Nil</td>
<td>0.11 L L$^{-1}$ h$^{-1}$</td>
<td>2.28 mol mol$^{-1}$ glucose</td>
<td>(Doi et al., 2010)</td>
</tr>
<tr>
<td>Swine wastewater treatment sludge</td>
<td>Sucrose</td>
<td>1.50</td>
<td>5.5</td>
<td>NA</td>
<td>1184 mg L$^{-1}$</td>
<td>Heat</td>
<td>3201.6 $\mu$ mol H$_2$ g$^{-1}$ vss h$^{-1}$</td>
<td>1.6 mol mol$^{-1}$ sucrose</td>
<td>(Maintinguer et al., 2008)</td>
</tr>
<tr>
<td>Mixed anaerobic culture</td>
<td>Maize silage</td>
<td>1.10</td>
<td>5.5</td>
<td>NA</td>
<td>200 g maize silage</td>
<td>Nil</td>
<td>Nil</td>
<td>7.65 mL g$^{-1}$ dry biomass</td>
<td>(Sträuber et al., 2012)</td>
</tr>
<tr>
<td>Mixed anaerobic culture</td>
<td>Cornstalk (CS)</td>
<td>0.10</td>
<td>7.0</td>
<td>NA</td>
<td>10.0 g L$^{-1}$</td>
<td>Heat</td>
<td>11.3 ml g$^{-1}$ CS h$^{-1}$</td>
<td>141.21 ml g$^{-1}$ dry biomass</td>
<td>(Ma et al., 2011)</td>
</tr>
<tr>
<td>Mixed anaerobic culture</td>
<td>Glucose</td>
<td>0.05</td>
<td>5.0</td>
<td>NA</td>
<td>5000 mg L$^{-1}$</td>
<td>Linoleic acid</td>
<td>0.02 L L$^{-1}$ h$^{-1}$</td>
<td>3.11 mol mol$^{-1}$ glucose</td>
<td>(Saady et al., 2012a)</td>
</tr>
<tr>
<td><strong>Semi-Continuous reactors</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anaerobic sludge (AnSBR)</td>
<td>Food waste</td>
<td>5.0</td>
<td>5.3</td>
<td>72.0</td>
<td>0.8 gVS L$^{-1}$</td>
<td>Heat</td>
<td>0.02 L L$^{-1}$ h$^{-1}$</td>
<td>-</td>
<td>(Jo et al., 2007)</td>
</tr>
</tbody>
</table>

*Table 2.5 Hydrogen production performance using different reactor systems via dark fermentation*
| Anaerobic seed sludge (AnSBR) | Sweet sorghum | 1.0 | 5.0 | 12.0 | 50 g sugar L⁻¹ d⁻¹ | Heat | 350 mmol L⁻¹ d⁻¹ | 0.6 mol mol⁻¹ glucose | (Saraphirom and Reungsang, 2011) |
| Mixed microbial communities (AnSBR) | Synthetic wastewater | 1.9 | 5.7 | 8.0 | 75 g COD L⁻¹ d⁻¹ | Acidification for 48 h at pH 3.0 | 0.19-0.23 L L⁻¹ h⁻¹ | 60-74 ml g⁻¹ COD | (Cheong et al., 2007) |
| Municipal wastewater (AnSBR) | Glucose | 3.0 | 5.0 | 72.0 | 5.0 g L⁻¹ d⁻¹ | Nil | 0.32 L L⁻¹ h⁻¹ | 180 mL g⁻¹ glucose | (Hwang et al., 2004) |
| Mixed anaerobe (ASBr) | Glucose + Xylose | 6.0 | 5.5 | 40.8 | 3.0 g L⁻¹ d⁻¹ | Linoleic acid | 0.04 L L⁻¹ h⁻¹ | 2.28 mol mol⁻¹ glucose | (Chaganti et al., 2013) |
| Anaerobic digestates (SCR) | Municipal solid waste | 1.0 | 6.4 | 21** | 11 g VS kg⁻¹ swmr d⁻¹ | Nil | 2800 NmL Kg⁻¹ d⁻¹ | 360 NmL H₂ g⁻¹ VSrem | (Valdez-Vazquez et al., 2005a) |
| WWTP (SCR) | Organic biowaste | 200 | 5.4 | 79.2 | 16.5 Kg TVS m⁻³ d⁻¹ | Nil | - | 36 L Kg⁻¹ VS added | (Cavinato et al., 2011) |
| Mixed sludge from H₂ fermentor, wastewater and compost | Starch-rich kitchen waste | 3.0 | 4.5 | 96.0 | 39 g COD L⁻¹ d⁻¹ | Nil | 0.09 L L⁻¹ h⁻¹ | 2.1 mmol H₂ g⁻¹ COD | (Wang et al., 2010) |

**Continuous reactors**

<p>| Swine wastewater (FBR) | Synthetic wastewater | 4.0 | 5.5 | 2.0 | 4 g L⁻¹ of glucose | Heat | 1.0 L L⁻¹ h⁻¹ | 2.52 mol mol⁻¹ glucose | (Barros et al., 2010) |
| MSTP (PBR) | Sucrose | 3.0 | 6.7 | 1.0 | 20 g COD L⁻¹ | Thermal | 1.2 L L⁻¹ h⁻¹ | 0.09 L g⁻¹ biomass | (Chang et al., 2002) |</p>
<table>
<thead>
<tr>
<th>Source</th>
<th>Reactor Type</th>
<th>COD (g L⁻¹)</th>
<th>pH</th>
<th>Mixed Liquor (d⁻¹)</th>
<th>Enrichment Method</th>
<th>VS Added</th>
<th>L V (g⁻¹)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Digested cow manure (CSTR)</td>
<td>Distillery wastewater</td>
<td>1.0</td>
<td>7.0</td>
<td>96</td>
<td>Enriched from batch studies</td>
<td>0.63 L d⁻¹</td>
<td>172.0 mL g⁻¹ VS added</td>
<td>(Qiu et al., 2011)</td>
</tr>
<tr>
<td>Hydrogenogenic culture (CSTR)</td>
<td>Wheat straw hydrolysate</td>
<td>1.0</td>
<td>5.2</td>
<td>72</td>
<td>20% v/v</td>
<td>0.2 L L⁻¹ d⁻¹</td>
<td>178 mL H₂ g⁻¹ sugar</td>
<td>(Kongjan et al., 2010)</td>
</tr>
<tr>
<td>Anaerobic digester (UASBR)</td>
<td>Glucose</td>
<td>5.0</td>
<td>6.5</td>
<td>12.0</td>
<td>10.0 g L⁻¹</td>
<td>3.42 L L⁻¹ d⁻¹</td>
<td>1.51 mol mol⁻¹ glucose</td>
<td>(Liu et al., 2012)</td>
</tr>
<tr>
<td>Kitchen waste composts (UASBR)</td>
<td>Chesse whey</td>
<td>4.6</td>
<td>5.0</td>
<td>24.0</td>
<td>Nil</td>
<td>1 L L⁻¹ d⁻¹</td>
<td>1.5 mol mol⁻¹ lactose²</td>
<td>(Castello et al., 2011)</td>
</tr>
</tbody>
</table>

SCR: semi continuous reactors; AnSBR: anaerobic sequential batch reactor; wmr: wet mass reactor; FBR: Fluidized bed reactor; PBR: Packed bed reactor; CSTR: continuous stirred tank reactor; UASBR: up-flow anaerobic sludge blanket reactor; WWTP: wastewater treatment plant; MSTP: municipal sewage treatment plant; NmL: Milliliters at 0 °C and 1 atm

* The letters in brackets represent the type of reactor used or mode of operation
** mass retention time (days)
° intermittent operation
² observed from the data presented in the figure / graph (approximately)
2.12.2 International status of biohydrogen production research

The potential of bio-H₂ production is widely known. At the 18th World Hydrogen Energy Conference in 2010, the major focus was on radical research and development of H₂ and fuel cell technologies. Researchers in the Netherlands and North America are currently working on the application of thermophilic and mesophilic bacteria for H₂ production in projects supported by their respective governments. The feedstock would consist primarily of biomass waste from molasses or potato processing industries, organic waste from the brewing industry and municipal solid waste. The use of energy crops such as SWG and CS has been mainly concentrated in North America due to their low cost and availability as a carbon source. In Japan and Korea, bio-H₂ fermentation is mainly focused on feedstock such as rice husks, wheat husks and food waste. Related research is underway globally, for example, studies using rice winery wastewater in China and paper sludge hydrolysate in Hungary are in progress. In the Netherlands present focus of this field is on the improvement of the combined two-stage H₂ and CH₄ fermentation systems.

2.12.3 Conclusion

Although research into H₂ production as an alternative source of energy for fuel and power seems to be more focused at the present time, methods to produce biofuels in an environmentally friendly manner and on a commercial scale are still not effective. Many research groups have focused their attention over the past decade on dark-fermentative H₂ production (DFHP) because of its potential for sustainable production from waste products (second and third generation feedstocks). Reports by these researchers have revealed that commercial DFHP will not be realistic in the near future. Further research involving pilot-scale or bench-scale studies of continuous mode systems are required with the objective of increasing both yields and rates of fermentative H₂ production. This includes evaluating the feasibility of processes using low value biomass over a long period and establishing a stable community of microflora for sustainable H₂ production. Well-designed research applications to study process variables and microflora involved in continuous H₂ production with large-scale reactor systems such as UASBR will play a major role in the development and expansion of large-scale bio-H₂ production systems.
The points mentioned above are emphasized in the present study, in anticipation that the results of research in this area will benefit the development of current understanding of DFHP.

2.13 References


CHAPTER 3: MATERIALS AND METHODS

3.1 Feedstock procurement and preparation

Pure sugars (glucose) and complex sugars from lignocellulosic material such as switchgrass (SWG) and corn stover (CS) were utilized as feedstocks. SWG was obtained from a grower in Leamington, ON. Corn stover was procured from a farmer (Windsor, ON) and from a pet store in the form of pellets (Pestells, Wilmot, ON). The raw material obtained from the farm was air-dried at room temperature to an equilibrium moisture content of < 3%, milled using a laboratory shredder (Retsch GmbH, Germany) to a particle size less than 5 mm by 2 mm for SWG and 3 mm dia for CS. The shredded biomass was homogenized and stored at 4°C until further use. For experiments involving CS, pellets from both sources was used on 1:1 weight basis for the pretreatment process.

3.2 Pretreatment of lignocellulosic biomass

3.2.1 Steam-explosion

Pretreatment of the SWG and CS was performed in a 4-L steam explosion reactor. The Zipperclave® stirred reactor (Autoclave Engineers, Erie, PA) was equipped with a 4-L reaction vessel (316SS & HASTELLOY®), a 3,300 RPM MagneDrive® magnetic mixer and a heating jacket designed to reach high temperature and pressure of 232 °C and 20.7 MPa, respectively. Initially, the biomass (shredded SWG or CS) was soaked with 1% wt/wt of H₂SO₄ for a 12 h period. The reactor was then charged with the biomass containing acid solution to a final biomass to water ratio of 1:10 (wt/wt). The contents of the reactor were allowed to mix thoroughly with a stirrer operating at 815 rpm. The reactor was heated to 190 °C for 10 min and then the reactor was rapidly cooled and depressurized by releasing the pressure valves (i.e., steam exploded). The exploded material was filtered and the pH of the hydrolysate was adjusted to neutral pH using 10 M NaOH. The hydrolysate was filtered using a 0.45 µm glass microfiber filter and stored at 4 °C. The reducing sugar content of the hydrolysate was analyzed by chemical methods, and the individual sugar composition (xylose, glucose, arabinose, mannose and galactose) was estimated by analytical methods.
3.2.2 Resin treatment of liquid hydrolysate

The filtered hydrolysate was treated with a resin (Amberlite polymeric adsorbent XAD-4 resin (Rohm and Haas (Philadelphia, PA))) to remove furans (furfural and 5-hydroxymethyl furfural (HMF)), phenols, and acetic acid. Removal of fermentation inhibitors was accomplished by adding 500 mL of the XAD-4 resin to a 5 L of hydrolyzed liquor with a contact time of 4 to 6 h. The resin treated liquor was filtered through a 0.45 µm glass microfiber filter under vacuum conditions and the solution was stored at 4 °C. The stored resin treated liquor was filtered before feeding to the culture. The water soluble extract (hydrolysate) was analyzed for sugars, acetic acid and sugar degradation products (i.e., furfural and HMF) before and after resin treatment.

Regenerating the resin (desorption of the furan and phenolic compounds) was accomplished by treating 500 mL resin with 1L 10 to 20 % H₂O₂, followed by adding 1L 1% NaOH and 1L 1% HCl for a minimum duration of 8 h for each chemical treatment at room temperature. The resin was washed with a copious amount of distilled water followed by Milli-Q (MQ) water after each treatment [MQ: water that was passed three to four times through a NANO pure water purification system (Millipore, Barnstead, USA; 18.0 ± 0.1 MΩ-cm)].

3.3 Culture source

The cultures were obtained from the following two industrial wastewater treatment facilities: 1) an upflow anaerobic sludge blanket reactor (UASBR) at a baby food processing wastewater treatment facility (Cornwall, Ontario) (designated as culture A) and 2) an UASBR treating effluent from a brewery (Guelph, Ontario) (designated culture B). The cultures were stored at 4 °C and fed glucose (1 g L⁻¹) every 30 days. The volatile suspended solids to total suspended- solids ratio (VSS/TSS) (APHA, 1999) and the volatile fatty acids (VFAs) concentration were used to characterize the cultures prior to seeding the reactors. The cultures VSS levels maintained for studies in batch reactors were 10 g VSS L⁻¹ and for studies in the continuous reactors the level was 30 g VSS L⁻¹. During culture acclimation, the reactors (total volume 10 L, working volume 8.5 L) were operated in a sequential batch mode with a 14 d HRT. The fill time, settling time, decant time and reaction time for each cycle in sequencing batch mode is as follows: 0.03 d,
0.45 d, 0.02 d and 6.5 d and the volume decanted per cycle was kept constant at 4.25 L and fed 5 g L\(^{-1}\) of glucose or a mixture of glucose and xylose (1:1). Prior to conducting studies with lignocellulosic hydrolysate, the cultures were acclimated to the 1:1 ratio glucose and xylose mixture. The pH of the reactors was in the range of 6 to 7.8 over the 7 day period. The procedure followed for reactor maintenance was adapted from Ray et al. (2008).

### 3.4 Batch studies

All the batch studies were conducted using 160 mL serum bottles with a 50 mL working volume containing basal medium and culture of 2000 mg VSS L\(^{-1}\). The composition of the basal medium was adapted from Wiegant and Lettinga (1985) (see Table 3.1). The batch reactors were prepared in an anaerobic glove box with an atmosphere containing approximately 76% N\(_2\), 20% CO\(_2\) and 4% H\(_2\). The culture pretreatment was accomplished using linoleic acid (LA). The amount of LA injected into the bottles varied as per the experimental conditions described in the experimental design (Chapter 6). The initial pH was adjusted inside the glove box according to the pH levels described in the experimental design using 1 M HCl or 1 M NaOH. The bottles were sealed with Teflon\textsuperscript{®} lined silicone rubber septa and aluminum crimp caps. Prior to removing the bottles from the anaerobic chamber, 20 mL of the gas mixture was injected into the headspace to avoid the formation of a negative pressure during liquid and gas sampling. The reactors were maintained at 37 \(\degree\)C, an initial pH of 5.5 and mixed using an shaker (Lab line instruments, Model 3520) at 200 rpm for 24 hours prior to adding the substrate. The total liquid volume (50 mL) in each reactor was maintained by removing a volume of liquid equivalent to the total volume of the substrate stock solutions which were added. Gas and liquid metabolites were analyzed on a daily basis.

A stock solution of LA (95% purity; 50 g L\(^{-1}\)) (99.9% purity) (Sigma Aldrich, ON) was prepared in accordance with the methods described by Rinzema et al. (1994). The procedural details are outlined in section 3.8.6. The stock solution containing furan derivatives (furfural and HMF; 50 g L\(^{-1}\)) and the stock solution of glucose (100 g L\(^{-1}\)) (Spectrum Chemicals, CA) were prepared with Milli Q water.
### Table 3.1 Composition of basal medium

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration (mg L(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nutrient composition</strong></td>
<td></td>
</tr>
<tr>
<td>Sodium bicarbonate (NaHCO(_3))</td>
<td>6000</td>
</tr>
<tr>
<td>Ammonium bicarbonate (NH(_4)CO(_3))</td>
<td>70</td>
</tr>
<tr>
<td>Potassium chloride (KCl)</td>
<td>25</td>
</tr>
<tr>
<td>DI-potassium hydrogen phosphate (K(_2)HPO(_4))</td>
<td>14</td>
</tr>
<tr>
<td>Ammonium sulfate ((NH(_4))(_2).SO(_4))</td>
<td>10</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>10</td>
</tr>
<tr>
<td>Magnesium chloride (MgCl(_2).4H(_2)O)</td>
<td>9</td>
</tr>
<tr>
<td><strong>Resazurin (Indicator)</strong></td>
<td>1</td>
</tr>
<tr>
<td><strong>Trace element</strong></td>
<td></td>
</tr>
<tr>
<td>Ferrous chloride (FeCl(_2).4H(_2)O)</td>
<td>25</td>
</tr>
<tr>
<td>Ethylenediaminetetraacetic acid (EDTA)</td>
<td>1</td>
</tr>
<tr>
<td>Manganese chloride (MnCl(_2).4H(_2)O)</td>
<td>0.5</td>
</tr>
<tr>
<td>Cobalt chloride (CoCl(_2).4H(_2)O)</td>
<td>0.15</td>
</tr>
<tr>
<td>Ammonium molybdate (NH(_4))(_6)MnO(_7).4H(_2)O</td>
<td>0.09</td>
</tr>
<tr>
<td>Zinc chloride (ZnCl(_2))</td>
<td>0.05</td>
</tr>
<tr>
<td>Boric acid (H(_3)BO(_3))</td>
<td>0.05</td>
</tr>
<tr>
<td>Nickel(II) chloride (NiCl(_2).6H(_2)O)</td>
<td>0.05</td>
</tr>
<tr>
<td>Cupric chloride (CuCl(_2).4H(_2)O)</td>
<td>0.03</td>
</tr>
</tbody>
</table>

#### 3.5 Continuous reactor studies

An illustrative schematic diagram of the UASBR used in this research is provided in Figure 3.1. A granular culture (source B, see section 3.3) was used to seed the reactor (except for the study described in chapter 4 in which both granulated and flocculated cultures were used. The volatile suspended solids (VSS) and total suspended solids (TSS) were approximately 10 g L\(^{-1}\) and 12.5 g L\(^{-1}\), respectively, at start of the experiments (except chapter 5 and 9, where the VSS concentration used up was 12 g L\(^{-1}\)). After seeding a reactor with culture, in order to maintain the anaerobic conditions the reactors were sparged with nitrogen (99.99% purity, Praxair, ON) for approximately 10 min. The reactors were first operated in sequencing batch mode with a 24 h hydraulic retention time (HRT). The fill time, settling time, decant time and reaction time for each cycle in sequencing batch mode is as follows: 20 min, 30 min, 10 min and 11 h and the volume decanted per cycle was kept constant at 4.25 L. Operating in sequencing batch mode was conducted to acclimate the cultures to a feed containing 5 g L\(^{-1}\) pure sugars at
experimental pH. After attaining stable glucose degradation, the reactors were changed to continuous operation at 37±2°C.

The experiments were conducted in duplicate using two UASBRs (designated as reactor R1 and R2). Each reactor had a working volume of 8.5 L (total volume 9.5 L; internal dia, 12 cm and height 84 cm) were configured with extended internal recirculation to assist granule fluidization. All experiments were conducted in duplicate. Gas production was monitored with a tipping bucket gas meter Speece (1976) and the pH was monitored using a pH probe (PHP-700 series, Omega).

![Figure 3.1 Schematic representation of operation of a UASBR](image)

Figure 3.1 Schematic representation of operation of a UASBR

The flow rate of the influent (basal medium and the substrate refrigerated at 4 °C prior to feeding) was adjusted according to the HRT and the organic loading rate (OLR) defined in the experimental design. The feed tank containing basal medium was purged with N2 at a low flow rate to maintain anaerobic conditions. The composition of the basal medium was similar to that presented in Table 3.1, except for the amount of sodium bicarbonate (NaHCO3), which was varied to maintain pH levels in the reactor.
3.6 Experimental plan

Hydrogen production studies were conducted in batch and continuous reactors. All studies were conducted using mixed anaerobic cultures at $37\pm2\,^\circ C$. The experimental plan is shown in Table 3.2. All culture pretreatment described in Table 3.2 was accomplished using LA. The LA concentration used in each study is defined in each chapter. In case of batch studies, LA was injected into the serum bottles and incubated for 24 hours. In case of the continuous reactor studies, the cultures were pretreated with LA while operating the reactor in batch mode. The initial LA concentration used during pretreatment in continuous flow reactor studies is provided in the respective chapter. After batch LA treatment, the reactor was switched to continuous operation. A detailed description of the experimental design for each objective is outlined in the respective section of the thesis chapter.

All experiments in continuous reactors were conducted in duplicates, while batch experiments were conducted in triplicates. The protocol/methodology followed and data analysis are described in sections 3.7 to 3.12.
Table 3.2 Experimental plan for hydrogen production studies using batch and continuous reactors

<table>
<thead>
<tr>
<th>Culture source</th>
<th>Aim of study</th>
<th>Parameters studied</th>
<th>Analysis Conducted during research</th>
<th>Chapter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture B</td>
<td>H₂ production from flocculated and granulated cultures in UASBR using glucose</td>
<td>Culture pretreatment and HRT</td>
<td>Experimental analysis: Gas (H₂ and CH₄); liquid metabolites (VFAs and alcohols), residual sugars, COD, hydrogenase assay, pH, VSS and TSS</td>
<td>4</td>
</tr>
<tr>
<td>Culture B</td>
<td>Effect of Organic loading rate (OLR) and hydraulic retention time (HRT) on H₂ production in UASBR using pure glucose</td>
<td>OLR and HRT</td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Both culture A and B</td>
<td>Effect of lignocellulose derived inhibitors on H₂ production and a prelude study with lignocellulosic hydrolysate</td>
<td>Culture pretreatment, furan concentrations</td>
<td>Microbial analysis: Microbial characterization using T-RFLP</td>
<td>6 (batch studies)</td>
</tr>
<tr>
<td>Culture B</td>
<td>Optimization of operational parameters using detoxified lignocellulosic hydrolysate in UASBR for enhanced H₂ yield</td>
<td>pH, HRT and culture pretreatment</td>
<td>Data Analysis: Electron or COD Metabolic Flux, PCA, cluster analysis, CCA and Tukey’s</td>
<td>7</td>
</tr>
<tr>
<td>Culture B</td>
<td>Effect of operational parameters on H₂ production using detoxified switchgrass hydrolysate</td>
<td>Culture pretreatment, HRT and N₂ purging</td>
<td>Modeling: Statistical modeling of experimental data conducted for chapter 6 (6.2 and 6.3)</td>
<td>8</td>
</tr>
<tr>
<td>Culture B</td>
<td>H₂ production potential from raw hydrolysate from steam pretreated corncob on a continuous operating reactor with varying OLR</td>
<td>HRT, OLR, culture pretreatment and toxic inhibitors present in hydrolysate</td>
<td></td>
<td>9</td>
</tr>
</tbody>
</table>

Notes:
1. Culture sources A and B, see section 3.3.
2. Hydraulic retention time = HRT; Organic loading rate = OLR; Chemical oxygen demand = COD; principal component analysis = PCA; Principal co-ordinate analysis = PCoA; Canonical correspondence analysis = CCA; Terminal restriction fragment analysis = T-RFLP.
3. Culture pretreatment was accomplished by adding linoleic acid (LA)
3.7 Chemical analysis

3.7.1 Characterization of lignocellulosic biomass

The lignocellulosic biomass (SWG and CS) was ground to powder using a laboratory mixer prior to analysis (see section 3.1 for details). All chemicals and standards used for characterization were analytical grade. All analyses were conducted in triplicate using a dry solids basis.

3.7.1.1 Moisture content

The moisture content in the SWG and CS was determined by drying 1 gram of biomass in the oven at 105 °C for 24 h. The moisture content was calculated from the weight loss of the biomass.

3.7.1.2 Elemental analysis

The lignocellulosic biomass was analyzed for elemental composition of carbon, hydrogen, nitrogen and sulfur. The analysis was performed using oven dried SWG and CS which in a CHNS/O analyser (Perkin-Elmer 2400 Series) at Department of Chemistry and Biochemistry, University of Windsor.

3.7.1.3 Cellulose

Estimation of the amount of cellulose was conducted in accordance with Updegraff (1969). A 3 mL acetic/nitric reagent (consisting of a mixture of 80% acetic and concentrated nitric acid in a ratio of 10:1) was added to 0.5 g of oven dried biomass and vortexed for 2 min. The contents were heated to 100 °C for 30 min (during this time the cellulose was acetolysed to form acetylated cellodextrin). The reaction mixture was cooled to room temperature, and centrifuged (Beckman Coulter, Inc., USA) at 3000 rpm for 20 min. The supernatant was discarded and the residue washed with MQ. The residues were treated with 67% H₂SO₄ (10 mL) for 1 h. One mL of the above reaction mixture solution was diluted by a factor of 100) and used for further analyses. Pure cellulose was used as the standard for calibration and treatment of the standard samples was performed in accordance with the procedure previously described for oven dried biomass. The mass of cellulose in the standard samples was varied from 20 to 200 µg. The cellulose treated samples were not diluted.
One mL of the solution from the test samples and 10 mL of fresh chill anthrone reagent (200 mg anthrone in 100 mL concentrated H₂SO₄) was heated in a water-bath at 90 °C for 10 min. The color intensity of the samples was measured using a UV Spectrophotometer (CARY 50 scan (Varian, CA)) at 630 nm. The blank sample used in the analysis was comprised of MQ water which was treated in the same manner as the standard calibration samples. The amount of cellulose present in the test samples (dried biomass) was then calculated from the calibration graph.

3.7.1.4 Hemicellulose

Estimation of the hemicellulose content in the biomass was carried out in accordance with methods described by Goering and Van Soest (1970).

Neutral detergent fiber (NDF)

To 1 g of oven dried biomass, 10 mL of cold neutral detergent solution (prepared as outlined by Goering and Van Soest (1970)), 2 mL of decahydronaphthalene and 0.5 g sodium sulfite were added and heated in a reflux for 60 min. The contents were cooled, vacuum filtered and washed with hot water using a sintered glass crucible. Finally, unwanted particles and chemicals were removed using 2 washings with 100 mL of acetone. The residue was dried at 105 °C for 8 h and then weighed. The difference in weight before and after treatment is the NDF content in the biomass.

Acid detergent fiber (ADF)

One g of oven dried biomass was refluxed with 100 mL acid detergent solution (2% w/v of cetyl trimethyl ammonium bromide in 1 N H₂SO₄) and 2 mL of decahydronaphthalene for 1 hour to dissolve all acid soluble contents in the biomass. The contents were cooled, vacuum filtered and washed with hot water through a sintered glass crucible. Unwanted particles and chemicals were removed with 2 washings with 100 mL acetone. The residue was dried at 105 °C for 8 h and weighed. The weight of the remaining residue is the ADF content in the biomass.

The hemicellulose content of the biomass is calculated from the difference in the neutral and acid fiber contents of the biomass (NDF-ADF).
3.7.1.5 Lignin

The total lignin content of the biomass is estimated by delignification of the biomass with acid-chlorite treatment. The procedure is based on the method described by Hubbell and Ragauskas (2010). To 5 g of oven-dried biomass, 100 mL of 2% sodium chlorite, adjusted to pH 3.0 with glacial acetic acid were added and heated in a at 70°C water bath for 2 to 3 h with occasional stirring using a glass rod. The contents were cooled and vacuum filtered using a sintered glass crucible to remove the dissolved lignin in the filtrate. After the washing the residual biomass with a solution containing 3 g L⁻¹ sodium sulfate in MQ water, the residue was washed again with 2 or 3 times with MQ water. The residue in the filter crucible was dried in an oven for 2-3 hours and the lignin content in the biomass was calculated from the weight loss in the biomass. The weight of the remaining biomass corresponds to the holocellulose (total cellulose, comprising cellulose and hemi-cellulose) content of the biomass.

3.7.1.6 Klason lignin

The klason lignin is also known as acid insoluble lignin. The klason lignin content was estimated according to methods by Goering and Van Soest (1970). 0.5 g of the residue obtained from the ADF treated with cold 72% H₂SO₄ for 2 h with occasional stirring. The contents were diluted to 4% with water and the suspension was heated in a boiling water bath for 1 h. The mixture was cooled and filtered using 0.45 µm glass fiber filters. In order to obtain an acid-free residue, the filtered residue was washed with a surplus amount of MQ water. The residue was dried at 105 °C for 8 h and weighed. The weight difference between before and after oven drying to the initial biomass content taken for the ADF analysis was used to calculate the fraction of the klason lignin in the lignocellulosic biomass.

The residue obtained after klason lignin estimation was placed in a crucible and placed in a muffle furnace at 550 °C for 2 h. The remaining inorganics after ashing was placed in a desiccator and the weight of the content determined. The ash content was calculated from weight obtained after ashing to the initial biomass taken for ADF analysis.
3.7.2 Characterization of pretreated liquor

After steam explosion, the liquor was adjusted to a neutral pH 7.0 using 10 M NaOH and filtered using 0.45 µm glass fiber filter paper. The filtrate was then passed through a filter consisting of a syringe filter holder (Ø 25 mm) (PAL Sciences, MI, USA) fitted with a 0.45 µm polypropylene membrane (GE Osmonics, MN) to remove suspended solids. This filtered liquor was filtered using a pack bed polypropylene cartridge with a 20 µm polyethylene frit (Spe-ed Accessories, PA) filled with an ion exchange resin (Chelex 100, Bio-Rad, California) to remove any dissolved metals present in the sample. The samples were diluted by a factor of 50 with MQ water and stored at 4 °C. The reducing sugars, filtered COD and phenols content of the sample were determined to characterize the liquor.

3.7.2.1 Reducing and total sugars

The amount of reducing sugars in the biomass was estimated by the dinitrosalicylic acid (DNSA) method described by Miller (1959). The total sugar content was estimated by the anthrone method described by Hedge and Hofreiter (1962).

Steam exploded liquor samples (2 mL) from section 3.7.2 were mixed with 2 mL of DNSA reagent prepared according to Miller (1959). The contents were heated in a water bath at 100 °C for 8 minutes. Standards containing glucose and xylose (alone and in combination) with concentrations ranging from 100 to 500 µg were treated similarly as the samples. Blank samples containing the mixture of MQ and DNSA were used for calibrating zero. The contents were then cooled in cold water bath and absorbance (optical density) was measured at 540 nm using a UV Spectrophotometer (CARY 50 scan (Varian, CA)).

The sugar content of the biomass (total sugars) was estimated by hydrolyzing 1 mL of steam exploded liquor with 0.1 mL of 2.5 N HCl in a water bath at 100°C. The contents were cooled and neutralized with 3 M NaOH. The volume of the solution containing the sample was made up to 50 mL using MQ and centrifuged at 3000 rpm for 20 min. One mL of the sample from the supernatant was mixed with 4 mL of freshly prepared anthrone reagent. Standards containing glucose (100 to 500 µg) were similarly prepared and used for calibration. The contents of the sample were heated in a boiling water bath.
for 8 min. The contents of the tube were cooled and the color intensity (green to dark green) was measured at 630 nm using a UV Spectrophotometer (CARY 50 scan (Varian, CA)). A blank sample comprised of anthrone reagent and MQ was used to calibrate the instrument to zero before assessing the standards and test samples.

3.7.2.2 Chemical oxygen demand

The chemical oxygen demand (COD) of the influent substrate (steam exploded liquor/ detoxified liquor, see section 3.2.1 and 3.2.2) was measured using a closed reflux calorimetric method according to *Standard Methods* (APHA, 1999). The COD was measured for both filtered and unfiltered samples (influent substrate).

2.5 mL of sample (filtered or unfiltered influent substrate) was placed in a 16 mm Ø COD tube digester and 1.5 mL of 0.25 N potassium dichromate (K$_2$Cr$_2$O$_7$) digestion solution was added to each sample. Next, 3.5 mL of sulfuric acid reagent (containing a mixture of 0.55 g AgSO$_4$ in 100 g H$_2$SO$_4$) was added and mixed with a vortex. The standards (50 to 500 µg of COD equivalent) were similarly prepared to develop a calibration curve using potassium hydrogen phthalate (KHP). A known quantity of KHP (425 mg) equivalent to 500 mg of COD was used as a benchmark. The blank sample (for calibrating zero) consisted of MQ water, K$_2$Cr$_2$O$_7$ digestion solution and sulfuric acid reagent. The COD tubes, sealed with screw caps, were placed in a COD temperature bath at 150 °C for 2 h. The digested contents were cooled and the absorbance was measured at 600 nm using a UV Spectrophotometer (CARY 50 scan (Varian, CA)).

3.7.2.3 Phenol

The total phenols in the liquor samples before and after resin treatment (see section 3.2.1 and 3.2.2) was calorimetrically determined using Folin–Ciocalteu (FC) reagent with catechol as the standard (Singleton and Rossi, 1965).

5 mL of the diluted sample, 5 mL of MQ and 1 ml of FC reagent were added to a 50 mL test tube. The sample containing tubes were incubated for 15 minutes at room temperature, after which 10 mL of 20% sodium carbonate (Na$_2$CO$_3$) was added and mixed well in order for the reaction to proceed. The standards containing catechol were prepared with concentrations ranging from 20 to 100 µg. The standards were treated in
the same way as the test samples. The reaction was carried out at 30 °C for a period of 90 min. The color intensity was measured at 730 nm using a UV Spectrophotometer (CARY 50 scan (Varian, CA)).

3.7.2.4 Biological oxygen demand

Biological oxygen demand (BOD) in the hydrolyzed and detoxified liquor samples after resin treatment was determined using the standard protocol described by Adams (1990). All the reagents for the dilution water were prepared according to the methods described in the standard protocol.

One mL of phosphate buffer, 1 mL of 1 M magnesium sulfate, 1 mL of 1 M calcium chloride and 1 mL of 1 M ferric chloride were added to a 1000 mL volumetric flask. The flask was filled with distilled water to the 1 L mark and purged with air at a flow rate of 50 mL min\(^{-1}\) and then closed with the stopper. The content was then transferred to BOD bottles (330 mL working volume). The BOD bottles were closed with stoppers. One bottle without seed inoculum was stored in a BOD incubator maintained at 20±0.5°C. Another blank (B) with 2 mL of seed inoculum, obtained from a local domestic sewage plant, was used as a control without substrate. Diluted liquor samples (dilution factor (DF) = 0.1) (0.5 mL) adjusted to neutral pH was added with 2 mL of the seed inoculum. Glucose was used as a standard with 0.5 mL of 4000 mg L\(^{-1}\) stock solution. Blank or control, standards and samples were prepared in replicate and the dissolved oxygen content was measured at day 1, day 5, day 7 and day 20. The BOD\(_5\) to COD ratio is presented in the results (Table F.2, Appendix F).

3.7.3 Characterization of fermented samples

3.7.3.1 pH

The pH of samples taken at the end of the batch experiments and the pH of the effluent during continuous operation were measured using a VWR SR40C, Symphony pH meter. For continuous operation of the UASBR, the pH was monitored using a pH probe (PHP-700 series, Omega).
3.7.3.2 Total and volatile suspended solids

The volatile suspended solids (VSS) and total suspended solids (TSS) were measured at the beginning and the end of each experimental condition under batch as well as continuous systems. The VSS and TSS in the effluent were also measured to quantify the biomass washout from the continuous reactor system. All of these measurements were carried out according to the procedures described in *Standard Methods* (APHA, 1999).

3.7.3.3 Chemical oxygen demand

The estimation of the COD for the effluent samples was performed for filtered and unfiltered samples. The COD of the samples was estimated by diluting the samples until the concentration of the sample was within the calibration range. This method is similar to the procedure outlined in section 3.7.2, with the exception that the standards for COD estimation were prepared using filtered and diluted basal medium and the blank sample for calibrating zero was prepared with basal medium instead of MQ water.

3.8 Analytical methods

All of standards were prepared in triplicate to generate the calibration curves provided in Appendix A. Variation of ±5 % from the calibration standards was considered acceptable.

3.8.1 Sugars analysis in the feed solution

The sugar composition of the feed was analyzed for glucose, xylose, arabinose, mannose and galactose content using ion-exchange chromatograph (IC). The samples were processed as described in section 3.7.2 and stored in 5.0 mL polypropylene vials (Dionex, Oakville, ON). The samples were analyzed for simple sugars before and after resin treatment of the steam exploded liquor using a DX-600 IC (Dionex, Oakville, ON) equipped with a GP 50 gradient pump, an AS 40 automated sampler, an ED 50 electrochemical detector and a 25-μL-sample loop. The instrument was configured with 15 cm x 3 mm CarboPac™ PA 20 analytical column and a CarboPac™ PA 20 guard column (Dionex, Sunnyvale, CA). The isocratic method used at a flow rate of 0.2 mL min⁻¹ with 50% 40 mM NaOH and 50% H₂O.
The standards used for calibration were comprised of mixed sugars. The standards prepared with concentrations ranging from 0 to 500 mg L\(^{-1}\) were prepared from a stock solution of 5000 mg L\(^{-1}\). Standards prepared with individual sugars were analyzed with standards with mixed sugars to identify the peak retention time of each individual sugar.

### 3.8.2 Volatile fatty acids analysis

The VFAs levels in liquid samples withdrawn from batch reactors and the samples collected from the effluent of the continuous reactor using a 0.5 mL Hamilton Gastight (VWR, Canada) syringe were diluted with 4.5 mL of MQ water. The samples were centrifuged and filtered as outlined in section 3.7.2. and stored in 5.0 mL polypropylene vials (Dionex, Oakville, ON). The IC analysis was carried out in accordance with Veeravalli et al. (2013).

The VFAs produced from glucose or glucose/xylose fermentation studies were quantified using a DX-500 IC (Dionex, Oakville, ON) equipped with a CD 20 conductivity detector, ASRS suppressor, a GP 40 gradient pump, an AS 40 automated sampler and a 25-µL-sample loop. The IC was configured with a 24 cm x 4 mm diameter IonPac AS11-HC analytical column and an IonPac AG11-HC guard column (Dionex, Sunnyvale, CA). Three eluents were MQ, 5 mM NaOH, and 50 mM NaOH and the total flow rate was 1 mL min\(^{-1}\). The analytical method was the same as the procedure described by Lalman and Bagley (2000). The samples were further diluted with MQ by 1:1 ratio prior to analysis.

VFAs in the feed and reactor effluents for experiments conducted with lignocellulosic sugars (Chapter 7, 8 and 9) were analyzed by high performance liquid chromatography (HPLC) using a Dionex Ultimate 3000 HPLC equipped with a Dionex Ultimate 3000 auto sampler. The method of analysis was adapted from Moon et al. (2013). The instrument was configured with a photo-diode array (PDA) detector, and the analysis was conducted under isocratic conditions using an analytical column: 3.0 × 100 mm Agilent Eclipse plus C18 column in an oven set at 55 °C. The eluent (90% H\(_3\)PO\(_4\) solution set at pH 3 and 10% methanol) flow rate was set at 0.3 mL min\(^{-1}\). The instrument was set to scan at 210 nm.
Standards were prepared using a VFAs (lactate, acetate, propionate, formate and butyrate) mixture and concentrations ranging from 0 to 60 mg L\(^{-1}\). The dilutions for standards were prepared from a stock solution of 5000 mg L\(^{-1}\) using a dilution medium (filtered basal media diluted with MQ at 1:10 ratio).

### 3.8.3 Sugar and alcohol analysis

The residual total sugars and the alcohols in liquid samples withdrawn from the batch reactors and the samples collected from the effluent of continuous reactor systems were processed and stored before analysis as described in section 3.8.2. The analysis in IC was carried out in accordance with Veeravalli et al. (2013).

The stored samples were analyzed using a DX-600 IC (Dionex, Oakville, ON) equipped with a GP 50 gradient pump, an AS 40 automated sampler, an ED 50 electrochemical detector and a 25-μL-sample loop. The instrument was configured with a 25 cm x 4 mm CarboPac™ MA-1 column (Dionex, Sunnyvale, CA). The isocratic method of elution was used at a flow rate of 0.4 mL min\(^{-1}\) using 480 mM NaOH.

Standards were prepared from a glucose plus alcohols (ethanol, i-proapnol, propanol, butanol and i-butanol) mixture with concentrations ranging from 0-500 mg L\(^{-1}\). Standards were prepared from a 5000 mg L\(^{-1}\) stock solution plus a solution prepared from filtered basal media diluted with MQ water in a ratio of 1:10.

### 3.8.4 Furan analysis

The hydrolyzed liquor samples (from section 3.2) were analyzed to determine the levels of furan compounds, such as furfural and HMF. The fermented samples were analyzed for furfural and HMF together with furoic acid and furfuryl alcohol (furan degradation byproducts). The samples were processed and stored as described in section 3.7.2. The stored samples were analyzed using a Dionex Ultimate 3000 HPLC equipped with a Dionex Ultimate 3000 auto sampler. The instrument was configured with a photo-diode array (PDA) detector, and the analysis was conducted under isocratic conditions using an analytical column: 3.0 × 100 mm Eclipse plus C18 column (Dionex, Sunnyvale, CA) with the oven set at 50°C. The eluent (80% of a 0.5% H\(_3\)PO\(_4\) solution and 20% methanol) flow rate was set at 0.2 mL min\(^{-1}\). The instrument was set to scan at the
following 3 different wavelengths: 280 nm for furfural and HMF, 215 nm for furoic acid and 252 nm for furfuryl alcohol.

Standards were prepared using a mixture of furan derivatives with concentrations ranging from 0-100 mg L\(^{-1}\). The diluents for the standards were prepared using the dilution medium (filtered basal media diluted with MQ water in a 1:10 ratio). The stock solution used to prepare the standards was 5000 mg L\(^{-1}\).

3.8.5 Gas analysis

Gas analysis was carried out in accordance with method described by Veeravalli et al. (2013). Gas samples (25 µL) (containing H\(_2\), methane and carbon dioxide) from the head space of a tipping bucket gas meter or batch reactors were analyzed using gas chromatography (GC). In the case of batch and continuous reactors, gas samples were removed using a 50 µL Hamilton Gastight syringe (VWR, Canada) and injected into a Varian-3600 gas chromatograph (Varian, Palo Alto, CA) configured with a TCD and a 2 m long 2 mm I.D. Carbon Shin column (Alltech, Deerfield, IL). The GC injector, detector, and oven temperatures were set at 100\(^\circ\) C, 200\(^\circ\) C, and 200\(^\circ\) C, respectively. The nitrogen (99.999%, Praxair, ON) carrier gas flow rate was set at 15 mL min\(^{-1}\).

Gas standards were prepared in 160 ml serum bottles. The bottles were purged with pure nitrogen gas (99.999%) (Praxair, ON) for 3 minutes and capped with aluminum crimp caps with Teflon-lined septas. Known quantities of H\(_2\), CH\(_4\) and CO\(_2\) were injected into the serum bottles. Prior to injecting a gas sample, a corresponding amount of gas was withdrawn from the bottles to ensure 1 atmosphere pressure within the bottles was maintained after injecting the gas standard. The gas standards were prepared and analyzed according to the peak areas obtained within the experimental range.

3.8.6 LCFA extraction and analysis

The method of LCFA extraction was based on work reported by Lalman and Bagley (2000) and Saady et al. (2012). Liquid samples withdrawn from the reactor while purging with nitrogen to obtain uniform mixing of the biomass and the fermentation medium in the reactor. Thus obtained sample was stored in a sealed 20 mL serum vial. One mL of the sample from the serum vial was placed in a 5 mL vial containing 3 mL of 1:1:1
Hexane:MTBE:chloroform (EM Science, USA), approximately 0.1 g NaCl (ACP Chemicals, Montreal, Quebec) and 2 drops of concentrated H$_2$SO$_4$ (EMD Chemicals, USA). The serum vials were capped with aluminum crimp sealed caps with Teflon-lined septa. The vials were mixed for 1 h at 37 °C in an orbital shaker at 200 rpm. After 1 h, the vials were centrifuged at 4000 rpm for 15 minutes after which 1 mL of the organic layer was removed using a 2.5 mL Hamilton Gastight (VWR, Canada) syringe. The extracted LCFAs were stored in 1.5 mL amber glass HPLC vials capped with Teflon-lined septa and stored at -4 °C.

The standards were treated the same manner as the test samples. Standards of varying concentrations (0 to 2000 mg L$^{-1}$) were prepared from a stock solution (10000 mg L$^{-1}$) containing a LCFAs mixture. The mixture contained the following LCFA: caproic acid (C6:0, >99%), caprylic acid (C8:0, >99%), capric acid (C10:0, >99%), lauric acid (C12:0, >99%), myristic acid (C14:0, >99%), palmitic acid (C16:0, >99%), stearic acid (C18:0, >99%), oleic acid (C18:1) and linoleic acid (C18:2, >95%) (TCI, USA). The LCFA stock solution was prepared using a saponification technique described by Rinzema et al. (1994). The amount of NaOH added was based on the weight of the LCFAs (see Table 3.3).

<table>
<thead>
<tr>
<th>LCFA</th>
<th>NaOH added (g g$^{-1}$ LCFA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caproic acid (C6)</td>
<td>0.310</td>
</tr>
<tr>
<td>Caprylic acid (C8)</td>
<td>0.278</td>
</tr>
<tr>
<td>Capric acid (C10)</td>
<td>0.233</td>
</tr>
<tr>
<td>Lauric acid (C12)</td>
<td>0.200</td>
</tr>
<tr>
<td>Myristic acid (C14)</td>
<td>0.175</td>
</tr>
<tr>
<td>Palmitic acid (C16)</td>
<td>0.156</td>
</tr>
<tr>
<td>Stearic acid (C18)</td>
<td>0.141</td>
</tr>
<tr>
<td>Oleic acid (C18:1)</td>
<td>0.142</td>
</tr>
<tr>
<td>Linoleic acid (C18:2)</td>
<td>0.143</td>
</tr>
</tbody>
</table>

The percent LCFA recovered was calculated from calibration curves. The LCFA standards were prepared using pure hexane as the solvent. The calibration standards were prepared from a stock solution of 5000 mg L$^{-1}$ containing a mixture of 9 LCFAs.
dissolved in n-hexane. The calibration curves and the percent recoveries for the 9 LCFAs are shown in Appendix A.

The stored test samples were analyzed using a Varian 3800 GC equipped with a flame ionization detector (FID) and a split/splitless injector. The instrument was configured with a DB-FFAP 30 m × 0.25 mm × 0.25 µm analytical column (J and W Scientific, USA). The injector and the FID were maintained at 240 °C and 250 °C, respectively. The analytical program was based on work reported by Saady et al. (2012). The oven temperature was programmed as follows: 1) 100 °C for 2.0 minutes; 2) increase the temperature to 240 °C in 15 °C min⁻¹ gradients and 3) hold at 240 °C for 8.67 minutes. The total analysis time was 20 minutes. Helium was the carrier gas at a constant pressure of 30 psi and a flow rate set at 5 mL min⁻¹. The split injector was off for 0.01 min, then on at a split ratio of 70:1 until the end of the program’s duration (i.e., 20 minutes).

3.9 Enzymatic assay

The enzymatic assay was carried out to quantify the hydrogenase activity in mixed microbial cultures under continuous operation. The procedure was adapted from Pendyala et al. (2012). The sample were withdrawn from the UASBR for the analysis with sparging the bioreactors with N₂ and collected in a 5 mL serum vial purged with N₂ in the head space.

3.9.1 Hydrogenase activity: Hydrogen evolution assay (HEA)

In vitro assays of H₂ production were performed using reduced methyl viologen (MV) (99% purity) (Sigma, ON) as an artificial electron donor. A cell extract solution was prepared by adding 0.3 mL of mixed culture from the USABR to 2.1 mL of distilled water, 0.3 mL of 1.0 M phosphate buffer (pH 7.0) and 0.3 mL of a 10% Triton X-100 solution (ACP Chemicals Inc., QC). The reaction mixture (approximately 2.0 mL) contained 0.1 mL of the cell extract solution, 1.76 mL of 20 mM phosphate buffer (pH 7.0), and 40 µL of oxidized MV (20 mM). Sodium dithionite (ACP chemicals Inc., QC) at a concentration of 2.5 mg per mL was added to initiate the reaction, and then, the reaction mixtures were incubated for 1 h at 37 °C. Headspace gas samples from the serum
bottles were injected into the GC to determine the amount of H$_2$ produced. The evolution specific activity (ESA) was reported as $\mu$moL of H$_2$ produced h$^{-1}$ mg$^{-1}$ VSS.

### 3.9.2 Hydrogenase activity: Hydrogen uptake assay (HUA)

The in-vitro H$_2$ uptake assay was performed using oxidized benzyl viologen (BV) (TCI America, OR) as an artificial electron acceptor. The steps used during the assay were as follows:

Two mixtures 1) cell mixture and 2) electron acceptor mixture were used for conducting the assay. A cell mixture solution under anaerobic conditions (N$_2$ atmosphere) was prepared by adding 0.3 mL of the cell extract broth to 1.8 mL of anaerobic MQ water, 0.3 mL of 1 M phosphate buffer (pH 6.0) and 0.3 mL of dithiothreitol (DTT) (TCI America, OR). An electron acceptor solution was prepared under anaerobic conditions by adding 0.4 mL of 40 mM BV to 2.3 mL of anaerobic MQ water and 0.3 mL of 1 M phosphate buffer (pH 6.0). H$_2$ was sparged into the solutions 1 and 2 for 5 min to replace the N$_2$ in the head space and sealed the cuvettes with septas (Skidmore, 2010). After 4 min, continuing the H$_2$ sparging to the cell solution 0.3 mL of a Triton X-100 (10%) solution (Sigma Aldrich, ON) was added. An anaerobic cuvette of 5 mL volume sealed with Teflon coated septa and a screw cap, was taken and purged with H$_2$ for 3 min to replace the gas in the cuvette with H$_2$. A 2 mL of the electron acceptor solution with 0.67 mL of the cell solution was added to this 5 mL closed cuvette while incubating at 37 °C in a water-bath. The cuvette was then removed and OD was measured at 546 nm (CARY 50 scan (Varian, CA)) for 10 minutes at 0.1 sec intervals. The initial slope of the OD versus time (min) plot was used to compute the total activity ($\mu$mol H$_2$ min$^{-1}$). The uptake specific activity (USA) was reported as $\mu$mol of H$_2$ consumed h$^{-1}$ mg$^{-1}$ VSS (Shenkman, 2003; Skidmore, 2010).

### 3.10 Microbial characterization

The microbial characterization was carried out using Terminal Restriction Fragment Length Polymorphism (T-RFLP) following the method developed and described by Chaganti et al. (2012).
3.10.1 Genomic DNA extraction

A well-mixed sludge (microbial) sample (0.4 ml) was added to a 2 ml sterile tube containing approximately 250 mg of zirconia/silica beads, 0.4 ml cetyl trimethylammonium bromide (CTAB) extraction buffer [comprised of 20% CTAB (wt/vol) (Sigma, Toronto, ON) in 1.4 M NaCl with 480 mM potassium phosphate buffer at pH 8.0] and 0.4 ml of phenol–chloroform–isoamyl alcohol (25:24:1 (pH 8.0)). The mixture was subjected to three freeze (-80°C) and thaw cycles. Bacterial cells in the sample were lysed by homogenizing for 45s in a Thermo Savant Bio 101/FP120 Fast prep homogenizer at a speed setting of 6.5. Phase separation was achieved by centrifugation (16,000×g) for 10 min at 4°C. The clear aqueous upper phase was transferred to micro-centrifuge tubes and re-extracted by mixing with an equal volume of chloroform–isoamyl alcohol (24:1). This was followed by centrifugation (10,000×g) for 10 min. Nucleic acids were then precipitated from the extracted aqueous layer with 0.6 vol of iso-propanol for 10 min at room temperature. The sample was then centrifuged (10,000×g) at 5°C for 20 min. Nucleic acid pellets were washed in 70% (v/v) ice-cold ethanol and air dried before re-suspension in 50 µl sterile MQ water.

3.10.2 PCR and T-RFLP profiling

Nested-PCR of the microbial 16S rRNA gene was performed using the 6-carboxyfluorescein (FAM) labeled 5’ end of the forward primer B8F (5’/-5IRD700/AGAGTTTGATCCTGGCTCAG-3’) (Edwards et al., 1989) with the reverse primer Eub-539R (5’- ATCGTATTACCGCGGCTGCTGGC-3’). Similarly, archaeal 16S rRNA genes were amplified with forward primer Arc-112F (5’/-5IRD700/GCTCAGTAACACACGTGG-3’) and Arc-533R (5’-TTACCGCGGCGGCTGGCA-3’) reverse primer. PCR mixtures (25 µl) containing 10.2 mM Tris buffer, 2.3 mM MgCl2, 50 mM KCl, 2% DMSO, 5 µg BSA, 0.2 mM of each 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 30s, 72°C for 30s, and a final elongation step of 72°C for 1 min. Purified PCR products (3 µl) (using QIAquick spin columns (Invitrogen)) were digested with 2.5 U of restriction enzyme (Hae III, Hha I, MSP-I and Hinf I) in a total volume of
20 µL for 2 h at 37°C. *Hae* III and *Hha I* was selected because they produce several short terminal restriction fragments (T-RFs) (<300 bases) from most bacteria. The digested restriction products (1 µl) were mixed with 3 µL of stop solution (LI-COR, Inc., Lincoln, NE); the samples, along with the size markers (50-700 base pairs, LI-COR, Inc., Lincoln, NE), were denatured at 95°C for 2 min, and then rapidly chilled with ice. The denatured samples were loaded onto a 6.5% polyacrylamide gel (KBPlus™, LI-Cor, Inc., Lincoln, NE) and separated by 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). The software application, Gene ImagIR 4.05 (Scanalytics, Inc., Rockville, MD), was used to estimate the fragment sizes and relative abundances (band intensity).

### 3.10.3 Phylogenetic assignment of terminal restriction fragments (T-RFs)

T-RFLP data generated by digestion of sludge DNA samples with restriction enzymes (*Hae* III) were formatted according to Phylogenetic Assignment Tool (PAT; [https://secure.limnology.wisc.edu/trflp/](https://secure.limnology.wisc.edu/trflp/)) requirements, and analyzed on-line using the default fragment bin tolerance window setting. Phylogenetic assignment was performed using a modified database consisting of the default database generated from microbial community analysis (MiCA; [http://mica.ibest.uidaho.edu/](http://mica.ibest.uidaho.edu/)) plus experimentally determined T-RFs for micro-organisms identified from the 16S rRNA gene clone library analysis described by Chaganti et al. (2012). T-RFs that differed by ±1 bp in different profiles were considered to be identical, and fragments smaller than 40 bp were excluded from the analysis.

### 3.10.4 Pyrosequencing

The variations in the mixed microbial communities fed glucose at different loading rates at constant HRT (see section 5.3.8.3) were analysed using 454 pyrosequencing.

After DNA isolation from the samples multiplex amplicon sequencing was performed on all the samples. The V5 and V6 regions of bacterial 16S rRNA genes were amplified using the fusion primers E 786F (5’-GATTAGATACCCTGG TAG-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 containing 10.2 mM Tris buffer, 50 mM KCl, 2% 2.3 mM MgCl₂, 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 melting agarose gel followed by QIAquick gel extraction kit (Qiagen) and quantified using Nano Vue. Equal amounts of the barcoded 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 Bioanalyzer 2100 (Agilent Technologies, USA).

The obtained pyro sequencing data after the initial process by the GL FLX software, RDP Pryo-sequencing 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 a confidence threshold of 80%.

3.11 Flux balance analysis

A flux balance analysis (FBA) was conducted using data obtained from the analysis of metabolites (both gas and liquid) produced from the substrate fermentation. The metabolic reaction network and stoichiometries were adapted from the model reported by Chaganti et al. (2011), (see Figure 3.2 for model and Table 3.4 for the stoichiometric reactions) in order to estimate the electron fluxes diverted to various metabolites over the fermentation pathway.

The flux balance analysis was majorly used in estimating the amount of H₂ consumed from the actual H₂ produced (R12, Table 3.4) under different experimental conditions. The H₂ consumption is majorly affected by homoacetogenic (R17, Table 3.4) and hydorgenotrophic methanogen (R29, Table 3.4). In either case 4 moles of H₂ are consumed to produce 1 mole of acetic acid or methane. The other source of H₂
consumption includes the propanol or i-propanol flux (R21, Table 3.4). The valerate and caproate were not detected in the liquid metabolite, therefore net H₂ consumption was simply calculated by considering only R17, R21 and R29. The difference between the actual H₂ produced (observed H₂ yield, R13, Table 3.4) and the model predicted H₂ yield (R12, Table 3.4), gives the total H₂ consumed. The FBA was conducted using MetaFluxNet software, Version 1.8.6.2. The inputs included the external substrate (glucose equivalents) and the measured by-products (i.e., gases, VFAs, and alcohols). H₂ production was selected as the objective function.

Figure 3.2 Metabolic reaction network of the model used in flux balance analysis (Adapted from Chaganti et al. (2011))
<table>
<thead>
<tr>
<th>Rxn no.</th>
<th>Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>R01</td>
<td>Glucose (ext) → Glucose</td>
</tr>
<tr>
<td>R02</td>
<td>Glucose → Biomass</td>
</tr>
<tr>
<td>R03</td>
<td>Glucose → Residual glucose</td>
</tr>
<tr>
<td>R04</td>
<td>Glucose + 2NAD⁺ → 2 Pyruvate + 2 NADH</td>
</tr>
<tr>
<td>R05</td>
<td>NADH + CO₂ → NAD⁺ + HFo</td>
</tr>
<tr>
<td>R06</td>
<td>NADH + Pyruvate → HLa + NAD⁺</td>
</tr>
<tr>
<td>R07</td>
<td>HLa → HLa (ext)</td>
</tr>
<tr>
<td>R08</td>
<td>HLa + NADH → HPr</td>
</tr>
<tr>
<td>R09</td>
<td>HPr → HPr (ext)</td>
</tr>
<tr>
<td>R10</td>
<td>Pyruvate + CoA + 2Fd²⁺ → AcetylCoA + CO₂ + 2 Fdred (Fd⁺)</td>
</tr>
<tr>
<td>R11</td>
<td>NADH + 2Fd²⁺ ↔ NAD⁺ + 2 Fdred</td>
</tr>
<tr>
<td>R12</td>
<td>2 Fdred + 2H₂ → H₂ +2Fd²⁺</td>
</tr>
<tr>
<td>R13</td>
<td>H₂ → H₂ (ext)</td>
</tr>
<tr>
<td>R14</td>
<td>HPr + 6 H₂ → HVa</td>
</tr>
<tr>
<td>R15</td>
<td>AcetylCoA → HAc + CoA</td>
</tr>
<tr>
<td>R16</td>
<td>HAc → HAc (ext)</td>
</tr>
<tr>
<td>R17</td>
<td>4 H₂ + CO₂ → HAc</td>
</tr>
<tr>
<td>R18</td>
<td>AcetylCoA + 2 NADH → EtOH + 2NAD⁺ + CoA</td>
</tr>
<tr>
<td>R19</td>
<td>2 AcetylCoA → AcetoacetylCoA + CoA</td>
</tr>
<tr>
<td>R20</td>
<td>AcetoacetylCoA → Acetone + CoA + CO₂</td>
</tr>
<tr>
<td>R21</td>
<td>Acetone + H₂ → PrOH/i-ProH</td>
</tr>
<tr>
<td>R22</td>
<td>Acetone → Acetone (ext)</td>
</tr>
<tr>
<td>R23</td>
<td>AcetoacetylCoA + 2 NADH → ButyrylCoA + 2NAD⁺</td>
</tr>
<tr>
<td>R24</td>
<td>ButyrylCoA → HBu + CoA</td>
</tr>
<tr>
<td>R25</td>
<td>HBu → HBu (ext)</td>
</tr>
<tr>
<td>R26</td>
<td>HBu + 6H₂ → HCa</td>
</tr>
<tr>
<td>R27</td>
<td>ButyrylCoA + 2NADH → BuOH + 2NAD⁺ + CoA</td>
</tr>
<tr>
<td>R28</td>
<td>HAc → CO₂ + CH₄</td>
</tr>
<tr>
<td>R29</td>
<td>CO₂ + 4H₂ → CH₄ + 2H₂O</td>
</tr>
<tr>
<td>R30</td>
<td>CH₄ → CH₄ (ext)</td>
</tr>
</tbody>
</table>

### 3.12 Statistical analysis

#### 3.12.1 Principal component analysis

A principal component analysis (PCA) was conducted, using the PAST, version 2.15 (Hammer et al., 2001), to identify relationships between the different fermentation conditions for each phase of the experiment. A PCA reduces the large number of interlinked variables to a simplified structure that can be plotted in the two dimensional plane as score and loading plots. The clusters of interconnected sample points can be...
visualized and the relevant underlying patterns be identified (the patterns of grouping are based on the similarities in the dataset that might be due to common underlying factors [i.e., principal components]. The PCA was used to identify the variables that accounted for major variance in the fermentation profile under different operating conditions and group the samples based on their similarity in the metabolite distribution. A bi-plot was used to represent the observations and variables (the byproducts) in the same two-dimensional plot. The gas and liquid metabolite values were used as the inputs to study the effect of operational conditions on the metabolic profile of the mixed microbial culture.

3.12.2 Statistical analysis of microbiological data

The replicates of the microbial samples obtained from reactors (R1 and R2) were aligned based on band size and intensity using the moving average algorithm in the T-align software application (Smith et al., 2005). A PCA or principal coordinate analysis (PCoA) was used to analyse the data from the T-RFLP profiling. A PCoA was conducted using the PAST software package to visualize dissimilarities in the microbial profiling of the mixed microbial community under different operating conditions. PCoA is a multidimensional scaling tool, which takes the dissimilarity matrix as an input to display the distance between the groupings of the microbial cluster in the lowest possible dimensional space. The T-RFs obtained with the restriction enzyme (Hae III) under different conditions were given as inputs (in terms of the intensity of the bands). Terminal restriction fragments smaller than 50 base pairs (bp) were excluded from the analysis. A multivariate cluster analysis (MCA) was also performed to relate the samples, based on the T-RFLP profile based on their similarity. The clustering algorithm used was paired group algorithm with the arithmetic mean to determine the possible linkages between different samples present. According to Kosman and Leonard (2005), an appropriate coefficient of similarity needs to be used for clustering, the samples based on similarity between individuals and among different clusters, because based on similarity coefficients the clustering among the individuals may vary. Among the similarity coefficients occurrence based measure is preferred for samples showing large diversity (Duarte et al., 1999). In the current study, cluster analysis was carried out using

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Kulczynski similarity index. The Kulczynski index was used in quantifying the similarity within the species, which compares the different clusters represented at the nodes to determine the homogeneity based on the absence or presence of the T-RFs (Anderson et al., 2011). The formula for calculating this coefficient index is as follows:

\[
    kuleczynski = \frac{C(N1+N2)}{2(N1+N2)}
\]  

(3.1)

Where, C = species present in both areas; N1 = total number of species in first sample; N2 = total number of species in second sample

3.12.3 Canonical correspondence analysis

A canonical correspondence analysis (CCA), is a multivariate statistical analysis used in elucidating the relationship between the environmental factors and the species abundance in the samples (ter Braak, 1986). This method is widely used in ecology samples and aquatic systems so as to arrive a relationship in the factors and the environmental species that is associated with the factor variables. Similar factors influence on these systems show high correlation within the samples. The CCA also helps in identifying the major gradient and the distribution pattern of the samples in the triplot. Here the fermentation byproducts were used as an input for the environmental factors and the species (Hae III digest of T-RFs) represented by the bandwidth with relative intensity was used as environmental species. The CCA was conducted using the PAST (with the options provided in the package) to relate the microbial species with the fermentation byproducts obtained under different conditions.

3.12.4 Optimization study

Based on preliminary studies conducted in batch reactor and continuous reactor systems, optimization of the factors influencing H₂ production from detoxified lignocellulosic sugars obtained from steam explosion of SWG was carried out in UASBR. The factors that were adjusted included: HRT, pH and LA concentration. A response surface methodology using the Box–Behnken experimental design was used to optimize the factors (Box and Behnken, 1960). The design matrix used included 14 experiments, with two center points. The quadratic polynomial equation was used to
predict the optimum conditions for H₂ production. Minitab (Version 16, Minitab Inc., State College, PA) software applications were used for analysis of the design and for generating the contour plots. An analysis of variance (ANOVA) was performed to test the significance of the fit of the full quadratic model and evaluate the approximation of the response surface model. The detailed methodology of the model’s development and the factor level chosen is presented in section 7.2.

3.12.5 Other statistical analysis

Data presented in section 6.3.7 was modeled using regression analysis. Fitting the data to a regression equation was performed using Sigma Plot (Systat Software, Inc., IL). The ‘goodness of fit’ of the model equation was evaluated using the coefficient of determination (R²) and F value statistics. The Anderson-Darling test was used to test the normal distribution of the residuals. A statistical significance comparison test was conducted using Tukey’s test comparison procedure at a 95% confidence level (Box et al., 1978).

3.13 Quality assurance and quality control procedures

Various Quality assurance and Quality control (QA/QC) protocols were followed during the research to ensure accuracy and the precision in the data obtained from chemical and analytical methods.

1) All the glasswares used in experiments were washed and cleaned by soaking them in a hypochlorite containing the soap solution for at least 24 hours. Thereafter, the glasswares were washed with tap water followed by rinsing in distilled water, and were oven dried at the 180 ºC for 3 hours. (Exceptions: plastic materials such as IC vials were air dried at room temperature after rinsing with DI water, HPLC vials were ultrasonicated for 15 min to clean and remove dirt inside the vials).

2) Disposable glass Pasteur pipettes (VWR, Canada) were used for weighing the chemicals in preparing the stock solution. The needles used for preparing different dilutions of standards were maintained separately to avoid cross contamination.
3) All the chemicals used for the standards were of HPLC grade (>98% purity). All other chemicals used were of analytical grade.

4) Refrigerated stock solutions sealed in 20 mL serum vials were used in preparation of standards and all the dilutions from this stock solution were carried out using MQ or dilution medium (basal medium and MQ) in a clean oven dried 5 or 20 mL serum vials.

5) 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 checking their relative change in the area under the curve to the standard run at the time of preparation of the stock solution. Alternatively, the COD method was used in checking the quality of standards. Knowing the theoretical oxygen demand for the standard, the quality of the standard is estimated. If the difference is greater than 5% then fresh stock solutions were used.

6) 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.

7) The HPLC and IC reliability was checked by shooting standards 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.)

8) 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.
9) Standards (gas standards) for the GC were prepared fresh at the time of calibration and the deterioration 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 frequently and fresh set of standards of three different known compositions is run on a quarterly basis to check the quality of the column by comparing their deviation with the previous standard peak area.

10) Detection limits of the instrumental methods of analysis for each analytical compound were analyzed by preparing standards of lower concentrations. The detection limits for each instrument are given in the Appendix B.

11) The pH probe was calibrated on the daily basis before measuring the pH of the samples. And fresh pH buffers were replaced once in three days.

12) The glass cuvette used for the spectrophotometer were cleaned with MQ water and wiped with Kimwipes® disposable wipers before and after usage and placed in iso-propanol solution to keep free from bacterial contamination on the cuvette walls.

13) All of the batch scale and pilot scale experiments were conducted in replicates, in order to test the reproducibility associated with the process and determine the errors associated with the sampling and analysis.

Note: The results of QA/QC are presented in Appendix B.

3.14 References


CHAPTER 4: EFFECT OF CULTURE TYPE AND LINOLEIC ACID ON HYDROGEN PRODUCTION IN AN UP-FLOW ANAEROBIC SLUDGE BLANKET REACTOR USING MIXED MICROFLORA

4.1 Introduction

Research on hydrogen (H\textsubscript{2}) production from organic waste through biological processes has gained momentum over the past decade. Bio-H\textsubscript{2} serves as an attractive alternative energy source because of the clean energy source and sustainability compared with non-biological H\textsubscript{2} production methods such as steam reforming, thermochemical and gasification processes (Das and Veziroglu, 2001). The biological processes of photolysis, photo and dark fermentation are under investigation, however, H\textsubscript{2} production rates (HPR) are higher in dark fermentation (Levin et al., 2004).

Utilizing mixed cultures to produce H\textsubscript{2} from renewable waste is advantageous over employing pure culture because they can operate under non-sterile conditions, they can be acquired from natural sources, they are suitability for converting a variety of mixed substrates and they can produce a wide variety of products spectrum volatile fatty acids (VFAs) and alcohols (Guo et al., 2010; Kleerebezem and van Loosdrecht, 2007). Different reactor systems have been such as an upflow anaerobic sludge blanket reactor (UASBR) (Yu and Mu, 2006), continuous stirred tank reactor (CSTR) (Show et al., 2007), anaerobic fluidized bed (AFB) reactor (Wu et al., 2003), anaerobic baffled reactor (ABR) and a trickling biofilter system have been employed for producing H\textsubscript{2}. However, because of their enhanced cell retention and treatment efficiency, UASBR are preferred over the other reactor configuration (Jung et al., 2011). Several studies have been carried out in UASBR using H\textsubscript{2} producing granules (HPG) (Chang and Lin, 2007; Kotsopoulos et al., 2006) since, Fang et al. (2002) first reported stable performance in UASBR with HPG.

Inoculum selection and the type of pretreatment have been reported to affect H\textsubscript{2} production in different reactor configurations. According to Chaganti et al. (2012) and Kim et al. (2003), the retention of a desired micro-flora for H\textsubscript{2} production in
bioreactors is due to the influence of these different factors. Pretreated inoculum in reactors with a continuous feed yielded stable H₂ production (Kongjan et al., 2010; Kotsopoulos et al., 2006), implying that a continuous process is practical and feasible for H₂ production in comparison with batch and semi-continuous processes (Hawkes et al., 2007).

In mixed culture fermentation, the H₂ produced is consumed by different H₂ consumers such as the hydrogenotrophic methanogens and homoacetogens (Abreu et al., 2011; Lovley and Klug, 1983). Hence, it is necessary to inhibit the activity of H₂ consumers in order to obtain a higher H₂ yield from mixed cultures. There are several inhibitory pretreatment methods employed for the inhibiting methanogenic activity with heat treatment as the most commonly employed method (Lay et al., 1999; Oh et al., 2003; Wang et al., 2007a). However, applying heat treatment in full-scale reactors as a pretreatment method is impractical. Furthermore, heat treatment also eliminates the non-spore forming H₂ producers such as Enterobacter spp. (Redwood et al., 2009). Hence, chemical methods of inhibition such as the use of 2-bromoethanesulfonate (Valdez-Vazquez et al., 2005), chloroform and acetylene (Zhao et al., 2010), or long chain fatty acids (LCFAs) (Ray et al., 2010) are preferred pretreatments but in some cases use of chemical inhibitors are impractical. Among the known chemical inhibitors, use of LCFAs is of more realistic because of their renewable source and ecofriendly nature i.e., bio-degradable. LCFAs are degraded to acetic acid and H₂ by acetogenic bacteria via beta-oxidation (Weng and Jeris, 1976). Palatsi et al. (2009) reported a possible mechanism for LCFA inhibition, where LCFA binds to the cell surface and subsequent stoppage of nutrient transport through the membrane. This is why flocculated cultures are more susceptible to LCFA inhibition than granulated cultures, since in the flocculated cell structure, more membrane surface area is exposed for chemical interaction (Hwu et al., 1996).

Granular cultures with higher cell retention times are preferred over immobilized systems for continuous H₂ production (Show et al., 2008). Zhang et al. (2008) reported that the granulated culture in a continuous system resulted in an enhanced HPR that was over 10 times higher than that of suspended culture. However, it should be noted that the mass transfer of the substrate into the microbial culture is effective with flocculated cultures in CSTR, although washout of biomass at lower hydraulic retention times (HRT) could be problematic (Chen et al., 2001; Lee et al.,
Hence, it is important to study the influence of culture type on H\textsubscript{2} production because of differences in the ability of the biomass to be retained at a lower HRT or higher loading rates. Many studies have indicated that the inocula type and the reactor configuration can play an important role in H\textsubscript{2} production (Danko et al., 2008; kaparaju et al., 2009; Saady et al., 2012). A comparison of the performance of different H\textsubscript{2} producing systems, including the inoculum type and the methods of inhibition applied is shown in Table 4.1. When comparing the performance of microorganisms in suspended or granular mixed culture systems, it is important to study them under the same operational conditions.

Table 4.1 Comparison of hydrogen production performance of continuous systems with different culture types

<table>
<thead>
<tr>
<th>Reactor configuration</th>
<th>Biological growth mode</th>
<th>Culture Pretreatment</th>
<th>HRT (h)</th>
<th>H\textsubscript{2} yield (mol mol\textsuperscript{-1} substrate)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>UASBR</td>
<td>Granular</td>
<td>Bromoethan o-sulfonate (BES)</td>
<td>26.7</td>
<td>2.47±0.15</td>
<td>(Kotsopoulos et al., 2006)</td>
</tr>
<tr>
<td>UASBR</td>
<td>Granular</td>
<td>Heat shock at 105 °C</td>
<td>17</td>
<td>1.19±0.05</td>
<td>(Wang et al., 2007b)</td>
</tr>
<tr>
<td>EGSB</td>
<td>Granular + Immobilization</td>
<td>-</td>
<td>2</td>
<td>3.47*</td>
<td>(Guo et al., 2008)</td>
</tr>
<tr>
<td>CSTR/ UASBR</td>
<td>Suspended/ Granular</td>
<td>Heat shock at 90 °C</td>
<td>8/6</td>
<td>0.3 and 1.3*</td>
<td>(Jung et al., 2010)</td>
</tr>
<tr>
<td>CSTR</td>
<td>Suspended</td>
<td>-</td>
<td>6</td>
<td>1.40*</td>
<td>(Chang et al., 2011)</td>
</tr>
</tbody>
</table>

* indicates mol mol\textsuperscript{-1} hexose

Although there have been various studies of assessing inhibited mixed cultures in batch and continuous reactors inoculated with granulated and/or flocculated (suspended) culture have been reported (Abreu et al., 2011; Danko et al., 2008). Using linoleic acid (LA) to inhibit flocculated and granulated culture for continuous H\textsubscript{2} production has not been examined. Linoleic acid, an unsaturated LCFA, is an effective methanogenic inhibitor during fermentative H\textsubscript{2} production (Chowdhury et al., 2007). The objective of this study was as follows: 1). Evaluate the H\textsubscript{2} production performance of pretreated flocculated and granulated cultures in UASBRs 2) Study LA degradation in continuously operated cultures and 3) Employ terminal restriction fragment length polymorphism (T-RFLP) to characterize the microflora for each experimental condition.
4.2 Materials and methods

The experiment was conducted using granular and flocculated cultures using culture B with an initial VSS concentration of 10 g L\(^{-1}\) (see sections 3.3 for inoculum source and maintenance). The flocculated cultures were prepared by crushing the granular cultures and passing the through a mesh of sieve number 200. The experiment was conducted at 37 °C at an operating pH of 5.0±0.2 for three different HRTs (48, 36 and 24 h) (see section 3.5 for reactor operation). Glucose (5 g L\(^{-1}\)) was selected as the substrate for this study. The reactor was converted from the sequential batch mode to continuous operation after 7 days (indicated by start period in Figure 4.1). The parameters selected for the study was based on the previous studies conducted by Chaganti et al. (2013) using similar culture source operating under sequential batch mode. The current study was conducted for more than 60 days using flocculated and granular culture. Experiments were conducted in duplicate using two UASBRs (designated as reactor R1 and R2). At each experiment condition shown in Table 4.2, experiments were repeated at least 7 times using reactors R1 and R2.

Table 4.2 Hydrogen and methane production performance in continuous operation of UASBRs containing flocculated and granulated cultures under different HRTs with and without LA

<table>
<thead>
<tr>
<th>HRT (h)</th>
<th>Culture Type</th>
<th>H(_2) production rate (^a) (L L(^{-1}) d(^{-1}))</th>
<th>CH(_4) production rate (^b) (L L(^{-1}) d(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>LA</td>
<td>Control</td>
</tr>
<tr>
<td>48 Flocculated</td>
<td>0.13±0.07(^{B,C})</td>
<td>0.38±0.04(^{C})</td>
<td>0.36±0.04(^{B})</td>
</tr>
<tr>
<td>Granular</td>
<td>0.24±0.06(^{B})</td>
<td>0.39±0.02(^{C})</td>
<td>0.39±0.08(^{A,B})</td>
</tr>
<tr>
<td>36 Flocculated</td>
<td>0.36±0.09(^{B})</td>
<td>0.88±0.21(^{B})</td>
<td>0.30±0.03(^{B})</td>
</tr>
<tr>
<td>Granular</td>
<td>0.40±0.05(^{B})</td>
<td>0.71±0.06(^{B})</td>
<td>0.46±0.07(^{A})</td>
</tr>
<tr>
<td>24 Flocculated</td>
<td>0.86±0.11(^{A})</td>
<td>1.67±0.25(^{A})</td>
<td>0.26±0.04(^{B,C})</td>
</tr>
<tr>
<td>Granular</td>
<td>0.90±0.09(^{A})</td>
<td>1.53±0.07(^{A})</td>
<td>0.25±0.04(^{B,C})</td>
</tr>
</tbody>
</table>

\(^a,b\) The data are the mean values at each HRT (the “±” denotes standard deviation for \(n \geq 7\)). \(^A, \, B, \, C\) indicate statistically different means in the same column.

All the chemical and analytical methods used in this study are described in sections 3.7.3 and 3.8, respectively. Characterization of the LCFA degradation in the continuous UASBR systems treated with, 1000 mg LA L\(^{-1}\) (3.56 mM LA) was carried out in this study. The LCFA extraction methods are outlined in section 3.8.6. The microbial methods used for identifying the pattern in the cultures are as outlined in
sections 3.10. The statistical methods include a principal component analysis (PCA)-biplot used for correlating the samples under different conditions to their metabolic profile and a principal coordinate analysis (PCoA) used for correlating the diversity in microbial profile. All statistical methods are described in section 3.12.

The experimental plan is described in Table 4.2, along with the performance outcomes (HPR and CH₄ production rate (MPR)).

4.3 Results

4.3.1 Continuous hydrogen and methane production using flocculated culture

Continuous H₂ production was carried out in UASBRs fed 5 g glucose L⁻¹ at pH 5.0. The data in Table 4.2 show that in the control cultures the HPR increased with decreasing HRTs from 48 h to 24 h. However, note a significant amount of CH₄ was detected for all of the HRTs examined in this study. An increase in HPR (≥ 90%) and decrease in MPR (85%) was observed in LA inhibited culture compared to the corresponding untreated control cultures operating at the same HRT (Table 4.2). In control cultures, the decrease in CH₄ yield was 28% (from 1.01±0.13 to 0.72±0.10 mol mol⁻¹ glucose) with a decrease in HRT suggested that CH₄ production was not suppressed even at pH 5.0 (Figure 4.1a). With pretreatment (inhibition by LA), the CH₄ yield was reduced to 0.17±0.04 mol mol⁻¹ glucose at the end of operating at a 48 h HRT (day 40) and the CH₄ yields were negligible with further decreases in the HRT. The H₂ yield in the LA inhibited cultures increased from 1.24±0.14 mol mol⁻¹ glucose and reached a peak of 3.16±0.22 mol mol⁻¹ glucose at 24 h HRT on day 57; however, the yield decreased to 2.28±0.20 mol H₂ mol⁻¹ glucose with further operation (Figure 4.1a).

4.3.2 Continuous hydrogen and methane production using granulated culture

In granular cultures, increasing H₂ production with decreasing the HRT was similar to the trend observed for the flocculated cultures (Table 4.2, Figures 4a and b). The maximum H₂ yield obtained from untreated control (C) cultures was 1.50±0.07 mol mol⁻¹ glucose at a 24 h HRT. A maximum H₂ yield and HPR of 2.48±0.08 mol mol⁻¹ glucose and 1.56±0.07 L L⁻¹ d⁻¹, respectively, were observed in LA treated cultures operating at a 24 h HRT (Figure 4b and Table 4.2). The CH₄ yields (mol mol⁻¹ glucose) in the control cultures decreased from 1.21±0.10 to 0.34±0.06 when the HRT decreased from 48 to 24 h (Figure 4b).
control cultures operating at 48 and 36 h, a 1.5 fold increase in the H\textsubscript{2} yield was observed in LA treated cultures operating at the same HRT conditions (Figure 4b). The CH\textsubscript{4} yield observed in LA treated cultures ranged from 0.12 to 0.22 mol mol\textsuperscript{-1} glucose, which is approximately 35-60\% less in comparison to the control cultures operating at similar HRTs.

Figure 4.1. Hydrogen and methane yields under different HRT for control (C) conditions and after treatment with linoleic acid (LA): a) flocculated culture b) granulated culture

Notes: The H\textsubscript{2} and CH\textsubscript{4} yields plotted shows average values for duplicate reactors R1 and R2.
4.3.3 Substrate utilization

The effectiveness of the substrate utilization is characterized from the residual level present in the effluent and the byproduct produced during the fermentation. In addition to the gaseous products produced, the soluble metabolite products (SMP) included volatile fatty acids such as acetic acid (HAc), propionic acid (HPr) and butyric acid (HBu) as well as alcohols such as ethanol (EtOH) and i-propanol (i-PrOH). In the current study, glucose conversion was more than 95±2% in both the granulated and flocculated cultures throughout the experiment (> 60 days of operation). This large conversion indicates that the reactor operation was suitable for microbial fermentation (data not shown).

4.3.4 Soluble metabolite profile and electron balance

The metabolite profile obtained during continuous dark fermentation using glucose as the substrate is shown in Figures 4.2a and b for flocculated and granulated culture, respectively. HAc and HBu were the major SMPs present throughout fermentation. The HAc and HBu levels, 4 to 15 mM, detected in the untreated flocculated culture were stable during operation at each HRT. With the addition of LA, the HAc levels increased gradually from 15.8±0.8 mM on day 43 to 41.2±3.2 mM on day 56 of reactor operation (Figure 4.2a). When the reactor operation was extended further, the HAc level slowly decreased to 26.9±1.4 mM on day 65. In addition, the HBu concentration decreased over this period with the level decreasing from 12.6±1.6 mM on day 43 to 4.5±0.2 mM on day 64 in LA inhibited flocculated cultures.

In flocculated cultures, the EtOH levels reached 9±2 mM in the untreated control samples and were reduced by approximately 75% after adding LA. The EtOH levels in the control flocculated cultures on an average increased to 10±1 mM (between days 23-27) and in the LA treated cultures operating at 24 h HRT the EtOH levels decreased to 4.1±0.4 mM (between days 63-68). The other byproducts observed such as HPr and i-PrOH were found to decrease following LA treatment when compared to the control conditions under the same HRT. The HPr levels in the LA treated flocculated cultures was in the range of 0.5±0.0 to 2.0±0.2 mM, which is approximately 30% less compared to the HPr levels observed in the untreated
flocculated culture. With the addition of LA, the i-PrOH levels reduced initially and stabilized to 2.6 ± 0.2 mM during 61-68 days of the operation.

In the case of granulated culture (shown in Figure 4.2b), consistent increases in the HAc levels were observed as HRT was reduced for the control conditions. Following LA treatment, the HAc levels remained stable until day 40 when HRT was reduced to 36 h, after which there was a sweeping increase in HAc levels over a period of 10 days, from 12.5±0.8 mM on day 41 to 36.5±2.0 mM on day 53. The HAc level then stabilized (between the 54th and 63rd day of the operation and at a 24 h HRT) in the range of 32.9 to 35.3 mM. There was no evident trend observed in the HBu levels detected in untreated granular culture as HRT was reduced. The HBu levels in control samples was in the range of 3.6±0.5 mM to 7.1±0.7 mM, whereas in the LA inhibited granulated culture, HBu was detected primarily in the range of 7.1±1.0 mM to 12.1±1.2 mM. These levels were higher than the levels observed in LA inhibited flocculated culture. The HPr levels were found to vary from 2.6±0.3 mM to 5.8±0.7 mM in both untreated and LA inhibited granular cultures at different HRTs examined in this study.

The EtOH and i-ProOH levels in the granular control cultures increased when the HRT was decreased from approximately 3 mM to 7 mM. In the LA treated granular cultures, the alcohol levels reached a maximum of approximately 3.2 mM.
Figure 4.2 Soluble metabolite distribution during anaerobic fermentation at different HRTs with: (a) flocculated culture (b) granulated culture

Notes:
1. The operating conditions at each stage are specified over the arrow, where the #s represent the HRT and the letters ‘C’ and ‘LA’ correspond to untreated control and LA-treated culture.
2. Acetic acid = HAc; propionic acid = HPr; butyric acid = HBu; isopropanol = i-PrOH and ethanol = EtOH.
3. The error bars represent the standard deviation for n = 4
The electron-equivalent balance as the percent electron equivalents (e⁻ equiv) distributed from glucose fermentation under the various experimental conditions is shown in Figure 4.3. The total e⁻ equiv balance for the fermentation byproducts ranged from 89±7% to 104±8% for all the conditions examined in this experiment. The fs value (fraction of e⁻ equiv from glucose to biomass) was assumed to be constant as 10% of the e⁻ equiv from the initial glucose (Rittmann and McCarty, 2001). In the untreated flocculated and granular cultures, the largest electron sink was in CH₄, followed by HBu and HAc, which each accumulated 10-15% of e⁻ equiv on average. The electron sink in the HPr was smaller and was measured at levels varying from 5-7%, while i-PrOH and EtOH accounted for 13-20% of e⁻ equiv.

Following treatment with LA, differences in the electron distribution to metabolites were observed in both the granular and flocculated cultures. In the case of LA treated flocculated culture, a large fraction of the electrons was transferred to H₂ at 36 h and 24 h HRT with the HAc levels varying between 13 and 35%. This result support the idea that HAc-type of fermentation is associated with higher H₂ yield. Initially, the major electron sink observed was HBu was with 30% of the available e⁻ equiv derived from glucose; however, then the HAc increased in a linear fashion with a corresponding decrease in the HBu e⁻ equiv to 18% (Figure 4.3). This suggests that acid (HAc through acetyl-CoA and HBu) formation is favorable for H₂ production, whereas alcohol production is an electron consuming reaction involving NADH (see section 3.11 for reactions). As there was an accumulation of H₂ e⁻ equiv along with HAc e⁻ equiv, the fermentation byproducts, i-PrOH, HPr and EtOH, could not compete for the electrons successfully, confirming that the electron sink for these metabolites would be less if the HAc and H₂ were the dominant byproducts of fermentation (Ren et al., 1997).

In the case of granular culture treated with LA, CH₄ production was markedly reduced by 80-87%, while percent reduction in electron sink diverted to CH₄ for LA treated flocculated culture is 83-95%. Note the deviation in suppression of methanogenesis in granular and flocculated cultures were less than 10%. The electron sinks in the CH₄ of LA treated granular cultures decreased from 7% to 3% with a reduction in the HRT. This suggest that with a further reduction in the HRT greater suppression of CH₄ production could be achieved.
In the LA treated granular cultures, the major electron sinks (26%) were in HBu at 48 h and 36 h HRTs and HAc (30%) at a 24 h HRT (Figure 4.3). The electrons diverted towards HPr were the same for LA treated granular culture as in the control culture. Low electron sinks were observed for EtOH and i-PrOH. The e⁻ sink due to the residual glucose remaining in the effluent was ≤ 5%.

Figure 4.3 Electron equivalent balance of fermentation byproducts from flocculated and granulated cultures

Notes: The data represent the mean values of runs obtained at steady state operation for each HRT, and the error bars represent the percent standard deviation for the total electrons distributed to the byproducts and biomass. Residual glucose = RG; Acetic acid = HAc; propionic acid = HPr; butyric acid = HBu; iso-propanol = i-PrOH and ethanol = EtOH.

4.3.5 Linoleic acid degradation

Linoleic acid degradation was observed in both flocculated and granulated cultures. The major long chain fatty acids resulting from this degradation that were detected were lauric acid (LUA), myristic acid (MA), palmitic acid (PA) and caproic acid (CA). In the flocculated cultures, PA and LUA were the major degradation by-products, and their maximum concentration at 1.15±0.1 mM (PA) and 1.2±0.1 mM (LUA) was observed following the completion operation at at 36 h and 24 h HRTs, respectively (Figure 4.4a). MA, the other major byproduct, with a maximum concentration of 0.74±0.12 mM was detected at the end of 24 h HRT. Only a small amount of CA (C6) was detected. In the granulated culture, the LA concentration at any time was approximately 20% less than that observed for the flocculated culture.
The major degradation byproducts observed in the granular cultures (Figure 4.4b) were MA (1.4±0.1 mM), PA (0.8±0.1 mM) and LUA (0.56±0.07 mM). The PA and LUA concentrations were approximately 30% less than the levels detected in the flocculated culture. Only trace amounts of stearic acid (SA) and oleic acid (OA) were detected in both the flocculated and granular cultures. There was some amount of LCFA that were washed out of the reactors in both flocculated and granulated cultures (data not shown). At the end of 48 h HRT the total LCFA present in the reactors was approximately 2.39 and 2.77 mM (equivalent of LA) in granulated and flocculated cultures, respectively.

![Figure 4.4 Linoleic acid (LA) degradation and its byproducts over the period of reactor operation (a) flocculated culture (b) granulated culture](image)

**Note:** The x-axis title ‘operating day’ refer to the time when the liquid sampled was removed for LCFAs analysis
4.3.6 Principal component analysis

A PCA was used to visualize the differences between the two types of culture (flocculated and granulated) before and after treatment with LA. The input data for the PCA included the glucose fermentation products. The dataset for the fermentation products consisted of 7 response variables (measures of gas and major liquid metabolites) and 12 samples or experimental conditions (i.e., 2 cultures x (3 HRTs without LA + 3 HRTs with LA)). The PCA reduced this complex dataset involving multiple variables to a small number of PCs. The first three PCs accounted for more than 94% of the total variability found in the dataset. Only the first and second PCs (PC1 and PC2) are shown in Figure 4.5, as these two components explained 80% of the total variance and the addition of more components did not markedly improve the strength of the model.

Figure 4.5 Principal component analysis of gas and soluble metabolite products (SMP) in granulated and flocculated cultures

Notes:
1. #s 24, 36 and 48 represent the HRT (h); Granular = G (circles); Flocculated = F (triangles); Control = C (open) and linoleic acid treatment = LA (closed).
2. Acetic acid = HAc; propionic acid = HPr; butyric acid = HBu; iso-propanol = i-PrOH and ethanol = EtOH, hydrogen = H₂, methane = CH₄.

A PCA biplot was used to visualize the relationship between the response variables and sample distribution within the two-dimensional plane (according to the relative influences of PC1 and PC2 on each data point). The PCA biplot presented in
Figure 4.5 shows that there is a relationship evident among the different operating stages for the reactor (i.e. grouping of the conditions under which similar metabolic profiles were observed). PC1 accounted for 59.58% of the total variability and was associated with the concentrations of CH₄, HPr and alcohols. PC2 accounted for a further 20.27% of the variability in the original data set and was primarily associated with HBu, HPr and i-PrOH.

Figure 4.5 shows that the control conditions for both cultures, flocculated (F-C) and granular (G-C), at all of the tested HRT (24, 36, and 48 h) were grouped together in the same (lower-right) quadrant of the biplot. The data points for the untreated granulated and flocculated cultures from the first stage of operation at 48 h HRT (labeled 48 G-C and 48 F-C) were positioned closer together on the plot and similar trends in the metabolite distribution were observed with a decrease in the HRT. The control cultures were primarily dominated by the presence of CH₄, EtOH, and to a lesser extent, i-PrOH. This is evident in the close proximity of the control samples with the loading vectors of these byproducts on the PCA biplot.

The addition of LA to the granulated cultures had a similar effects at longer HRT and only a small amount of variation was detected between the 48 h and 36 h HRT data set. Note LA treated granular cultures operating at 48 h and 36 h (36 G-LA and 48 G-LA) were clustered along with the LA treated flocculated cultures operating at 48 h (48-F-LA) in the upper half of the biplot in association with the metabolites such HBu and HPr. However, a reduction of HRT to 24 h placed the LA treated granular culture (24 G-LA) in a different quadrant (lower left) of the biplot along with LA treated flocculated cultures at 36 and 24 h HRT. The dominant metabolites associated with this grouping of LA treated samples were H₂ and HAc.

4.3.7 Microbiological analysis

Based on T-RFLP analysis with restriction enzyme Hae III a total of 99 different T-RFs ranging from 42–506 bp was revealed (Figure 4.6). Diversity in the fermentative microbial population was greater in the granular LA treated cultures in comparison to granular control cultures, except for cultures operating at 36 h HRT. However, in the flocculated cultures treated with LA showed less diversity in the microbial communities was observed when compared to the control cultures (Figure 4.6).
A PCoA was applied to the T-RFLP profiles from *Hae* III digestion for both of the culture types, including untreated control and LA treated cultures. PCoA of the T-RFLP profiles showed that the first three principal coordinates explained 55% of the variance in the data set. Among these three, the first two principal coordinates (PCoA1 and PCoA2) shown in Figure 4.7 accounted for 39.36% of the total variability in the dataset. The PCoA plot of the T-RFLP profiles using the enzyme *Hae* III dataset revealed 4 different clusters in the microbial pattern of the untreated flocculated and granulated cultures.

**Figure 4.6** Comparison of flocculated and granulated cultures based on the terminal restriction fragments generated by *Hae* III enzyme digestion in control and LA treated conditions

**Note:** #s 24, 36 and 48 represent the HRT (h); Granular =G; Flocculated =F; Control =C and linoleic acid treatment =L.
Figure 4.7 Principal coordinate analysis (PCoA) of T-RFLPs of Hae III digest in flocculated and granulated cultures

Note: #s 24, 36 and 48 represent the HRT (h); Granular = G (circles); Flocculated = F (triangles); Control = C (open) and linoleic acid treatment = LA (closed)

The control cultures from both flocculated and granulated cultures were observed in the bottom half of the PCoA plot. The granular control cultures were placed in close proximity to each other and clustered with the untreated flocculated culture operating at 24 h HRT. The LA treated flocculated (upper left quadrant) and granulated (upper right quadrant) cultures were grouped in different quadrants. The exception to this clustering was the LA treated granular culture operating at 48 h HRT (48-LA-G) which was grouped along with the LA treated flocculated cultures in the co-ordinate plane.

4.3.8 Evaluation of culture type: granulated vs flocculated

Evaluation of the granulated and flocculated cultures was conducted in the UASBR with HRT decreasing from 48 to 24 h. The results indicated similar performance in terms of H₂ production by both untreated cultures (i.e., control conditions that were not treated with LA). After LA treatment, the inhibited flocculated culture showed H₂ yields ranging from 2.28 to 3.16 mol mol⁻¹ glucose at 36 and 24 h HRT, whereas the granular culture treated with LA had lower H₂ yields in the range of 1.90 to 2.48 mol H₂ mol⁻¹ glucose at the same HRT conditions (see Figure 4.1). There were no significant difference observed in the HPRs for the flocculated and granulated cultures in both controls and LA treated cultures operating
under same HRT. This suggests that the culture type did not have a significant impact on the HPR for the HRTs conditions examined in this study (Table 4.2).

![Graph showing biomass concentration in reactor and effluent](image)

**Figure 4.8 Biomass concentration in reactor and effluent**

**Notes:**
1. The circles represent the granular cultures, with the open (○) corresponding to reactor VSS and the closed (●) corresponding to VSS in the effluent.
2. The diamonds represent the flocculated cultures, with the open (◇) corresponding to reactor VSS and the closed (●) corresponding to VSS in the effluent.
3. The VSS concentration in the effluent and reactor represent the concentration of biomass observed at that day.

The biomass content in the flocculated culture system was lower than in the granulated culture system. With the continuing operation of the system, a large amount of biomass was washed out of the flocculated system (Figure 4.8). Show et al. (2010) reported that a HRT equal to the critical washout point of suspended (flocculated) culture is required for granular culture in order to achieve higher HPR. In the current study, the biomass concentration of the flocculated cultures decreased from an initial concentration of 10 g VSS L\(^{-1}\) to 5.6±0.6 g VSS L\(^{-1}\), while a biomass concentration of 8.8±0.6 g VSS L\(^{-1}\) was maintained in granular cultures (Figure 4.8). This finding suggests that the non-hydrogen producing bacteria (non-HPB) such as methanogenic bacteria, HPr producing bacteria (PAB) or EtOH producing bacteria may remain intact in the granulated cultures indicating the fact that HRT plays an important role in the stability of granules. In comparison, non-HPB present in the flocculated cultures might be washed out with decreasing HRTs. Note the total percent of electron diverted to CH\(_4\), HPr, i-PrOH and EtOH was greater in granular
cultures by a range of 5 to 13% in all the HRTs conditions examined in this study. With decreasing the HRT from 48 h to 24 h, further washout of the biomass in the flocculated cultures was observed. This caused a decrease in the H$_2$ yield in comparison to the stable H$_2$ yield observed with using the granular cultures (Figure 4.1a and b). One possible reason for the decrease in H$_2$ yield could be due to the loss of H$_2$ producers along with non-H$_2$ producers over the time period.

4.4 Discussion

Several studies have examined methanogenic suppression during continuous H$_2$ production (Abreu et al., 2011; Hafez et al., 2011; Zhu et al., 2011). In past studies, the pretreatment of the culture (e.g. the application of heat, 2-bromoethanolsulfonate (BES) or chloroform) was investigated. However, using LCFAs to inhibit methanogens in full-scale bioreactors is a more practical approach because they are easily available from renewable sources, relatively inexpensive and they do not pose a major environmental hazard if discharged into receiving water bodies. Previous studies published on LCFA inhibition explored the impact of microbial treatment with biodegradable LCFA on the bacterial population (Hwu et al., 1998; Palatsi et al., 2009; Rinzema et al., 1994; Sousa et al., 2008). However, these reports focused on LCFA degradation, CH$_4$ inhibition and the recovery of methanogenesis. In the present study, the H$_2$ production potential using LA (C18:2) inhibited granular and flocculated cultures was examined in UASBR at long retention times. Among the LCFA, LA is known to enhance H$_2$ production from glucose and to suppress CH$_4$ production in the fermentation pathway (Ray et al., 2008).

The H$_2$ and CH$_4$ profiles presented in Figure 4.1a and b show that in both flocculated and granulated control cultures ≥ 70% decrease in the CH$_4$ yield was observed with decrease in HRT. Although a decrease in CH$_4$ production was observed in the control cultures during the period prior to LA treatment the resurgence of the CH$_4$ is possible under long term operation. Spagni et al. (2010) have reported that during long-term operation, CH$_4$ production was observed after 20 d of the reactor operation. Similar to the CH$_4$ yield observed in the control cultures operating at 24 h HRT in the current study, Yang et al. (2007) observed CH$_4$ yield of approximately 0.4 mol mol$^{-1}$ hexose with reduced pH systems operating in the range of pH 4 to 5 in a
CSTR operating at 24 h HRT using cheese whey as the substrate with concentration equal to 10 and 12 g L\(^{-1}\) COD.

In this study, the inhibition of methanogens by adding LA is similar to evidence provided by (Kim et al., 2004; Koster and Cramer, 1987). In the current study even after 60 days of operation, H\(_2\) yields > 2 mol mol\(^{-1}\) glucose was observed in both the flocculated and granulated LA inhibited cultures (Figures 4.1a and b). Under untreated control conditions, the granular culture HPRs were greater than the HPRs for flocculated culture operating at a 48 h HRT (Table 4.2). However, with the HRT reduced to 24 h, the H\(_2\) production performance of both cultures was similar, varying by less than 5\%. The reason that granular cultures may perform similar to that of flocculated cultures could be because the granulated systems were not operating at a HRT equal to the critical washout point of flocculated culture (Show et al., 2010).

A decrease in HRT was accompanied by an increase in the organic loading rate (OLR) with the influent substrate concentration remaining constant. The HPR observed in this study is low in comparison to studies using pure glucose reported in literature (Van Ginkel and Logan, 2005b; Wu et al., 2008). The results obtained using the mixed cultures in this study were comparable to those obtained in pure culture systems. For example, Ramachandran et al. (2011) reported an HPR equivalent to that obtained by the granular LA treated cultures (0.74 to 2.89 mmol H\(_2\) L\(^{-1}\) h\(^{-1}\)). The H\(_2\) yields obtained with LA treated cultures is comparable to that obtained by Van Ginkel and Logan (2005b) in the heat treated cultures operating with a low HRT and reduced OLR. Notice, these authors observed HPR equivalent to 3.5 L L\(^{-1}\) d\(^{-1}\) for cultures operating at 10 h HRT and 5.1 g glucose L\(^{-1}\). Therefore, the likelihood of increasing the HPR by decreasing the HRT is high. Studies by Wu et al. (2008) reported an HPR of approximately 22 L L\(^{-1}\) d\(^{-1}\) at a 4 h HRT using glucose and showed high biomass retention (up to 10 g VSS L\(^{-1}\)), whereas an HPR of approximately 10 L L\(^{-1}\) d\(^{-1}\) with a biomass concentration of 1.6 g VSS L\(^{-1}\) was observed for a suspended culture in a CSTR under the same operating conditions. Similar to results obtained by Wu et al. (2008), granular cultures showed high biomass retention over the range of HRTs tested in this study, however, no significant changes in HPR was observed between flocculated and granulated cultures operating under same conditions (Table 4.2; Tukey’s test for multiple comparisons of means, \(P<0.05\), (Box et al., 1978).
The HPR obtained using heat treated granular cultures in UASBR fed glucose were in range of 1.0 to 2.0 L L\(^{-1}\) d\(^{-1}\) (Spagni et al., 2010). Note Spagni et al. (2010) attributed a low HPR to increase in methanogenic activity within the duration of experiment conducted. In the current study, the low HPR is attributed to high HRT followed by product distribution in the fermentation pathway i.e. mixed acid and alcohol fermentation in addition to CH\(_4\).

VFA production is greatly influenced by pH in glucose fermentation (Zheng and Yu, 2004). The pH of the effluent varied between 4.6 and 5.0 throughout the course of fermentation in the current study. Glucose fermentation produces various byproducts along with the generation of H\(_2\). Equations 4.1 and 4.2 show that when HAc and HBu are end products of glucose fermentation, higher H\(_2\) yields can be attained.

\[
\text{C}_6\text{H}_12\text{O}_6 + 4\text{H}_2\text{O} \rightarrow 4\text{H}_2 + 2\text{CH}_3\text{COO}^- (\text{HAc}) + 2\text{HCO}_3^- +4\text{H}^+ \quad \Delta G_0^\circ = -206.3 \text{ kJ mol}^{-1} \quad (4.1)
\]

\[
\text{C}_6\text{H}_12\text{O}_6 + 2\text{H}_2\text{O} \rightarrow 2\text{H}_2 + \text{CH}_3\text{CH}_2\text{CH}_2\text{COO}^- (\text{HBu}) + 2\text{HCO}_3^- +3\text{H}^+ \quad \Delta G_0^\circ = -254.8 \text{ kJ mol}^{-1} \quad (4.2)
\]

The liquid byproducts produced from glucose fermentation correlated with the H\(_2\) yields under the operating conditions examined in this study. HAc and HBu are the major liquid by-products linked to H\(_2\) production, whereas EtOH production at high concentration is not favorable for H\(_2\) generation (Yan et al., 1988). In the current study, the HAc concentration was positively correlated with the H\(_2\) yield obtained from both types of cultures at various operating conditions. This trend is consistent with evidence provided by Kadar et al. (2004) using glucose, xylose, a mixture of glucose and xylose and a hydrolysate produced from paper. Note Kadar et al. (2004) studies were conducted with a thermophilic pure culture in a batch reactor. On day 57 (i.e., after the addition of LA), the flocculated culture reached a maximum H\(_2\) yield of 3.16±0.22 mol mol\(^{-1}\) glucose and the corresponding HAc concentration was 41.2±3.2 mM. In granular cultures, the H\(_2\) yield was maximum on day 59 with 2.48±0.21 mol mol\(^{-1}\) glucose and the corresponding HAc concentration was 35.9±2.9 mM.

Several reports have used the HBu to HAc ratio as an indicator of H\(_2\) production (Chen et al., 2001; Cheng et al., 2008). However, other studies have reported that the ratio alone cannot be used as an indicator of H\(_2\) production performance (Kim et al., 2006; Wang et al., 2007a). Studies by Van Ginkel and Logan (2005a) and Davila-
Vazquez et al. (2008) reveal that HBu is more toxic in comparison to HAc. Davila-Vazquez et al. (2008) reported HBu levels ≤ HAc levels for maximum H\textsubscript{2} yields observed using lactose and cheese whey powder as the feed. In comparison, Sung et al. (2004) reported that when the HAc to HBu ratio increased, the H\textsubscript{2} yield increased for a heat treated inoculum operated in batch reactors. Similar to the results obtained by Sung et al. (2004) in a pretreated inoculum, HAc concentrations increased in both cultures after the addition of LA compared to the other SMP concentration. Inhibition of methanogenesis by LA might have diverted more electron fluxes to HAc and H\textsubscript{2} instead of reduced end products such as HPr, EtOH and HBu. UASBR operating with flocculated or granulated cultures treated with LA was efficient for H\textsubscript{2} production under the conditions examined because of the byproduct distribution pattern. The HAc level was dominant followed by HBu and lower levels of EtOH production are similar to the trends reported (Ito et al., 2005; Lee et al., 2006).

In the case of granulated culture, the flow of electrons towards alcohol production was in the range of 12-17% (except for the 24 h HRT condition with LA inhibition). Ren et al. (1997) reported a significant amount of alcohol (ethanol) production from acidogenic culture under acidic conditions operating at an OLR ranging from 19 to 107 g COD L\textsuperscript{-1} d\textsuperscript{-1} with a molasses feed. These authors also observed that at pH levels of 4.5-5.0, HPr production was suppressed. The results from the current study (run at pH 5) also found that the HPr levels were relatively low. In the granulated cultures, 5-8% of the total electron equivalents from glucose was diverted to the formation of HPr while lesser amounts were utilized by the flocculated cultures (1-5%) (Figure 4.3).

The degradation mechanisms for LCFA are mainly due to the actions of LCFA-oxidizing bacteria and methanogenic archaea that live in a syntropic relationship with acetogens (Schink, 1997). The major degradation products of LA detected in the current study included PA, MA and CA. This is similar to data reported by Lalman and Bagley (2000). LUA was detected primarily in flocculated cultures (Figure 4.4). LUA is able to exhibit an increased toxicity effect in the presence of MA by working in synergistic manner on aceticlastic methanogens (Koster and Cramer, 1987). The synergistic effect of these fatty acids in combination on these cultures might be the reason for the H\textsubscript{2} production in the flocculated cultures to decrease on the days 62-67 (Figure 4.1a). In the flocculated cultures, the LUA reached a maximum of 0.63 mM
on day 60 while the concentration of MA was approximately 0.5 mM. Notice CH₄ production was increasingly suppressed in flocculated cultures treated with LA. The presence of PA can also affect the degradation of HBu and HPr to HAc (Salminen et al., 2000). The shift in the levels of HPr production observed in granulated culture with changes in HRT might be due to the presence of PA. Increasing HPr levels reaching approximately 5.2 mM was observed between day 38 to day 52 where the PA levels reached 0.8±0.07 mM. The HPr levels decreased to approximately 2.3 mM was coupled with a decrease in the PA levels to 0.42±0.05 mM between day 52 to day 60 of the reactor operation (Figures 4.2b and 4.4). In granulated cultures, high levels of MA and CA were detected in contrast to the low levels detected in the flocculated cultures.

The biplot illustrating the results of the PCA for the two major PCs (based on the data for gas metabolites and SMP under the various operating conditions) reveals that the byproduct distribution for the flocculated culture samples showed large variation between the inhibited and non-inhibited cultures (Figure 4.5). This is evident from the position of the samples in the two dimensional plane and their separation from the vectors of the biplot. The PCA showed that HRT is also associated with the metabolite distribution observed amongst the culture samples obtained under different operating conditions. This is confirmed by the grouping of the sample containing the cultures operated under similar treatment conditions in the biplot (Figure 4.5). In the case of untreated cultures with HRT of 48 h and 36 h, the clusters were grouped together, whereas when HRT was reduced to 24 h these elements were present at relatively distant proximity from the other control cultures. Clustering of conditions linked to longer HRT which was observed for the LA inhibited cultures indicated that HRT played an important role in metabolite distribution. Previous work by Wu et al. (2008) has shown that variation in HRT not only caused a change in the H₂ production profile, but also changed the metabolite distribution and the composition of the microbial population. These authors also showed that a change in the culture type altered the byproduct distribution. For example, in the suspended culture, the EtOH was dominant byproduct while in granular cultures HBu was dominant under similar operating conditions. Studies by Danko et al. (2008) reported that BES pretreated inoculum in batch reactors showed that composition of HAc were higher by 27% in flocculated cultures fed with glucose at mesophilic temperatures, whereas
granulated cultures showed that the HBu level increased by 10\% of the total SMP. The PCA results obtained from the response measures collected in this study showed that the LA inhibited granular cultures were associated with HBu fermentation at 48 h and 36 h HRTs (Figure 4.6). These results contradict those obtained by Saady et al. (2012) in batch reactors fed with LA, where the authors reported that the percent electron diverted to HBu were lower in granular cultures fed glucose in comparison to flocculated cultures fed with glucose plus LA.

The microbial diversity amongst samples of the different culture types and treatment conditions were analyzed using PCoA. Patterns indicating relationships amongst the microorganisms are visualized as clusters or groupings in the plot with first two principal coordinates (PCoA 1 and PCoA 2). The reason that the control cultures are grouped together (Figure 4.7) might be due to the presence of similar T-RFs (Figure 4.6). The control cultures showed presence of Bacillus sp. and Clostridium sp. in abundance; however, their relative percent was variable with the HRT. The Archaea data revealed methanogens belonging to Methanospirillum hungatei and Methanobacterium palustre was observed in abundance in both untreated flocculated and granulated cultures.

The reason that the LA treated granular and flocculated cultures are found grouped in separate quadrants of the 2-coordinate plane might be due to the presence of distinctive species (i.e., bandwidths distinguished by T-RFs with varying base pairs). The TRFLP showed that relative abundance of Clostridium sp. and Enterococcus sp. increased in flocculated cultures when the HRT was decreased from 48 h to 24 h in presence of LA. In comparison, in granulated cultures, Bacteroides sp. and Eubacterium sp. were observed in addition to Clostridium sp and Enterococcus sp.

In summary, adding LA at reduced pH to the two types of culture generally reduced the CH\textsubscript{4} and HPr levels with little variation in the composition of the microbial population (Figures 4.3 and 4.6). This is evident from the product distribution favoring H\textsubscript{2} and HAc production at low HRT for the LA treated cultures (Figures 4.3 and 4.5). The degree of stability of the microflora population with decreases in the HRT also confirms the presence of fermenting organisms that can produce H\textsubscript{2} in both flocculated and granulated cultures. The relationship between the microflora and H\textsubscript{2} production has been discussed by several authors (Chen and Lin,
In general, maintaining a stable reactor performance and an appreciable population of H$_2$ producing microflora is essential for the operation of continuous reactor systems. Loss of biomass might be the reason that the H$_2$ yield decrease in flocculated culture treated with LA at 24 h HRT (24-L-F). Studies by Pendyala et al. (2013) support the findings of current study where granular cultures is preferred because they contain a more diverse microbial population and are able to handle elevated COD.

4.5 Conclusions

Continuous H$_2$ production from the substrate, glucose, in UASBR inoculated with either granular or suspended culture under mesophilic conditions, and with and without the addition of the methanogenic inhibitor, LA, was studied, and the following conclusions were drawn:

1. Both granular and suspended culture performed in a similar fashion in control studies (i.e., without LA-treatment). The H$_2$ yield was 1.38±0.16 mol mol$^{-1}$ glucose in flocculated culture and 1.45±0.15 mol mol$^{-1}$ glucose in granulated culture at 24 h HRT after 25 days of continuous operation.

2. Adding LA induced an increase in H$_2$ yield in both flocculated and granulated cultures. An average H$_2$ yield (mol mol$^{-1}$ glucose) of 2.65±0.40 and 2.46±0.10, was obtained in LA fed flocculated and granulated cultures, respectively, at a 24 h HRT.

3. Based on electron flow distribution, control cultures (without LA) were associated with the production of CH$_4$ and alcohols such as i-PrOH and EtOH, whereas the cultures treated with LA were associated with the production of HBu in granulated cultures and HAc in flocculated cultures.

4. A metabolic shift to HAc-HBu type fermentation was observed with the addition of LA with low levels of other reduced end products such as HPr and alcohols.

5. Biomass retention was higher for granulated cultures than for flocculated cultures. This decrease in biomass concentration was accompanied by decrease in the H$_2$ yield from LA treated flocculated cultures operating at 24 h HRT.

6. LA degradation was observed in both the granular and flocculated cultures.
7. A PCoA of the T-RFLP data obtained from Hae III digestion revealed that there was less variation in the microbial composition of the control cultures under all operating conditions; however, variation in the microbial profiles between the LA treated flocculated and granular cultures was able to clearly distinguish the samples.

8. Control cultures showed an abundance of *Bacillus* sp. and *Clostridium* sp. in addition to methanogens belonging to *Methanospirillum hungatei* and *Methanobacterium palustre*.

9. The LA treated flocculated cultures showed abundance of *Clostridium* sp. and *Enterococcus* sp. while LA treated granulated cultures showed presence of *Bacteroides* sp. *Clostridium* sp. and *Eubacterium* sp.

10. For long-term continuous operation and achieving high HPR, granular culture is preferred to flocculated culture because of its ability to retain biomass with a diverse microflora population.

4.6 References


CHAPTER 5: EFFECT OF ORGANIC LOADING RATE AND HYDRAULIC RETENTION TIME ON CONTINUOUS HYDROGEN PRODUCTION USING GLUCOSE

5.1 Introduction

The world’s economy is largely based on non-renewable fossil fuels. Depleting reserves of these resource-based economic drivers have become increasingly apparent over the last decade. Significant research efforts have been invested in the development of future environmentally sustainable fuels. Hydrogen (H₂) is considered to be an alternative and renewable energy source. Hydrogen is a clean energy carrier with a high energy yield of 122 kJ g⁻¹ (Dunn, 2002; Thomas, 2000). Among the available H₂ production methods, dark-fermentative H₂ production is emerging as preferred process because of low temperature and pressure operational requirements (Rittmann and Herwig, 2012). Dark fermentation has the added advantages of combining waste treatment with fermentation of the substrate to produce H₂ (Castello et al., 2009) and also it has a high rate of H₂ production in comparison to photo-fermentation (Wang and Wan, 2009). The implementation of dark fermentative technology is of relatively low cost since the culture containing anaerobic fermentative bacteria does not require light and sterile operation conditions in comparison with pure bacterial cultures. Also, fermentative bacteria are capable of utilizing a wide variety of organic substrates (Das and Veziroglu, 2001; Hallenbeck, 2005).

Several process parameters controlling bio-H₂ fermentation processes includes factors such as pH, temperature, organic loading rate (OLR), hydraulic retention time (HRT), substrate type and reactor configuration (Li and Fang, 2007; Wang and Wan, 2009). In the dark fermentation process, OLR and HRT are the important factors influencing H₂ production in continuous flow bioreactors (Mohammadi et al., 2012; Zhang et al., 2012). Among the different factors, HRT plays a vital role in controlling H₂ production rate (HPR) by suppressing methanogenesis (Chen et al., 2001). However, the optimum HRT may vary depending on other process parameters. For example, Zhang et al. (2012)
reported that a 6 h HRT is optimal for glucose-fermenting halophilic H\textsubscript{2} producing bacterium with a maximum HPR of 9.5 mmol dm\textsuperscript{-3} h\textsuperscript{-1}. In comparison, studies by Kraemer and Bagley (2005) which reported a maximum HPR of 11.3 mmol dm\textsuperscript{-3} h\textsuperscript{-1} for a 10 h HRT with glucose as the substrate indicated that maximum HPR can be obtained at a lower HRT. Note, conditions such as substrate concentration pH and inoculum source were different for these studies. Studies by Wu et al. (2009) suggest that the optimal HRT reported in the literature is limited by the range of HRT’s investigated. Similarly, while a wide range of OLRs has been studied in different reactor systems, no optimum OLR could be defined for a particular system. Nevertheless, specifying a range of OLRs within which the system could operate effectively is of prime importance (Hafez et al., 2010). Many studies have reported contradictory effects of OLR on H\textsubscript{2} production. For example, higher OLR correlating to increasing the H\textsubscript{2} yield have been reported in some studies (Spagni et al., 2010; Zhang et al., 2004); however, in other studies, decreasing H\textsubscript{2} yield have been reported (Van Ginkel and Logan, 2005). Furthermore, many studies have shown increased H\textsubscript{2} yield at an optimum OLR and decreased yields after reaching the optimum OLR (Ren et al., 2006; Show et al., 2007). In general, the OLR is positively correlated with HPR (i.e., if the OLR is increased, then the HPR would also increase). This correlation has been reported in studies conducted by Wu et al. (2006) and Yu and Mu (2006).

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Gibb’s free energy ((\Delta G^0)) (kJ reaction(^{-1}))</th>
<th>Equation #</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\text{C}<em>6\text{H}</em>{12}\text{O}_6 + 4\text{H}_2\text{O} \rightarrow 2\text{C}_2\text{H}_3\text{O}_2^- + 2\text{HCO}_3^- + 4\text{H}^+ + 4\text{H}_2)</td>
<td>-206.3</td>
<td>5.1</td>
</tr>
<tr>
<td>(\text{C}<em>6\text{H}</em>{12}\text{O}_6 + 2\text{H}_2\text{O} \rightarrow \text{C}_4\text{H}_7\text{O}_2^- + 2\text{HCO}_3^- + 3\text{H}^+ + 2\text{H}_2)</td>
<td>-254.8</td>
<td>5.2</td>
</tr>
<tr>
<td>(4\text{H}_2 + \text{HCO}_3^- + \text{H}^+ \rightarrow \text{CH}_4 + 3\text{H}_2\text{O})</td>
<td>-135.6</td>
<td>5.3</td>
</tr>
<tr>
<td>(4\text{H}_2 + 2\text{HCO}_3^- + \text{H}^+ \rightarrow \text{C}_2\text{H}_3\text{O}_2^- + 4\text{H}_2)</td>
<td>-104.6</td>
<td>5.4</td>
</tr>
<tr>
<td>(\text{C}<em>6\text{H}</em>{12}\text{O}_6 + 2\text{H}_2 \rightarrow 2\text{C}_3\text{H}_5\text{O}_2^- + 2\text{H}_2\text{O} + 2\text{H}^+)</td>
<td>-359.2</td>
<td>5.5</td>
</tr>
<tr>
<td>(\text{C}<em>6\text{H}</em>{12}\text{O}_6 + 3\text{H}_2\text{O} \rightarrow \text{CH}_3\text{CH}_2\text{OH} + \text{C}_2\text{H}_3\text{O}_2^- + 2\text{H}_2 + 2\text{HCO}_3^- + 3\text{H}^+)</td>
<td>-215.7</td>
<td>5.6</td>
</tr>
<tr>
<td>(\text{C}<em>6\text{H}</em>{12}\text{O}_6 + 3\text{H}_2\text{O} \rightarrow \text{C}_2\text{H}_3\text{O}_2^- + 0.5\text{C}_4\text{H}_7\text{O}_2^- + 2\text{H}_2 + 2\text{HCO}_3^- + 3\text{H}_2 + 3.5\text{H}^+)</td>
<td>-230.5</td>
<td>5.7</td>
</tr>
</tbody>
</table>
Reduced H\textsubscript{2} yields in H\textsubscript{2} producing systems are attributed to high partial pressures within the system that accompany increase in loading concentration (Ruzicka, 1996). Table 5.1 lists typical dark fermentation reactions, which are involved in both the evolution and consumption of H\textsubscript{2} during dark fermentation of glucose. Other reasons of low H\textsubscript{2} yields might be due to the improper selection of operating conditions for cultivating H\textsubscript{2} producing cultures (Duangmanee et al., 2007; Kim et al., 2006) or due to the changes in the composition of the microbial populations under different loading conditions (Luo et al., 2008).

Maintaining optimum (high) biomass content (maintain the appropriate food: microorganism ratio at high OLR and low HRT) within the reactor system is necessary for an increased HPR (Kyazze et al., 2006). For this reason, high-rate reactors that can retain high biomass levels, such as the UASBRs, are preferred (Lettinga and Hulshoff, 1991). Mixed cultures used in dark fermentation are comprised of both H\textsubscript{2}-producing and H\textsubscript{2}-consuming microorganisms, such as methanogens and other non-H\textsubscript{2} producers, which consumes electron equivalents derived from substrate oxidation must be inhibited in order to obtain higher H\textsubscript{2} yields. Many microbial pretreatment methods were found to be effective in eliminating/suppressing these organisms. Heat (Wang and Wan, 2008) and acid and base treatments (Chang et al., 2002) are the major methods employed to eliminate non-H\textsubscript{2} producers and H\textsubscript{2} consumers. These pretreatment methods may affect the granular property of the sludge (Abreu et al., 2011). However, the simplest method to enhance H\textsubscript{2} yield by reducing competition between non-H\textsubscript{2} producers, H\textsubscript{2} consumers and H\textsubscript{2} producers is to adjust the operational parameters, such as pH, HRT and OLR, and monitor the impact of these variations on H\textsubscript{2} production. The effect of HRT and OLR on the elimination of H\textsubscript{2}-consuming or non-H\textsubscript{2}-producing organisms and the impact on biogas composition was recently studied (Hafez et al., 2011; Pakarinen et al., 2011; Spagni et al., 2010). However, genomic (microbial composition) data and hydrogenase activities were not reported in these studies to substantiate that increasing the organic loading suppressed these H\textsubscript{2}-consuming or non-H\textsubscript{2}-producing organisms within the system.
Hydrogenase is the enzyme that catalyzes the reversible oxidation of molecular H\textsubscript{2} in fermentative systems (2H\textsuperscript{+} + e\textsuperscript{-} \rightleftharpoons H\textsubscript{2}) (Vignais et al., 2001). Hydrogenases are grouped into two major categories (i.e., Fe-Fe and Ni-Fe hydrogenase) based on the metal content in the active catalytic center. Fe-Fe hydrogenase is present in many Clostridia, which are major H\textsubscript{2} producers and contribute only to H\textsubscript{2} production (Meyer, 2007), whereas Ni-Fe hydrogenase is present in H\textsubscript{2}-consuming facultative anaerobic bacteria and Archaea (methanogen) groups (Vignais and Colbeau, 2004).

Hence, this study aims at the following objectives. 1) To study the effect of OLR on H\textsubscript{2} production in conjunction with the suppression of methanogenesis 2) To examine the constitution of fermentation products and the types of fermentation that corresponds to the variation within the mixed microbial community structure induced by changes in the operating conditions. 3) To compare the HPR and hydrogenase activity at different HRTs and OLRs.

5.2 Materials and methods

The experiments were conducted in three different stages. Experiments were conducted in duplicate using two UASBRs (designated as reactor R1 and R2). At each experiment condition shown in Table 5.2, experiments were repeated at least 7 times using reactors R1 and R2. For operation of the UASBR see section 3.5. The inoculum source used in stage I, II and III include culture B with an initial biomass concentration of 10 g L\textsuperscript{-1} VSS (see section 3.3 for culture source and maintenance). In stage I, the effect of OLR was examined by varying the substrate concentration at a constant HRT (24 h). Experiments in stage II were conducted to examine the effect of OLR caused by the variation in HRT. Note, at the end of stage I, the amount of inoculum B was seeded in the reactors to increase the VSS concentration to 12 g L\textsuperscript{-1}, in order to achieve low HRT (1.5 h) and high OLRs (96 g L\textsuperscript{-1} d\textsuperscript{-1}) (Table 5.2). In stage III, the effect of linoleic acid (LA) was examined within the range of experimental conditions examined in stage II. Note, for experiments run in stage III the reactors were seeded with fresh inoculum with a VSS concentration of 10 g L\textsuperscript{-1}.

All the chemical, analytical and enzymatic methods used in this study are outlined in sections 3.7.3, 3.8 and 3.9, respectively. A pyrosequencing analysis was performed for
stage I samples and terminal restriction length polymorphism (TRFLP) was performed for stage II samples. The microbial analysis was conducted using the methodology described in section 3.10. The flux balance analysis using the metabolic flux model was used to quantify the H₂ consumption under the different experimental conditions in stage I and II (section 3.11). The hydrogenase flux, hydrogenase enzymatic activity and the microbial composition variation are shown with changes in OLR. Principal component analysis (PCA)-biplot was used for correlating the samples under different conditions to their metabolic profile. A principal coordinate analysis (PCoA) was also used for correlating the diversity in microbial profiles (obtained from T-RFLP analysis) under different experimental conditions in stage II. All the above statistical methods are described in the section 3.12. The experiments were conducted at 37 °C at an operating pH level of 5.0. The experimental conditions in stages I, II and III are presented in Table 5.2. In stage III, effect of increased OLR with an influent glucose concentration of 8 g L⁻¹ was studied on LA inhibited cultures with decreasing the HRT from 12 h to 6 h.

5.3 Results

5.3.1 Effects of organic loading rate and hydraulic retention time on hydrogen and methane yields

In the first stage of this study, the UASBR was operated under constant HRT (24 h). The reactor was operated at pH 5.0 with varying glucose loading rates (from 2 to 16 g L⁻¹ d⁻¹) over a period of 1464 h (61 days) (Figure 5.1). The results obtained indicate that the H₂ yield (per mol of glucose) increased from 0.75±0.09 at 2 g L⁻¹ d⁻¹ to 1.70±0.05 at 8 g L⁻¹ d⁻¹ (Table 5.2). There was a 10% decrease in the H₂ yield when OLR was increased further to 10 g L⁻¹ d⁻¹ and 12 g L⁻¹ d⁻¹; however, the decrease observed was statistically insignificant (Tukey’s test at p < 0.05, Table 5.2). Note, increasing the OLR to 16 g L⁻¹ d⁻¹, caused the H₂ yield to reach 1.0±0.04 mol mol⁻¹ glucose. Notable amounts of CH₄ reaching 0.97±0.09 mol mol⁻¹ glucose and 0.65±0.05 mol mol⁻¹ glucose were observed with lower glucose loading rates of 2 g L⁻¹ d⁻¹ and 4 g L⁻¹ d⁻¹, respectively. The CH₄ production decreased was observed with increase in the OLR. In comparison to the maximum CH₄ yield obtained at 2 g L⁻¹ d⁻¹ approximately, 94% decrease in CH₄ yield was observed at an OLR corresponding to 16 g L⁻¹ d⁻¹. In order to examine the biogas
production with increased OLR, the rates of H₂ and CH₄ production were monitored. Figure 5.1 presents only the HPR, as the CH₄ production rate was not comparable to the HPR at OLRs ranging from 6 to 16 g L⁻¹ d⁻¹ (i.e. CH₄ production rates were insignificant at these OLRs). The highest CH₄ production rate observed in this study was 0.13±0.02 L L⁻¹ d⁻¹ at 4 g L⁻¹ d⁻¹ (data not shown). The HPR increased with increased loading from 0.19±0.02 L L⁻¹ d⁻¹ at 2 g L⁻¹ d⁻¹ to 2.3±0.1 L L⁻¹ d⁻¹ at 14 g L⁻¹ d⁻¹. A further increase in the OLR to 16 g L⁻¹ d⁻¹ resulted in 15±1% decrease in the HPR.

Table 5.2 Experimental stages, operating parameters and experimental outcomes at different operating conditions for glucose fermentation in UASBRs

<table>
<thead>
<tr>
<th>Stage</th>
<th>Organic loading rate (g L⁻¹ d⁻¹)</th>
<th>HRT (h)</th>
<th>Substrate consumption (%)</th>
<th>H₂ yield (mol mol⁻¹ glucose)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage I (increasing OLR at a constant HRT)</td>
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</tr>
<tr>
<td>2</td>
<td>20</td>
<td>100±2A</td>
<td>0.75±0.09d</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>20</td>
<td>100±1A</td>
<td>1.23±0.15b</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>24</td>
<td>96±2A</td>
<td>1.56±0.12a</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>24</td>
<td>96±1A</td>
<td>1.70±0.05a</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>24</td>
<td>89±3A</td>
<td>1.65±0.04a</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>24</td>
<td>84±2B</td>
<td>1.57±0.05a</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>24</td>
<td>83±2B,C</td>
<td>1.32±0.04b</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>24</td>
<td>77±4D</td>
<td>1.00±0.04c</td>
<td></td>
</tr>
<tr>
<td>Stage II (increasing OLR with decreasing HRT)</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>8.0</td>
<td>24</td>
<td>93±1A</td>
<td>1.58±0.14c</td>
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<tr>
<td>9.6</td>
<td>20</td>
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<td>2.53±0.18a</td>
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<tr>
<td>12.0</td>
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<td>84±5A</td>
<td>2.43±0.18a</td>
<td></td>
</tr>
<tr>
<td>16.0</td>
<td>12</td>
<td>79±5A</td>
<td>2.12±0.07b</td>
<td></td>
</tr>
<tr>
<td>24.0</td>
<td>8</td>
<td>74±12A,B</td>
<td>1.64±0.06c</td>
<td></td>
</tr>
<tr>
<td>38.4</td>
<td>5</td>
<td>74±8B</td>
<td>1.42±0.07c</td>
<td></td>
</tr>
<tr>
<td>48.0</td>
<td>3</td>
<td>69±9B</td>
<td>1.34±0.06d</td>
<td></td>
</tr>
<tr>
<td>96.0</td>
<td>1.5</td>
<td>64±8B</td>
<td>1.29±0.04d</td>
<td></td>
</tr>
<tr>
<td>Stage III (increasing OLR in LA treated culture)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>12</td>
<td>97±3A</td>
<td>1.96±0.17b</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>8</td>
<td>93±2A</td>
<td>2.62±0.14a</td>
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<tr>
<td>32</td>
<td>6</td>
<td>92±3A</td>
<td>2.32±0.31a</td>
<td></td>
</tr>
</tbody>
</table>

Notes:
1. Samples from stage I, II and III with different superscripts (a, b, c, and d) differ significantly in their H₂ yields (p < 0.05).
2. Samples of stage I, II and III with different subscripts (A and B) differ significantly in their glucose consumption (p < 0.05).
3. Note, comparison should be made within each stage and not between stages.
4. The average and standard deviation are for n = 14. Two reactors (R1 and R2) operating under the same condition and each condition repeated 7 times.
Notes: The H$_2$ production rates and yields plotted shows average values for duplicate reactors R1 and R2. CH$_4$ production rates obtained were ≤ 0.2 L L$^{-1}$ d$^{-1}$.

In the second stage of this study, the reactor was operated at a reduced OLR (8 g L$^{-1}$ d$^{-1}$) at 24 h HRT after 61 days (1464 h) of reactor operation (i.e., after a decline in H$_2$ yield with increased OLR at the end of stage I). This change in the operating condition was accompanied by an increase in the H$_2$ yield to 1.58±0.14 mol mol$^{-1}$ glucose over a period of 240 h. The H$_2$ yield increased further and then remained stable in the range of 2.48±0.18 mol mol$^{-1}$ glucose as HRT was reduced to 16 h HRT and started decreasing thereafter (Stage II, Figure 5.2). The H$_2$ yield (mol mol$^{-1}$ glucose) decreased from 2.12±0.06 at 12 h HRT to 1.41±0.07 at 5 h HRT (Table 5.2 and Figure 5.2). A further decrease in HRT was accompanied with a reduction in the H$_2$ yield by 10±1% at each
HRT. Furthermore, CH₄ production was suppressed by > 99% by decreasing the HRT from 24 h to 1.5 h with simultaneous increase in the OLR from 8 g L⁻¹ d⁻¹ to 96 g L⁻¹ d⁻¹. A significant increase in HPR was observed with a decrease in HRT coupled with an increased OLR. The HPR increased from 1.57±0.14 L L⁻¹ d⁻¹ at 24 h HRT and an OLR of 8 g L⁻¹ d⁻¹ to 15.44±1.44 L L⁻¹ d⁻¹ at 1.5 h HRT with an OLR of 96 g L⁻¹ d⁻¹.

Figure 5.2 Operation parameters, hydrogen and methane production performance from glucose fermentation in UASBRs during stage II

Notes: The H₂ production rates and yields plotted shows average values for duplicate reactors R1 and R2. CH₄ production rates were ≤ 0.05 L L⁻¹ d⁻¹.
Figure 5.3 Operation parameters, hydrogen and methane production performance from glucose fermentation in UASBRs during stage III

Note: The H$_2$ production rates and yields plotted shows average values for duplicate reactors R1 and R2. CH$_4$ production rates were ≤ 0.03 L L$^{-1}$ d$^{-1}$.

Hydrogen production observed with LA treated granulated culture detected at HRTs ranging from 6 h to 12 h showed H$_2$ yields ≥ 2.0 mol mol$^{-1}$ glucose (stage III, Figure 5.3). The H$_2$ yield (mol mol$^{-1}$ glucose) obtained in LA treated cultures at 12 h and 8 h was 1.96±0.17 and 2.62±0.14, respectively. A reduction in HRT to 6 h did not significantly affect the H$_2$ yield in LA treated cultures. However, decreasing the HRT form 12 h to 6 h caused an increased in the HPR from 3.89±0.38 to 9.2±1.3 L L$^{-1}$ d$^{-1}$ (Table 5.2, Figure 5.3).
5.3.2 Substrate conversion

In this study, glucose was used as the model substrate. In stage I, the glucose removal efficiency varied according to the loading rate applied to the UASBR. Glucose removal was over 90% when the OLR was less than 10 g L$^{-1}$ d$^{-1}$. As the OLR increased in stage I, glucose removal rate decreased to 77% at 16 g L$^{-1}$ d$^{-1}$. However, glucose removal remained above 70% with further increase in OLR to 48 g L$^{-1}$ d$^{-1}$ (Table 5.2). During stage II, the glucose removal efficiency decreased from 93 to 64% with decreasing HRT. The glucose removal efficiencies obtained at HRTs from 12 to 24 h were statistically different from those obtained at HRTs below 12 h (Tukey’s test at p < 0.05, Table 5.2). The glucose removal efficiencies for the LA treated cultures were greater than 90% over the range of HRTs tested in this study (stage III, Table 5.2).

5.3.3 Soluble metabolite product distribution

Acetic (HAc) and n-butyric acid (HBu) were the major metabolites found among the volatile fatty acids (VFAs) detected. Traces of propionic acid (HPr) and lactic acid (HLa) were also present in the fermentation liquid by-products. Furthermore, alcohols, such as ethanol (EtOH) and iso-propanol (i-PrOH), were present among the soluble metabolites produced.

The electron equivalent (e$^-$ equiv) balances for all of the loading rates tested at 24 h HRT in stage I is shown in Figure 5.4a. At low OLRs, CH$_4$ was the major electron sink from substrate oxidation, accounting for 20±2 to 30±1% of the total substrate electrons. HAc and HBu were the major liquid soluble end-products accounting for 17 to 23% of e$^-$/equiv each (except at OLRs 14 and 16 g L$^{-1}$ d$^{-1}$). However, the electron sink for HBu was more constant, remaining at 23±1% of the e$^-$/equiv throughout the stage I. The desired end-product (H$_2$) varied, but accounted for only 7±0.5 to 13±0.5% of the total e$^-$/equiv during stage I of the experiment. This low level of H$_2$ can be explained by equations 5.3 to 5.7 (Table 5.1), which show that reduced end-products (e.g. HPr, EtOH and i-PrOH) are also produced in the fermentation broth as a result of glucose oxidation. The e$^-$/equiv diverted to biomass synthesis was assumed to be 10% of the initial glucose electrons (Rittmann and McCarty, 2001). The fraction of e$^-$/equiv in HPr decreased from 10±0.8% to 3±0.4%, in contrast to the fraction of e$^-$/equiv in i-PrOH that increased from 4±0.4% to...
13±0.9% as OLR increased during stage I. Similarly, the fraction of $e^-$ equiv in HAc decreased from 22±1% at 4 g L$^{-1}$ d$^{-1}$ to 11±0.8% at 16 g L$^{-1}$ d$^{-1}$, whereas the electron sink in the form of ethanol increased by 85±9% (Figure 5.4a).

![Figure 5.4](image)

**Figure 5.4** Electron distribution of fermentation byproducts using glucose as the substrate (a) Stage I and (b) Stage II (c) Stage III

**Notes:** Acetic acid = HAc; lactic acid = HLa; butyric acid = HBu; propionic acid = HPr; residual glucose = Res-glu; hydrogen = H$_2$; ethanol = EtOH; i-PrOH = iso-propanol and methane = CH$_4$. A 10% of electron equivalent to biomass was assumed in the electron distribution.
The fraction of e- equiv due to HLa was negligible (< 3%). The e- equiv balances observed during stage I ranged from 96±8% to 105±6%. Trace levels of iso-butanol and formate were detected during the first stage of reactor operation, but together these accounted less than 1% of the e- equiv (data not shown).

**Figure 5.4b** shows the electron distribution for glucose fermentation during stage II (increasing OLR with decreasing HRTs). In contrast to the first stage of this study (i.e., increasing OLR at constant HRT), the fraction of e- equiv from glucose to HBu in stage II decreased from 26±2% to 14±2% with decreasing HRT. The fraction of e- equiv in the form of HAc was maximal at 20 h and 16 h HRT (32±2%) and decreased gradually thereafter to 18±2%. The fraction of e- equiv diverted from glucose to EtOH increased from 5±1% at 24 h HRT to 12±1% at 1.5 h HRT.

The electron distribution during stage III in LA treated showed major e- sink in HAc (30.5±0.7%), H2 (20.6±1.8%) and HBu (17.0±1.8%) at 8 and 6 h HRTs (**Figure 5.4c**). The percent of e- sink in i-PrOH decreased with decreasing HRT while e- sinks EtOH increased by 3-4%.

5.3.4 **Flux balance analysis**

A flux balance analysis (FBA) was performed on the fermentation products (gas and soluble metabolites) obtained at different loading rates (OLRs of 2, 4, 8 and 16 g L\(^{-1}\) d\(^{-1}\)) in stage I and different HRTs (24, 12 and 1.5 h) corresponding to an OLR of 8, 16 and 96 g L\(^{-1}\) d\(^{-1}\) in stage II. The output from the model (H\(_2\) yield predicted; H\(_2\) consumed via methanogenesis, acetogenesis and i-PrOH formation and the experimentally observed H\(_2\) yield) is shown in **Figure 5.5**.

At low OLR of 2 and 4 g L\(^{-1}\) d\(^{-1}\), CH\(_4\) production occurred via hydrogenotrophic methanogenesis (**R29** flux, **Table 3.4, section 3.11**) and acetoclastic methanogenesis (**R28** flux, **Table 3.4, section 3.11**). H\(_2\) consumption via methanogenesis (**R29** flux) decreased from 1.8 (4 x 0.45) to 0.28 (4 x 0.07) mol H\(_2\) mol\(^{-1}\) glucose consumed, at 2 g L\(^{-1}\) d\(^{-1}\) and 8 g L\(^{-1}\) d\(^{-1}\), respectively. Note, the flux via acetoclastic methanogenic activity ranged from 0.5 to 0.05 mol mol\(^{-1}\) glucose (data not shown). Methane production was strongly suppressed by increased loading; however, complete suppression of the methanogenic activity was not observed. This is based on the trace amount of CH\(_4\)
produced from glucose at OLRs ranging from 6 to 96 g L$^{-1}$ d$^{-1}$ (Figures 5.1 and 5.2). The FBA revealed that, by increasing the OLR with simultaneous decrease in the HRT, hydrogenotrophic methanogenesis (R29 flux) was not observed.

Higher homoacetogenic activity (R17 flux, Table 3.4, section 3.11) was observed at an OLR of 16 g L$^{-1}$ d$^{-1}$ in stage I, where $4 \times 0.14 = 0.56$ mol H$_2$ mol$^{-1}$ glucose was consumed. In stage II, homoacetogenic activity (R17 flux) was observed to be maximum at 24 h HRT corresponding to 8 g L$^{-1}$ d$^{-1}$, in which $4 \times 0.08 = 0.32$ mol H$_2$ mol$^{-1}$ glucose was consumed.

By increasing the OLR with a constant 24 h HRT (Stage I), H$_2$ consumption was found to be accompanied by the i-PrOH production (R21 flux, Table 3.4, section 3.11). At OLRs of 14 and 16 g L$^{-1}$ d$^{-1}$, the H$_2$ consumption due to i-PrOH production (R21 flux) was 0.15 and 0.17 mol H$_2$ mol$^{-1}$ glucose, respectively. In stage II, H$_2$ consumption due to i-PrOH was not detected, while in stage III H$_2$ consumption was accompanied by i-ProH formation (data not shown). In LA treated cultures, no methanogenic or acetogenic flux from H$_2$ consumption was detected over the range of HRTs tested (data not shown).

![Figure 5.5](image)

**Figure 5.5** Comparison of hydrogen flux distribution in H$_2$ production and consumption during stage I and II using flux balance analysis

**Note:** Predicted H$_2$ yields is retrieved from (R12) hydrogenase flux., while H$_2$ consumption is calculated based on H$_2$ consumption due to homoacetogenesis (R17 flux), methanogenesis (R29) and iso-propanol formation (R21).
5.3.5 Hydrogenase activity levels

The H\textsubscript{2} evolution and uptake specific activities (ESA and USA, respectively) of hydrogenases were monitored at OLRs of 2, 4, 8 and 16 g L\textsuperscript{-1} d\textsuperscript{-1} in stage I and at 8, 16 and 96 g L\textsuperscript{-1} d\textsuperscript{-1} corresponding to HRTs of 24, 12 and 1.5 h in stage II, respectively. Figure 5.6 presents the activities of the hydrogenases in the cultures under different loading rates. In stage I (with constant HRT of 24 h), the maximum ESA of 13.2±1.4 µmol H\textsubscript{2} evolved mg\textsuperscript{-1} VSS h\textsuperscript{-1} was observed for an OLR of 16 g L\textsuperscript{-1} d\textsuperscript{-1} and the minimum ESA of 3.5±0.5 µmol H\textsubscript{2} evolved mg\textsuperscript{-1} VSS h\textsuperscript{-1} was observed at an OLR of 4 g L\textsuperscript{-1} d\textsuperscript{-1}. Note, the HPR corresponding to the maximum ESA in stage I was 1.97±0.05 L L\textsuperscript{-1} d\textsuperscript{-1}. The maximum level of USA of 45±5 µmol H\textsubscript{2} consumed mg\textsuperscript{-1} VSS h\textsuperscript{-1} was recorded at an OLR of 4 g L\textsuperscript{-1} d\textsuperscript{-1} and the corresponding consumption of the H\textsubscript{2} yield was 1.4±0.16 mol mol\textsuperscript{-1} glucose. The USA then decreased to 10.6±0.6 and 6.2±0.4 µmol H\textsubscript{2} consumed mg\textsuperscript{-1} VSS h\textsuperscript{-1} at OLRs 8 and 16 g L\textsuperscript{-1} d\textsuperscript{-1}, respectively. The maximum level of hydrogenase evolution activity in stage II of 22±2 µmol H\textsubscript{2} evolved mg\textsuperscript{-1} VSS h\textsuperscript{-1} was observed at an HRT of 12 h with a corresponding OLR of 16 g L\textsuperscript{-1} d\textsuperscript{-1}. In stage II, the activity levels of hydrogenases decreased at 96 g L\textsuperscript{-1} d\textsuperscript{-1} by 29±4% (ESA) and 92±10% (USA), in comparison to their corresponding maximum activity levels recorded in stage II at 16 and 8 g L\textsuperscript{-1} d\textsuperscript{-1}, respectively (Figure 5.6).

![Graph showing hydrogenase activity levels under different operating conditions](image-url)

Figure 5.6 Hydrogenase activity levels under different operating conditions (during stages I and II)
5.3.6 Principal component analysis

A PCA was applied to identify general patterns and grouping in the dataset for experimental results obtained at different OLRs. The data used as inputs for the PCA included percent equiv distributed to various byproducts of dark fermentation (e.g. VFAs, alcohols, residual glucose and gaseous products) obtained under different operating conditions. In this study, the first two principal components (PC1 and PC2) accounted for more than 83% of the total variability found within the dataset of fermentation byproducts.

![Figure 5.7 Principal component analysis based on byproducts formed during microbial metabolism](image)

**Notes:**
1. Only data from stage I and II is used for analysis.
2. The labels (#s) x, y in the plot represents the HRT (h) and OLR (g L\(^{-1}\) d\(^{-1}\)).
3. The open circles (○) correspond to stage I operating conditions and closed circle (●) correspond to stage II operating conditions
4. Acetic acid = HAc; butyric acid = HBu; propionic acid = HPr; residual glucose = Res. glu; hydrogen = H\(_2\); ethanol = EtOH; i-PrOH = iso-propanol and methane = CH\(_4\)

The position of the operating conditions within the biplot displays their relation to the variables and the component. For example, the operating conditions under constant HRT (24 h) with loading rate 2-8 g L\(^{-1}\)d\(^{-1}\) in the stage I was closely related to the PC 1 and
fermenting conditions at the higher OLRs (8-16 g L\(^{-1}\)d\(^{-1}\)) in stage I and stage II (38-96 g L\(^{-1}\)d\(^{-1}\)) were more associated with PC 2 (Figure 5.7). The variables CH\(_4\), HPr and HBu have loading values of 0.47, 0.46 and 0.40, respectively, in the first component whereas PC 2 was aligned with EtOH, i-PrOH, residual glucose and HBu with a load value of 0.48, 0.45, 0.18 and 0.14, respectively. Hydrogen and HAc were associated with PC3 with a loading value of 0.56 and 0.21 (data not shown). The load value signifies the correlation to the contribution of each variable to the PCs.

In the biplot shown in Figure 5.7, CH\(_4\) ordinate together with HPr and HBu showing they are highly correlated variables (Note, the cosine of angle between the variables indicates the correlation between variables). Whereas the H\(_2\) ordinate with HAc showing high correlation among them. Less correlation between EtOH and H\(_2\) is indicated by the angle > 90\(^\circ\) between H\(_2\) and EtOH. This indicates that a high H\(_2\) yield is related to the HAc production and EtOH production is linked to lower H\(_2\) yields.

Grouping of the loading conditions on the biplot (see dotted and continuous ellipse in lower right and left quadrant in Figure 5.7) reveals that metabolite distribution were influenced by the OLR and HRT. The association of low OLRs at long HRTs with the variables such as CH\(_4\), HBu and HPr indicates at long HRTs the substrate conversion efficiency (in terms of H\(_2\) production) would be less. Similar to the positioning of the stage I sample points, stage II conditions were positioned to the left side of the biplot. The HRTs between the 12-20 h indicates their association to H\(_2\) and HAc which suggest the optimal HRT for increased H\(_2\) production.

Overall, the PCA showed the major associated metabolites at each operating condition. Examination of the plot shows that the change in the OLR grouped the samples in the PC1:PC2 plot based on their fermentation profile.

5.3.7 Microbial analysis

5.3.7.1 Principal coordinate analysis

Figure 5.8 shows the difference between the microbial communities in reactor samples exposed to various OLRs with changes in HRTs during stage II. A PCoA of the T-RF data were conducted.
Figure 5.8 Principal coordinate analysis of T-RFs obtained from Hae III restriction enzyme for the samples collected in stage II

Note: #s in plot correspond to HRTs operated in stage II (see Table 5.2 for experimental design).

In stage II of the study, only samples collected at HRTs ≥ 5 h were microbiologically characterized. The PCoA (1st and 2nd coordinates) of the T-RF data showed a 75% variation in the dataset. The samples collected at higher HRTs (20 h and 16 h) were grouped together in the upper left quadrant. Notice these operating conditions also produced the maximum H₂ yields. Reducing the HRT further to intermediate levels (12 h or 8 h) induced marked changes in the microbial community structure. This is shown by the clustering of samples in the lower left quadrant of the coordinate plot. The culture analyzed at 5 h HRT showed further differentiation in the microbial community structure because it was separated from the samples treated with longer HRTs (Figure 5.8).

5.3.7.2 Microbial composition

The microbial composition of the biogas-producing community in culture samples from stage I are shown in Figures 5.9a and b. The figure depicts the taxonomic distribution of the microbial species belonging to different family obtained from pyrosequencing at the start and end of stage I (i.e., when the loading rates increased from 2 g L⁻¹ d⁻¹ to 16 g L⁻¹ d⁻¹ while the HRT is maintained at 24 h). The pyrosequencing results reveal that at the beginning of stage I, non-H₂ producers which was related mainly
to *Propionibacteriaceae* and *Synergistaceae* families composed a relatively high proportion (approximately 45%) of the culture. The percent of H$_2$ and ethanol-producing micro-organisms was approximately 34%, and were mainly related to *Clostridiaceae* and *Ruminococcaceae*; however, when the OLR increased to 16 g L$^{-1}$ d$^{-1}$ their dominance increased to 87%, while the HPr producers and other synergistic groups were reduced to 2% of the microbial community.

Figure 5.9 Population shift observed at (a) low (2 g L$^{-1}$ d$^{-1}$) and (b) high (16 g L$^{-1}$ d$^{-1}$) loading rate in stage I
The relative abundance of the microorganisms observed within the bacterial communities using T-RFLP during stage II is presented in Table 5.3. The data shows variation within the microbial population under 3 different HRTs (16 h, 8 h and 5 h).

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Relative abundance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. HRT: 16 h and OLR: 12 g L⁻¹ d⁻¹</strong></td>
<td></td>
</tr>
<tr>
<td><em>Parabacteroides</em> sp., <em>Moorella thermoacetica</em></td>
<td>23.8</td>
</tr>
<tr>
<td><em>Butyribrio</em> sp., <em>Clostridium</em> sp.</td>
<td>10.4</td>
</tr>
<tr>
<td><em>Butyribrio</em> sp., <em>Clostridium</em> sp.</td>
<td>8.9</td>
</tr>
<tr>
<td><em>Propionibacterium</em> sp., <em>Parabacteroides</em> sp.</td>
<td>7.2</td>
</tr>
<tr>
<td><em>Alkaliphilus</em> sp.</td>
<td>6.1</td>
</tr>
<tr>
<td><em>Propionibacterium</em> sp., <em>Parabacteroides</em> sp.</td>
<td>2.5</td>
</tr>
<tr>
<td><em>Clostridium</em> novyi</td>
<td>2</td>
</tr>
<tr>
<td><em>Butyribrio</em> sp., <em>Clostridium</em> sp.</td>
<td>2</td>
</tr>
<tr>
<td><em>Methylomonas</em> sp., <em>Lactobacillus</em> sp., <em>Eubacterium</em> sp.</td>
<td>2</td>
</tr>
<tr>
<td>Unidentified</td>
<td>7.7</td>
</tr>
<tr>
<td><strong>B. HRT: 8 h and OLR: 24 g L⁻¹ d⁻¹</strong></td>
<td></td>
</tr>
<tr>
<td><em>Bacteroides</em> sp., <em>Flavobacterium</em> sp., <em>Clostridium</em> sp.,</td>
<td>13.4</td>
</tr>
<tr>
<td><em>Bifidobacterium</em> sp.</td>
<td></td>
</tr>
<tr>
<td><em>Ethanoligenens</em> sp.</td>
<td>12.9</td>
</tr>
<tr>
<td><em>Parabacteroides merdae, Moorella thermoacetica</em></td>
<td>7.9</td>
</tr>
<tr>
<td><em>Alkaliphilus metalliredigens</em></td>
<td>6.7</td>
</tr>
<tr>
<td><em>Clostridium</em> cellulovorans, <em>Methylophilus methyloptrophus, Thermobaculum</em> terrenum</td>
<td>4.7</td>
</tr>
<tr>
<td><em>Bacillus</em> sp., <em>Clostridium</em> sp., <em>Desulfotomaculum</em> alkaliphilum</td>
<td>4.3</td>
</tr>
<tr>
<td><em>Thermoanaerobacter, Thioalkalivibrio</em></td>
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</tr>
<tr>
<td><em>Desulfotobacterium</em> sp., <em>Eubacterium</em> sp.</td>
<td>3</td>
</tr>
<tr>
<td><em>Thermanaerovibrio</em> acidaminovorans</td>
<td>2.5</td>
</tr>
<tr>
<td>Unidentified</td>
<td>11.8</td>
</tr>
<tr>
<td><strong>C. HRT: 5 h and OLR: 38.4 g L⁻¹ d⁻¹</strong></td>
<td></td>
</tr>
<tr>
<td><em>Ethanoligenens</em> sp.</td>
<td>27.9</td>
</tr>
<tr>
<td><em>Thermanaerovibrio</em> sp.</td>
<td>18.5</td>
</tr>
<tr>
<td><em>Alkaliphilus</em> sp.</td>
<td>13.7</td>
</tr>
<tr>
<td><em>Bacillus</em> sp., <em>Clostridium</em> sp., <em>Desulfotomaculum</em> alkaliphilum</td>
<td>6.9</td>
</tr>
<tr>
<td><em>Thermoanaerobacter</em> sp., <em>Thioalkalivibrio</em> sp.</td>
<td>5.3</td>
</tr>
<tr>
<td><em>Bacillus</em> sp., <em>Paenibacillus</em> azotofixans</td>
<td>3.4</td>
</tr>
<tr>
<td>Unidentified</td>
<td>6.1</td>
</tr>
</tbody>
</table>

**Note:** The table shows the list of microorganisms with relative abundance ≥2%.

At an HRT of 16 h, approximately 24% of the microbial population consisted of homoacetogens and mixed acid-producing organisms. Approximately 28% of the species
detected belonged to the class Clostridia (*Butyrovibrio, Clostridium, Alkaliphilus, Eubacterium*). Furthermore, about 7–9% of the population was HPr or HLa producing organisms belonging to the Actinobacteria or Bacilli classes. When the HRT was lowered to 5 h with a simultaneous increase in OLR to 38 g L\(^{-1}\) d\(^{-1}\), the relative abundance of H\(_2\), acid and ethanol producers belonging to the families *Ruminococcaceae, Clostridiaceae, Thermoanaerobacteraceae* and *Syntrophomonadaceae* increased and constituted approximately 65% of the population. The relative percent of homoacetogenic bacteria that were detected fell to less than 1% at a 5 h HRT (data not shown). The next major microflora under this condition was primarily composed of *Bacillus* sp., although other micro-organisms were detected (e.g. *Firmicutes* or unidentified species composed about 6% of the microbial population at HRT 5 h).

### 5.4 Discussion

Biogas production was monitored during different operational stages in UASBRs operating continuously and maintained at pH 5.0±0.2 with a glucose feed. The results obtained in this study revealed that operation the UASBRs at 24 h HRT and an OLR of 16 g L\(^{-1}\) d\(^{-1}\) in continuous mode at an acidic pH level of 5.0 is not adequate to suppress the H\(_2\) consumption and increase H\(_2\) production. The low H\(_2\) yields obtained at reduced OLRs were attributed to CH\(_4\) production and at higher OLRs low H\(_2\) yields were attributed to substrate inhibition (*Table 5.2, Figures 5.1 and 5.2*). Evidence of low substrate conversion efficiency and the substrate degrading efficiency was reported by Wu and Lin (2004) at high OLRs. Similarly, Spagni et al. (2010), observed low H\(_2\) yields at low OLRs due to high methanogenic activity. In the current study, on average, higher H\(_2\) yields were observed in stage II conditions. Notice the H\(_2\) yield reached a maximum of 2.53±0.18 mol mol\(^{-1}\) glucose at 20 h HRT at a corresponding OLR of 9.6 g L\(^{-1}\) d\(^{-1}\) (*Figure 5.2*). This observation is comparable to published reports with glucose that reported H\(_2\) yields greater than 2.0 mol mol\(^{-1}\) glucose (de Amorim et al., 2012; Hafez et al., 2009; Kotsopoulos et al., 2006). The maximum H\(_2\) yield obtained with a 24 h HRT in stage I was 1.70±0.05 mol mol\(^{-1}\) glucose, which corresponds to the H\(_2\) yields reported for complex substrates, such as cheese whey, sucrose and food waste (Castello et al., 2009; Kim et al., 2009; Kim and Shin, 2008). The H\(_2\) yields measured during stage I at constant
HRT are relatively low compared to other studies using pure glucose. This is likely because of the presence of H₂ consumers (evident from flux and CH₄ data, see Figures 5.1 and 5.5) and other non-H₂ producers, along with the H₂ producers in the mixed anaerobic culture (Figure 5.9). However, Van Ginkel and Logan (2005) observed increased biological H₂ production with reduced OLR, and reported a maximum H₂ yield of 2.8 mol mol⁻¹ glucose with a HPR of 2.94 L L⁻¹ d⁻¹ at 10 h HRT and a OLR of 6 g COD L⁻¹ d⁻¹. In the current study, as an outcome of the high HRT and low OLR applied in stage I, the maximum HPR observed was 2.34±0.06 L L⁻¹ d⁻¹. Close examination of the findings for stage I showed that the glucose feed concentration affected the H₂ yield. This observation is in accordance with work reported by de Amorim et al. (2012) and Kataoka et al. (1997). The effect of glucose concentration on the H₂ yield is clearly seen when the influent substrate concentration was increased from 2 to 6 g L⁻¹ at a 24 h HRT (Figure 5.1). However, concentrations above these levels showed no further improvement in the H₂ yield. Notice decreasing H₂ yields were observed with concentrations greater than 12 g L⁻¹ (Figure 5.1, Table 5.2). In supporting studies by Zhang et al. (2004), they observed that at a constant HRT (4.5 h) and with influent glucose concentrations of 5, 10, and 15 g COD L⁻¹, there was approximately 0.4 mol mol⁻¹ glucose increase in the H₂ yield with a maximum yield equals to 1.2 mol mol⁻¹ glucose.

The low H₂ yield observed could be attributed to high VFA concentrations (Van Ginkel et al., 2001). The total VFA concentration of 76±6.7 mM which was attained during stage I of the experiment corresponded to approximately 10 g L⁻¹ of COD equivalent (Figure E.1, Appendix E). Increase in the H₂ yield can be achieved by reducing the substrate concentration and the HRT. Van Ginkel and Logan (2005) have indicated that reducing the HRT could improve the H₂ yield and HPR at low organic concentrations. They also reported that this operational strategy also assisted in diluting the VFA levels in continuously fed reactor. In this study, decreasing the HRT from 24 to 20 h in stage II increased the H₂ yield by 60%. This yield was observed to remain stable as the HRT was reduced to 12 h with an H₂ yield greater than 2.0 mol mol⁻¹ glucose. However, with further decreasing the HRT further to 1.5 h, the H₂ yield was reduced to 1.29±0.04 mol mol⁻¹ glucose (Table 5.2). Similarly, Nasirian et al. (2010) observed that
decreasing the HRT from 14 to 12 h increased the H2 yield by 20%. However, they observed that decreasing the HRT to 10 h reduced the H2 yield by 41%.

Decreasing the HRT was able to improve the HPR and the selective growth of the H2 producing micro-organisms within the culture. Li et al. (2006) studied the effect of HRT on packed bed up-flow reactor systems and found that lowering the HRT from 30 h to 2 h increased the HPR from 0.46±0.04 to 6.17±0.39 L L⁻¹ d⁻¹ and the 16S rDNA analysis revealed that most of the species observed after HRT reduction were affiliated with an increase in Clostridium sp. In the stage II of this study, HPRs increased from 1.57±0.14 L L⁻¹ d⁻¹ (at 24 h HRT and an OLR of 8 g L⁻¹ d⁻¹) to 15.4±1.4 L L⁻¹ d⁻¹ (at 1.5 h HRT and an OLR of 96 g L⁻¹ d⁻¹). The H2 producing species belonging to Ethanoligenens sp., Clostridium sp. and Thermanaerovibrio sp. observed at low HRT (5 h) were primarily from different from those observed at 16 g L⁻¹ d⁻¹ and a 24 h HRT (Table 5.3, Figure 5.9b). The HPR obtained in stage III (Figure 5.3) with LA treated culture was greater than that obtained in stage II control cultures at similar operating conditions. Zhang et al. (2006) used heat treated inoculum to study the effect of HRT on bio-H2 production using glucose as the substrate. They reported an average yield of 1.85 mol mol⁻¹ glucose with a maximum HPR of 7.77±0.13 L L⁻¹ d⁻¹ at 6 h HRT and without eliminating the dominant H2 producing species.

The maximum H2 yield reported in the literature between 2 to 4 mol mol⁻¹ hexose was based on the type of substrate and also the activity of hydrogenase enzyme present in mixed microbial culture (Hallenbeck et al., 2012). Morimoto et al. (2005) observed a 1.7-fold increase in H2 yield with an over expression of the hydrogenase gene. In comparison, studies by Klein et al. (2010) concluded that the amount of hydrogenase content was not the limiting factor in determining the H2 yield from glucose. Increasing H2 yield was observed with suppression of the H⁺ uptake gene for hydrogenase in Clostridium sp. (Nakayama et al., 2008; Zhao et al., 2009). In stage I of the current study, an 85% decrease in the USA and a 250% increase in the ESA of hydrogenase were observed with increase in OLR to 16 g L⁻¹ d⁻¹ (Figure 5.6). The inhibition observed on hydrogenase with higher feeding rates by Ruzicka (1996) is similar to the results obtained in this study. A decrease in hydrogenase (both USA and ESA) activity levels was observed with
increased OLR to 96 g L\(^{-1}\) d\(^{-1}\) corresponding to 1.5 h HRT in stage II (Figure 5.6). Even though maximum HPR was obtained under this condition, a low H\(_2\) yield was obtained under this condition (Figure 5.2).

The decrease in methanogenic activity observed with increasing substrate concentration during stage I might be due to high VFA levels. Studies by Duangmanee et al. (2007) and Wang et al. (2009) revealed that the high VFA concentrations, which can occur at high OLRs/substrate concentrations, could be inhibitory to methanogens. In this study, a VFA concentration of 5.2 ± 0.64 g L\(^{-1}\) was achieved with an OLR of 12 g L\(^{-1}\) d\(^{-1}\) in stage I, which showed greater than 90% suppression in methanogenic activity (Figures 5.1 and Figure E.1, Appendix E). Fukuzaki et al. (1990) reported that the accumulation of HPr inhibited methanogens, whereas increasing levels of HBu decreased the H\(_2\) yield because of by-product inhibition at high substrate concentrations (Chin et al., 2003; Wu et al., 2010). Wu et al. (2010) showed increasing H\(_2\) yields from 1.8 to 2.2 mol mol\(^{-1}\) glucose was observed when the HBu composition in soluble products decreased from 75 to 63%. In this study, increasing levels of HPr and HBu with increasing OLR in stage I is likely linked to CH\(_4\) inhibition and low H\(_2\) yields, respectively (Figures 5.1 and Figure E.1, Appendix E).

The HRT was reduced from 24 h to 1.5 h in stage II to eliminate the growth and activity of organisms belonging to HPr producers, homoacetogens and simultaneously increase H\(_2\) production. The HPr levels decreased with a reduction in the HRT. An approximate 85% reduction in HPr production was observed in this study by operating the system at a low HRT 1.5 h compared to a 24 h HRT operation at 8 g L\(^{-1}\) d\(^{-1}\). However, complete elimination of HPr was not observed. This finding contradicts the results reported by Zhang et al. (2006), in which the authors successfully eliminated HPr producers by operating the CSTR at a 6 h HRT.

The total EtOH and HAc concentration reached 63% of the total soluble metabolites produced at OLRs ranging from 38.4 to 96 g L\(^{-1}\) d\(^{-1}\) (Figure E.1, Appendix E and see Table 5.2 for conditions). The theoretical maximum H\(_2\) yield per mol of glucose is 2.0 with HAc and EtOH as end products (Equation 5.6 in Table 5.1). Note, H\(_2\) yields in the
range of 1.3-1.4 mol mol\(^{-1}\) glucose was obtained in the current study at the HRT between 1.5 and 5 h (stage II, Table 5.2).

Adding LA and operating at low HRT favored HAc type fermentation with high H\(_2\) yields (Figures 5.3 and 5.4c) is consistent with the equation outlined for fermentative H\(_2\) production (Equation 5.1, Table 5.1). The average percent of electrons diverted to alcohol production in stage III were low in comparison to corresponding control cultures in stage II (Figures 5.4b and 5.4c) except for cultures operating at a 12 h HRT fed with LA. Under these conditions, the maximum percent e\(^{-}\) sink in alcohols (EtOH plus i-PrOH) reached 15±2%. Similarly, in studies by Chaganti et al. (2013), in sequential batch reactors operating at a 39 h HRT shows that for LA treated cultures fed glucose, the major e\(^{-}\) sinks were HAc (30%), HBu (23%) and i-PrOH (20%).

The pyrosequencing results revealed that the microbial community contained members belonging to Clostridiaceae (Clostridium sp.) for which HBu is considered to be a genus specific product (Andreesen et al., 1989). However, other species present such as C. beijerinckii, C. acetobutylicum and C. propionicum and belonging to the same genus indicated mixed acid and alcohol fermentation (Wiegel et al., 2006). The HPr levels observed in the fermentation by-products might be due to the family Propionibacteriaceae, which contained Propionibacterium sp. and Brooklawnia sp., are capable of producing HPr from various carbon sources. Narihiro et al. (2009) observed that these types of bacteria produced elevated levels of HPr during the treatment of food processing waste in an UASBR. In addition to these microorganisms, the culture also contained Synergistaceae (Aminobacterium sp., Cloacibacillus sp., Aminiphilus sp.), an organism capable of producing mixed acids in mixed anaerobic communities. Ganesan et al. (2008) reported that Synergistaceae are capable of degrading amino acids anaerobically and they can produce organic acids which can be consumed by methanogens. Ruminococcaceae were also identified that can produce H\(_2\), HAc and EtOH as major byproducts in dark fermentation (Pavloóstathis et al., 1988). An increase in the OLR was observed to cause a reduction in non-H\(_2\) producers (Figure 5.9). Therefore, only the species related to Ruminococcaceae and Clostridiaceae were retained with increasing the OLR. However, the composition of these micro-organisms remained the
same, thereby increasing their percent abundance in the bacterial community structure (data not shown). Liu et al. (2012) observed a similar pattern with increased OLR in an UASBR containing mixed anaerobic cultures grown in an attached growth system using activated carbon. The authors noticed an increase in percent abundance of the Ruminococcaceae and Clostridiaceae. The fermentation by-products observed in this current study showed elevated levels of HAc and EtOH in stage I at high OLRs. These observations are in agreement with the findings of Pavlostathis et al. (1988) and Ren et al. (1997) who observed fermentation of the EtOH and HAc production-type for an acidogenic culture at pH levels < 5.0.

The conditions from stage II with HRT decreasing from 20 h to 8 h were grouped together in the same quadrant of the PCA (Figure 5.7). In addition, the PCoA that was plotted using the microbial T-RFs data shows clustering were based on the substrate loading rate and the HRT operated (Figure 5.8). The predominant groups of organisms at a 16 h HRT belonged to Parabacteroides sp. (a mixed acid-producing group) and Moorella thermoacetica (a homo-acetogenic group). Tan et al. (2012) reported that Parabacteroides sp., as an obligate-anaerobe in the wastewater of a paper mill, was capable of assimilating a variety of substrates, such as glucose, lactose, sucrose, and cellobiose, to produce organic acids. Similarly, Moorella thermoacetica is considered a model acetogenic bacterium capable of producing HAc from carbon sources, such as glucose, CO₂, CO, etc. (Pierce et al., 2008). However, in the current study, no significant homoacetogenic (R17 flux) activity was observed in stage II. The resurgence of Propionibacterium sp. clearly indicates that reducing the OLR may allow the non-H₂ producing populations to reestablish themselves. The H₂-producing populations were primarily composed of Butyrivibrio sp. and Clostridium sp., which constituted for 22.4% of the microbial community at a 16 h HRT. The presence of these microorganisms indicates HAc and HBu type fermentation is possible under this operating conditions (Moat et al., 2002). The fermentation by-products detected under these experimental conditions also supports this evidence. Both HAc and HBu contributed about 67% of the soluble metabolite byproducts. Interestingly, Alkaliphilus sp. belonging to the Clostridia class was observed at 16, 8 and 5 h HRTs (Table 5.3). These species are strict anaerobes
that are capable of reducing metals, such as iron and cobalt use HAc and HLa as their preferred electron donors (Ye et al., 2004). When the HRT was reduced to 5 h while the OLR was increased to 38.4 g L\(^{-1}\) d\(^{-1}\), the EtOH fermentation-type of H\(_2\)-producing bacteria (e.g. *Ethanoligenens* sp.) were dominant. Studies demonstrating EtOH type of fermentation in a continuous reactor system fed molasses wastewater reportedly showed *Ethanoligenens* sp. as the dominant microorganism (Ren et al., 2007). The second major microorganism detected was *Thermanaerovibrio* sp. This group of microorganism which consisted of *T. acidaminovorans* are capable of metabolizing sugars or organic acids such as HAc (Baena et al., 1999). Baldursson (2006) reported that *T. acidaminovorans*, which were isolated from Icelandic hot-springs and can grow under thermophilic conditions, could be potential H\(_2\) producers. In comparison, Saady et al. (2012) reported that *T. acidaminovorans* was the dominant species detected in H\(_2\)-producing anaerobic granular cultures grown in batch reactors under mesophilic conditions and fed with glucose. The other H\(_2\) or acid producing communities that had a major presence under low HRTs belonged to *Firmicutes* (consisting of *Clostridium* sp., *Bacillus* sp., *Themoanaerobacter* sp., etc.). All of these are considered as potential H\(_2\) producers in mixed anaerobic communities (Hniman et al., 2011; Huang et al., 2010). With the relative abundance of *Clostridium* sp. decreasing from stage I to stage II in response to simultaneous adjustments to HRT and OLR, decreased H\(_2\) yield was observed with HRT \(\leq 5\) h (Figure 5.3). According to Huang et al. (2010), operating at a lower HRT than the optimal retention time for suppressing H\(_2\) consumers may also inhibit the growth of H\(_2\) producers, such as *Clostridium* sp..

### 5.5 Conclusions

In summary, this study demonstrates the effects of OLR and HRT on bio-H\(_2\) production. An increase in H\(_2\) yield with a concurrent decrease in CH\(_4\) yield was observed when the OLR was increased from 2 to 8 g L\(^{-1}\). At a 24 h HRT, a maximum H\(_2\) yield of 1.70±0.05 mol mol\(^{-1}\) glucose was obtained for cultures operating at 8 g L\(^{-1}\) d\(^{-1}\), with the corresponding CH\(_4\) yield of 0.18±0.06 mol mol\(^{-1}\) glucose. This CH\(_4\) yield is 82% less than the maximum CH\(_4\) yield obtained at an OLR of 2 g L\(^{-1}\) d\(^{-1}\).
A decrease in HRT along with a corresponding increase in OLR was required for the subsequent suppression of methanogens to produce an appreciable increase in H$_2$ yield i.e., < 2.0 mol mol$^{-1}$ glucose at HRTs ranging from 20 to 12 h. The increase in OLR with a decrease in HRT had a positive impact on the HPR and a maximum HPR of 15.4±1.4 L L$^{-1}$ d$^{-1}$ was observed with a 1.5 h HRT (corresponding to an OLR of 96.0 g L$^{-1}$ d$^{-1}$). Elevated solvents levels which decreased the H$_2$ productivity by up to 40% was observed in reactors operating with high glucose loading.

The FBA in control cultures revealed increasing OLR by reducing the HRT up to 12 h, suppressed H$_2$ consumption activity with increase in H$_2$ yield, while LA treated cultures showed increased suppression of H$_2$ consuming flux with H$_2$ yields $\geq$ 2.0 mol mol$^{-1}$ glucose for HRTs below 12 h.

Increasing the relative abundance of Clostridiaceae and Ruminococcaceae, potential H$_2$ and EtOH producers, was a result of increasing the OLR at a 24 h HRT in stage I. However, changes to the HRT in stage II caused a shift in the composition of the microflora, with an abundance of Parabacteroides sp., Ethanoligenens sp., Clostridium sp., Thermanaerovibrio sp. and Alkaliphilus sp. The presence of these organisms exhibit different functions in the pathways for H$_2$, EtOH and mixed acid fermentation. Note that species belonging to Bacteroidetes were reduced to less than 1% at a low HRT of 5 h.

The study suggests that both the optimum substrate level and retention time are required to establish stable H$_2$ production. Adding a methanogenic inhibitor such as LA is important for establishing stable operation with an increasing the H$_2$ yield (> 2.0 mol mol$^{-1}$ glucose).

5.6 References


CHAPTER 6: EFFECT OF FURANS AND LINOLEIC ACID ON METABOLIC SHIFTS IN HYDROGEN FERMENTATION

6.1 Introduction

Lignocellulosic biomass is an underutilized low value renewable resource with an estimated global annual production of 4.63 billion tons (Yokoyama, 2008). Utilizing this abundant resource for fermentative hydrogen (H\textsubscript{2}) production could potentially lead to the development of an economical biohydrogen production process (Kotay and Das, 2008; Sims et al., 2010). Because of their sugar composition, low value biomass such as corn stover (Zhang et al., 2007), sugarcane bagasse (Patra et al., 2008), switchgrass (Keshwani and Cheng, 2010) and wheat straw (Chen et al., 2007) could serve as viable feedstocks for biohydrogen production. However, lignocellulosic materials are not readily usable because of their complex structure and recalcitrant nature. A major goal of the biofuels industry is to develop pretreatment technologies which can produce chemicals and fuels from lignocellulosics; however, to date, only a few technologies can efficiently use this biomass source (Bothast and Saha, 1997; Wheals et al., 1999; Zaldivar et al., 2001).

Among the different technologies, steam explosion is used extensively to pretreat lignocellulosic biomass (Kumar et al., 2009). During pretreatment, lignocellulosic residues release free sugars and other chemicals such as furfural, 5-hydroxymethylfurfural (HMF), vanillin, syringaldehyde, organic acids and other phenolic compounds (Klinke et al., 2004; Larsson et al., 1999; Palmqvist and Hahn-Hagerdal, 2000).

A key challenge for producing biofuels from sugars in a liquor produced from pretreatment is to alleviate the microbial inhibitory effects caused by furans (furfural and HMF). Optimum production of sugars with minimum levels of furans is dependent on optimizing the pretreatment conditions. During pretreatment, furfural and HMF are produced from pentose and hexose dehydration, respectively. The levels of furfural and HMF produced are dependent on the nature of the lignocellulosic raw materials, the operating conditions and the treatment process. Many microorganisms modify these
compounds as a means to reduce their toxic effects. Furfural is converted into furfuryl alcohol and furoic acid (Liu et al., 2005) while HMF is either converted to 5-hydroxymethyl furfuryl alcohol (Boopathy et al., 1993) or 2,5-bis-hydroxymethylfuran (Liu et al., 2004). Evidence describing the effects of HMF and furfural and other furan derivatives on pure cultures have been reported by several researchers (Cao et al., 2010; Pienkos and Zhang, 2009; Sakai et al., 2007). According to Pienkos and Zhang (2009), the inhibition trend for *E. Coli* LY01, a recombinant ethanologenic strain, is as follows: hydroxybenzaldehyde > vanillin > syringaldehyde > furfural > HMF > ethanol.

Hydrogen producing mixed microbial cultures are negatively impacted by furans and furan derivatives. In comparison to H₂ producing controls (1.67 mol mol⁻¹ xylose), low H₂ yields ranging from 0.34 to 1.39 mol H₂ mol⁻¹ xylose have been reported for cultures inhibited with furan derivatives, phenolics and lignin (Quéméneur et al., 2012). Inhibition studies demonstrating the larger inhibitory effect caused by furan derivatives in comparison to phenolics have been described by Quéméneur et al. (2012). According to these researchers, H₂ yields for cultures fed furan derivatives and phenolics were 0.40-0.51 mol mol⁻¹ xylose and 1.28-1.39 mol mol⁻¹ xylose, respectively.

Many studies have shown H₂ production from sugars using mixed anaerobic microbial communities (Abreu et al., 2012; Chaganti et al., 2012). Anaerobic microbial communities are a mixture of hydrolytic microorganisms, acidogens, acetogens and methanogens. Two essential populations which can affect the H₂ yield are grouped as H₂ consumers (methanogens) and H₂ producers. In methanogenic reactors, H₂ consumers and H₂ producers operate in synchrony to maintain low H₂ levels and hence, provide stable thermodynamic conditions (Stams, 1994). Uncoupling this syntrophic condition between H₂ consumers and H₂ producers by employing different reactor operating strategies and/or applying stressing agents can lead to increased H₂ yields. Inorganic acids, 2-bromoethanesulfonic acid (BESA) and long chain fatty acids (LCFAs) such as linoleic acid (LA) are microbial inhibitors and their use as microbial chemical stressing agents have been reported in many studies (Lee et al., 2009; Pendyala et al., 2012; Ray et al., 2010; Zhu and Béland, 2006). LCFAs are renewable chemicals, utilizing them to inhibit methanogenesis could be an economical approach for maximizing H₂ production when compared to thermal and other chemical methods. According to Ray et al. (2010),
LA is an excellent H₂ uncoupler and hence, a methanogenic inhibitor. Oleic acid (OA) is another LCFA which has been reported to inhibit H₂ consumers and subsequently increase the H₂ yield in mixed anaerobic communities fed glucose and xylose (Chaganti et al., 2012).

Chemical inhibitors impose their effect at threshold levels on specific microbial populations. For example, LA, furfural and HMF are effective in uncoupling the syntrophic interaction between H₂ producers and H₂ consumers at threshold levels (Belay et al., 1997; Chowdhury et al., 2007). Several studies have provided evidence showing H₂ production using LA inhibited cultures fed glucose (Chowdhury et al., 2007; Ray et al., 2010). Hence, one objective of this study was to examine the impact of feeding mixtures containing glucose, furfural and HMF to two mixed anaerobic microbial communities not inhibited and inhibited with LA. Another objective was to identify threshold furfural and HMF levels above which the inhibition of H₂ production occurs.

6.2 Materials and methods

All experiments were conducted in 160 mL serum bottles with 50 mL working volumes at 37 °C and an initial pH of 5.5. Two different culture sources belonged to cultures A and B, outlined in section 3.3 were used in this experiment to study the effect of the fermentation inhibitors from two different source and generalize their effects. The cultures were maintained in a bench scale reactor (3.5 L working volume, with VSS concentration of 10 g L⁻¹), operated under sequencing batch mode fed 5 g L⁻¹ of glucose at an HRT of 14 days with the pH range observed to be between 6.7 to 7.8. The preparation of bottles for experimentation is outlined in section 3.4. The experiments were conducted using glucose as a substrate and varying ratios of furan as the fermentation inhibitor (total 1 g L⁻¹) with LA inhibited cultures. A furan control set, without addition of LA was run in parallel, for experimental design see Table 6.1.

In addition, experiments with steam exploded corn stover (CS) liquor and resin treated liquor (outlined in section 3.2.2) as a carbon source for fermentation was examined. The sugar composition of the CS hydrolysate (both resin treated and raw steam exploded hydrolysate) is presented in Appendix F, Table F.2.
Table 6.1 Experimental design conditions to study the effect of furans on the different culture source

<table>
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<th>Concentration of parameters (g L(^{-1}))</th>
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<td>B-RCS-L</td>
<td>Resin treated CS</td>
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</table>

Notes:
1. A and B represent the culture sources described in Section 3.3.
2. X: xylose; CS: corn stover; RCS: resin treated corn stover; C: control cultures and L:LA treated cultures
3. The substrate concentration in the bottles at start up were 5.0 g\(\text{hexose L}^{-1}\).
4. Initial pH was 5.5

The furfural and HMF concentration chosen for the experiments was based on concentrations reported by Boopathy (2009) and Sanchez and Bautista (1988). The LA concentration (2 g L\(^{-1}\)) and pH (5.5) were adapted from work reported by Chaganti et al. (2011). All analytical and chemical methods were followed as outlined in sections 3.7 and 3.8. The microbial characterization was carried out as described in section 3.10.

Tukey’s test at a 95% confidence interval was used to determine the statistical difference between the \(\text{H}_2\) yields for the cultures receiving LA and furan (furfural and HMF mix). Fitting the data to a model equation was performed using Sigma Plot (Systat Software, Inc., IL). The ‘goodness of fit’ of the model equation was evaluated statistically using the coefficient of determination \((R^2)\) and F value. The Anderson-
Darling (AD) test was used to assess the normal distribution of the residuals. A principal component analysis-biplot was used to study the underlying patterns in the electron distribution among the culture source treated with different stress conditions. Canonical correspondence analysis (CCA), a statistical tool widely used in ecological and environmental studies, was used to evaluate the impact of different factors on the composition of mixed microbial communities. In addition, a non-parametric clustering technique (multivariate cluster analysis) was used to determine similarities between the T-RFs. All the statistical methods are described in section 3.12.

6.3 Results and discussion

6.3.1 Hydrogen production by two cultures fed furans and glucose

No statistical significant difference in the H₂ yield was observed for cultures fed 1 g L⁻¹ of furan and 2 g L⁻¹ LA. On day 7, a maximum H₂ yield of 1.89±0.27 mol mol⁻¹ glucose was observed for culture A fed LA (2 g L⁻¹), furfural (0.75 g L⁻¹) and HMF (0.25 g L⁻¹) (Expt.# A-2, Table 6.1) while for culture B fed the same inhibitors at the same levels, the maximum H₂ yield was 1.75±0.22 mol mol⁻¹ glucose (Expt.# B-2, Table 6.1) (Figure 6.1a). Studies by Nissilä et al. (2012) have shown a H₂ yield of 0.8 mol mol⁻¹ glucose for mixed anaerobic cultures operating at pH 6 and fed a hydrolysate from dry conifer.
Figure 6.1 Hydrogen production from (a) model lignocellulosic compounds (b) lignocellulosic (corn stover) hydrolysate

Notes:
1. Experimental design defining each test condition is given in Table 6.1.
2. Error bars in the bar graph represent the standard deviation for n = 3.

Relative to the maximum yield, adding furfural (1 g L\(^{-1}\)) plus HMF (1 g L\(^{-1}\)) caused the H\(_2\) yield on the day 7 to decrease by 32±4\% (1.28±0.12 mol mol\(^{-1}\) glucose) and 48±6\% (0.91±0.12 mol mol\(^{-1}\) glucose) in cultures A and B, respectively (Expt.#, Table 6.1, Figure 6.1a). These results suggest that beyond the threshold furfural and HMF levels, the inhibitory effect likely affected not only the H\(_2\) consumers but also the H\(_2\) producers.

Studies describing the impact of furans or lignocellulosic hydrolysates on H\(_2\) producing mixed anaerobic cultures are limited and further work is required to establish the full impact on these chemicals on microorganisms. Although data on the fermentation of lignocellulosics residues and hydrolysates to methane showed evidence of impact on anaerobic microorganisms, no comprehensive study has described the impact of furan and furan derivatives on specific populations. Note according to Nissilä et al. (2012),
microorganisms belonging to the *Bacteroidetes, Firmicutes and Proteobacteria* phyla were involved in converting a birch and conifer hydrolysate into H₂.

The Tukey’s test at a 95% confidence interval revealed that the H₂ yields were statistically similar for cultures A and B treated with LA. This analysis indicates the ratio of furfural to HMF at 1 g L⁻¹ did not affect the H₂ yield (Figure 6.1a, Table 6.1). Data from this work contradicts work reported by Cao et al. (2010) and Mussatto and Roberto (2004), where the former observed inhibition in H₂ yield observed at 1 g L⁻¹ HMF was greater when compared to 1 g L⁻¹ furfural and the later observed a larger negative synergistic effect when a mixture of the inhibitors was compared to each inhibitor.

Evidence showing the impact of LA is based on data for experiments conducted using a feed containing glucose plus 0.5 g L⁻¹ furfural and 0.5 g L⁻¹ HMF (Expt. C-1, Table 6.1) in comparison to cultures fed glucose, 2 g L⁻¹ LA, plus 0.5 g L⁻¹ furfural and 0.5 g L⁻¹ HMF (Expt. # 3, Table 6.1). On day 7, the H₂ yield for culture A and culture B receiving 0.5 g L⁻¹ furfural and 0.5 g L⁻¹ HMF were 20±3% (1.46±0.15 mol mol⁻¹ glucose) and 62±15% (0.67±0.38 mol mol⁻¹ glucose), respectively, less in comparison to cultures operating under conditions with maximum yields.

The H₂ yields in this study are comparable to the yields obtained by Cao et al. (2009) for *Thermoanaerobacterium thermosaccharolyticum* W16 fed acid hydrolyzed corn stalk liquor. These researchers reported a H₂ yield range of 0.71 to 2.24 mol mol⁻¹ sugar for a feed containing furans concentration ranging from 0.21 to 0.94 g L⁻¹ and a culture operating at 60 °C and pH 7. Hydrogen production studies by Fangkum and Reungsang (2011) have provided some evidence showing the effects of furans in lignocellulosic liquor. They reported using a hydrolysate containing (g L⁻¹) glucose (1.46), xylose (9.10), arabinose (0.72) acetic acid (HAc) (1.30) and furfural (0.22). Fangkum and Reungsang (2011) showed a H₂ yield of 1.48±0.22 mol mol⁻¹ sugar consumed for heat treated elephant manure operating at 55 °C and maintained at a pH of 5.5. Notice studies reported by Cao et al. (2009) and Fangkum and Reungsang (2011) were conducted under thermophilic conditions while this work was performed using mesophilic cultures. Datar et al. (2007) studied H₂ production from corn stover hydrolysate at 35 °C, these authors observed the furfural concentrations obtained at high severity conditions (steam
explosion at 220 °C for 5 min) resulted in approximately 50% reduction in cumulative H₂ production with high lag time of 32 h.

The control cultures fed no LA and no furan showed no significant amount of H₂ produced, while LA fed cultures with no furan produced H₂ yields (mol mol⁻¹ glucose) up to 2.12±0.14 and 1.96±0.18 in cultures A and B, respectively. The effect of addition of furan is seen by comparing the C-1 and C-2 cultures where addition of furan increases the H₂ yield by suppressing the methane production (Table 6.2).

6.3.2 Hydrogen production from corn stover

No appreciable amount of H₂ was produced by the cultures fed with xylose alone (Figure 6.1b), one possible reason for apparently low level of H₂ production might be due to the co-occurrence of H₂ consumers with mixed microflora (Dinamarca and Bakke, 2012). The maximum H₂ yield obtained using culture B fed with pure xylose (X); CS hydrolysate or furan removed (RCS) hydrolysate over the period of 7 days is shown in Figure 6.1b (Note: Except for LA treated culture fed with a CS hydrolysate, maximum H₂ yield observed at day 7, while for LA treated cultures fed CS hydrolysate maximum H₂ yield was observed in day 4).

The results reveal that the H₂ yield obtained from LA-treated culture was greater than that of control culture in both the samples fed with pure sugar (xylose) (B-X) and with resin treated hydrolysate (B-RCS). The H₂ yields per mole of hexose from the LA-treated cultures fed with xylose and RCS were 2.68±0.36 and 2.25±0.17, respectively. The results obtained in cultures fed xylose and LA, is comparable with previously reported yields obtained from LCFA-treated cultures fed with xylose (Chaganti et al., 2012). Similarly, the molar H₂ yield obtained from LA-treated culture samples fed resin treated hydrolysate was 20% greater than that obtained by Yang et al. (2010) from samples treated with an acid (HCl) followed by enzymatically hydrolyzed corn cob (a part of corn stover). For the control cultures (no LA) fed directly with steam exploded hydrolysate, the H₂ yield (per mole hexose) reached a maximum yield of 1.74±0.23 on day 7, whereas for the LA-treated culture samples, the maximum H₂ yield obtained on day 4 (from first injection of hydrolysate) was 1.73±0.22, and there was no significant amount of H₂ detected during the second injection of the hydrolysate with the LA treated cultures (data not shown).
6.3.3 Impact of furans and their degradation byproducts

The impact of furans and their degradation byproducts on microorganisms have been reviewed by Almeida et al. (2009). HMF and furfural can affect the metabolism of many microbial populations.

Microorganisms relieve these chemical stresses by converting HMF and furfural into less inhibitory compounds as long as the initial concentrations are not beyond a threshold inhibitory level (Boyer et al., 1992). In this study, furfural degradation byproducts, furoic acid and furfuryl alcohol, were detected in the fermentation broth (Figure 6.2). HMF degradation was observed; however, its degradation byproducts such as 5-hydroxymethylfurfuryl alcohol (HMF alcohol) were not detected. At threshold levels, inhibitors such as LA and furans are able to affect the metabolic pathways of dominant anaerobic microorganisms and the distribution of fermentation byproducts (Borole et al., 2009; Ray et al., 2010)

Figure 6.2 Conversion of furfural to furfuryl alcohol and furoic acid for different concentrations of furfural described in Table 6.1 a) Culture A and b) Culture B

Note: Error bars in the bar graph represent the standard deviation for n = 3
The distribution of metabolites from glucose fermentation was affected by the presence of furans or furans plus LA. Furoic acid was the major byproduct detected in controls without LA. Although HMF was degraded, the degradation rate was less in comparison to furfural. The ease of HMF degradation in comparison to furfural is similar to data reported by Larsson et al. (1999) for \textit{Saccharomyces cerevisiae}.

In the presence of 1 g L\textsuperscript{-1} furans (Expts. #1-5, Table 6.1), 54.8±3.7\% of the electron equivalents were diverted to the total VFAs in culture B. In comparison, increasing the furan level beyond 1 g L\textsuperscript{-1} (Expt. #6, Table 6.1) in culture A caused 40.0±5.0\% of the electrons equivalents to be diverted into VFAs (Table 6.2). This decrease in electron diversion could be due to differences in the microbial populations as well as variations in the inhibitory effects on H\textsubscript{2} producers in the two cultures.

6.3.4 \textbf{Electron distribution under different conditions}

The concentration and distribution of VFAs and alcohols are useful indicators for monitoring H\textsubscript{2} production. The major VFAs detected were HAc, propionic acid (HPr) and butyric acid (HBu) while ethanol (EtOH) and i-propanol (i-PrOH) were the key alcohols (Table 6.2). Based on the percent electron equivalents, in the presence of feeds containing furans plus LA, the average VFA levels in culture A (36.5±2.4\%) was less than culture B (52.3±6.2\%) while the average alcohol levels in culture A (34±11\%) was greater than in culture B (12±5\%) (Table 6.2). The distribution of electron equivalences is likely linked to synergistic interactions between LA and furans under low pH conditions or LA and low pH conditions. Evidence of LA inhibition at pH 5.5 on methanogens and the redirection of electron equivalents to reduced metabolites was reported by Chaganti et al. (2012) and Ray et al. (2010).

The HAc concentration was approximately the same for cultures fed 1 g L\textsuperscript{-1} furans plus LA. In cultures A and B, HAc production accounted for 12.5±0.4\% and 16.7±1.1\% of the total electron equivalents. Relative to the controls (C-1), HAc formation in cultures A and B was greater by 25±2\% and 29±4\%, respectively. At elevated furan levels of 2 g L\textsuperscript{-1}, the low HAc levels produced in cultures A and B indicate inhibition of acetogenic microorganisms.
Table 6.2 Fraction of electron sinks under different test conditions in fermentative bio-hydrogen production

<table>
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<tr>
<th>Expt#</th>
<th>H₂</th>
<th>CH₄</th>
<th>HLa</th>
<th>HAc</th>
<th>HPr</th>
<th>HBu</th>
<th>i-PrOH</th>
<th>EtOH</th>
<th>Res. Glu</th>
<th>Biomass</th>
<th>Total</th>
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<td>A-1</td>
<td>15.5±3.3</td>
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<td>0.0±0.0</td>
<td>12.0±3.5</td>
<td>7.5±1.0</td>
<td>14.4±1.4</td>
<td>23.5±1.3</td>
<td>17.0±1.4</td>
<td>0.0</td>
<td>10.0</td>
<td>100.0±12.1</td>
</tr>
<tr>
<td>A-2</td>
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<td>0.0±0.0</td>
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<td>8.5±0.9</td>
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<td>23.9±1.6</td>
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</table>

Notes:
1. a Biomass - 10% of the e equivalent from glucose is assumed to be converted into biomass.
2. HAc = acetic acid, HPr = propionic acid, HLa = lactic acid, HBu = butyric acid, i-PrOH = iso-propanol, EtOH = ethanol and Res. Glu = residual glucose.
3. The Expt# represents the experimental conditions described in Table 6.1
4. The mean ± standard deviation is for n = 3
In cultures A and B, the quantity of electron equivalents diverted to propionate production was 9±2% and 23±3%, respectively. The HPr levels in culture B controls (C-1) and those fed furan plus LA were approximately the same. Relative to the control (C-1) for culture A, more HPr (51±5% (Table 6.2)) was produced in the furan plus LA fed cultures. The percent electrons diverted to butyrate in cultures A and B fed furan plus LA was approximately the same in both cultures (15±2% (Table 6.2)).

In culture A, the EtOH levels produced were greater in comparison to culture B. In culture A, conditions favoring the growth of EtOH producing microorganisms was likely responsible for the difference in metabolite levels. Notice at a threshold (A-6 and B-6) furan level (1 g L⁻¹ HMF plus 1 g L⁻¹ furfural), the quantity of electron equivalents diverted to i-PrOH formation was substantially reduced in cultures A and B. The inhibition of enzymes responsible for i-PrOH production by the presence of furans might be responsible for this decrease.

Under stressful conditions, microorganisms adapt by implementing numerous mechanisms. In the presence of chemical agents such as HMF and furfural, anaerobic mixed cultures avoid the stress conditions by converting these chemicals into less toxic compounds. The formation of different metabolites at varying levels in two cultures fed the same substrates could be associated with chemical potential fluctuations in microenvironments, differences in the type and quantity of microorganisms and availability of co-factors and adenonucleotides. In microenvironments within cultures A and B, pH variations, metabolite type and concentration as well as microbial populations will affect the metabolic pathways. Under elevated H₂ levels, microorganisms relieve stressful conditions by the production of reduced metabolites (Dabrock et al., 1992; Lay et al., 2012). Metabolic switching between pathways is a strategy used by microorganisms to relieve the effects of inhibitory agents (Kim et al., 1984).

6.3.5 Principal component analysis

PCA is a tool used to visualize the variance in a data set obtained from multiple samples. The data set analyzed were taken from the measured outputs for the different conditions under which cultures A and B were subjected to during H₂ fermentation. These variables included the substrate, gas and liquid metabolites (H₂, CH₄, HLa, HAc, HPr, HBu, formate, i-PrOH and EtOH). In addition to the PCA biplot shown in Figure
a PCA using log transformation of these variables is shown in Figures G.1a and b, Appendix G to test the distribution of the samples in the two dimensional plane and study the treatment effect using ANOVA on the factor scores obtained.

Figure 6.3 Principal Component plot (bi-plot) showing the grouping of samples from cultures A and B tested under various conditions based on their gas and liquid metabolites

Notes:
1. Only the first and second principal components are shown.
2. Only experiments 1 to 6 and ‘C-1’ in Table 6.1 were used for PCA analysis and the letters ‘A’ and ‘B’ denote the culture source.
3. CH\textsubscript{4} = methane; H2 = hydrogen; HAc = acetic acid; HPr = propionic acid; HLa = lactic acid; HBu = butyric acid; EtOH = ethanol and i-PrOH = iso-propanol.

The clusters shown in Figure 6.3 are associated with the gas and liquid byproducts. PC1 and PC2 accounted for 67% of the total variability present in the data set. Based on the loading values, PC1 correlated with butyrate (0.71) and ethanol (0.81) while PC2 was correlated with H\textsubscript{2} (0.87) and i-propanol (0.76). Both cultures (A and B) fed mixtures of different furfural and HMF ratios (1 g L\textsuperscript{-1} total), 5 g L\textsuperscript{-1} glucose and 2 g L\textsuperscript{-1} LA were clustered into two groups. This trend indicates the ratio of furfural to HMF did not affect the byproduct distribution for culture A and B when the total furan concentration was set at 1 g L\textsuperscript{-1}. However, at 2 g L\textsuperscript{-1} furans (1:1 ratio), cultures A and B were clustered and associated with butyrate production. Notice at 2 g L\textsuperscript{-1} LA, culture A was linked to alcohol
and H₂ production while oxidized byproducts were associated with culture B (Figure 6.3 and Table 6.2).

Controls (C-1 cultures A and B) linked to methane production were clustered into one group. At a threshold furan level of 1 g L⁻¹, methane production was not inhibited in both cultures. This trend indicates common microbial population between the two cultures which are involved in methanogenesis. With increasing stress conditions, the cultures metabolic pathways were expressed differently and diverged into the production of different metabolites. Evidence supporting the influence of stressing agents such as pH and LA has shown to vary the distribution of electron fluxes from glucose to byproducts (Chaganti et al., 2011). Similarly, studies by Quershi et al. (2012) have shown the effects of furans (furfural and HMF) on acetone, ethanol and butanol production by Clostridium beijerinckii P260. At 1.5 g L⁻¹ furfural plus 1.0 g L⁻¹ HMF, they observed a 50% decrease in alcohol productivity.

6.3.6 Canonical correspondence analysis

In the CCA plot, the length of the vector indicates the extent to which the community structure can be explained by a given environmental variable, while the angle between the vectors provides an indicator of the correlation between environmental variables. On the tri-plot, the variables (gas, VFAs and alcohol) are shown by dashed lines and the T-RFs with band intensity ≥20% are shown by open squares.

The first two axis of the CCA explains approximately 47.5% of the species variation in the samples analyzed (Figure 6.4). Component 1 was mostly associated with methane and butyrate whereas component 2 was associated with HAc, HPr and i-PrOH. Note correlations between the variables and the species were weak with 0.04 on the first axis and 0.02 on the second (Table 6.3). The CCA plot showed that LA treated cultures were primarily associated with HAc, HPr, H₂ and i-PrOH while the control cultures were linked with methane and butyrate.
Figure 6.4 Canonical correspondence analysis based on the metabolites and the species abundance in cultures A and B

Notes:
1. Triangle represents control (C-1) cultures A (▲) and B (△)
2. Circle represents LA treated cultures A (●) and B (○)
3. Square (□) corresponds to T-RF bands with ≥ 20% relative intensity
4. CH₄ = methane; EtOH = ethanol; HPr = propionic acid; i-PrOH = iso-propanol; HAc = acetic acid; HBu = butyric acid.

Table 6.3 Summary of canonical correspondence analysis ordination

<table>
<thead>
<tr>
<th>Axes</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>Total inertia</th>
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</tr>
<tr>
<td>Cumulative percentage variance of species data</td>
<td>24.6</td>
<td>47.5</td>
<td>68.2</td>
<td>83.8</td>
<td>93.9</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

Species which were abundant but less sensitive (correlated) to the factors were closely associated with the cultures within the cluster. Only control cultures (not treated with LA) were located in different quadrants. Species associated with the variables included Propionibacterium sp., Clostridium sp., Flavobacterium sp., Bacillus sp., and Eubacterium sp. Clostridium sp., are known to produce byproducts such as HAc, HBu, EtOH and HPr (Minton and Clarke, 1989). Apart from the liquid byproducts, Clostridium sp. are H₂ producers and are able to survive stressful conditions (Pendyala et al., 2012;
Quéméneur et al., 2012; Quershi et al., 2012). *Flavobacterium* sp. is able to grow and ferment feedstocks containing furans (Fangkum and Reungsang, 2011; Lopez et al., 2004). The presence of *Propionibacterium* sp. is strongly associated with the detection of HPr in cultures treated with LA (Table 6.2). Ren et al. (2007) reported that *Propionibacterium* sp. are facultative anaerobes which could grow on a wide pH range from 5-9 and showed HPr type of fermentation in mixed anaerobic communities. *Bacillus* sp. and *Thermoanerobacter* sp. are known to produce H\(_2\) and ethanol from lignocellulosic feed stock (Bala-Amutha and Murugesan, 2013; Klinke et al., 2001). Klinke et al. (2001) have reported the effect of the fermentation inhibitors in hydrolysate from wheat straw using *Thermoanerobacter* sp. These authors have shown that *Themoanaerobacter* sp. were less affected by the furoic acid. The presence of *Moorella thermoacetica* indicated acetogenic activity which is considered as a known acetogenic bacterium capable of producing HAc from carbon sources such as glucose, CO\(_2\) and CO (Pierce et al., 2008).

### 6.3.7 Modeling the experimental data

Interaction effects of LA, furfural and HMF on the response variable are shown in the contour plots (Figure 6.5a and b). The shape of the contour shows a combined effect caused by furan inhibitors (furfural and HMF) on H\(_2\) production. For culture A, when the individual concentration of furfural and HMF were in the range of approximately 0.75 to 0.95 g L\(^{-1}\) and 0.8 to 1.0 g L\(^{-1}\), respectively, the peak H\(_2\) yield attained was approximately, 1.8 mol mol\(^{-1}\) glucose (Figure 6.5a). In the case of culture B, the H\(_2\) yield attained was approximately, 1.8 mol mol\(^{-1}\) glucose when the individual furfural concentration was in the range of approximately 0.8 to 0.9 g L\(^{-1}\) and 1.6 mol mol\(^{-1}\) glucose when HMF concentration was in range of 0.7 to 1.0 g L\(^{-1}\) (Figure 6.5b). When the individual concentration of furfural and HMF reached approximately 1 g L\(^{-1}\), the H\(_2\) yield attained in culture A and B were 1.3 mol mol\(^{-1}\) glucose and 1.0 mol mol\(^{-1}\) glucose, respectively (Figure 6.5a and b). Model equations for the H\(_2\) yield as a function of the furfural, HMF and LA concentrations were developed for cultures A and B (equation (6.1 and 6.2)).
Figure 6.5 Contour plot showing the effect of furfural and HMF on the hydrogen yield for (a) culture A and (b) culture B

\[ H_2 \text{ yield (A)} (mol \ mol^{-1} \ glucose) = 0.752 + 0.01[\text{Furfural}] g \ L^{-1} \]
\[ - 0.980[\text{HMF}] g \ L^{-1} + 0.542[\text{LA}] g \ L^{-1} \]  
\[ (6.1) \]

\[ H_2 \text{ yield (B)} (mol \ mol^{-1} \ glucose) = 0.520 - 0.242[\text{furfural}] g \ L^{-1} \]
\[ - 0.358[\text{HMF}] g \ L^{-1} + 0.674[\text{LA}] g \ L^{-1} \]  
\[ (6.2) \]

Table 6.4 ANOVA results for the model equations 6.1 and 6.2 (cultures A and B)

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>A</td>
<td>B</td>
<td>A</td>
</tr>
<tr>
<td>Regression</td>
<td>3</td>
<td>1.769</td>
<td>1.026</td>
<td>0.590</td>
<td>0.342</td>
</tr>
<tr>
<td>Residual</td>
<td>5</td>
<td>1.440</td>
<td>0.0255</td>
<td>0.288</td>
<td>0.0085</td>
</tr>
<tr>
<td>Total</td>
<td>8</td>
<td>3.210</td>
<td>1.052</td>
<td>0.401</td>
<td>0.175</td>
</tr>
</tbody>
</table>

Notes:
1. The F-value is the mean square due to regression divided by the mean square due to the residual.
2. DF = degrees of freedom; SS = sum of squares; MS = mean square
The model’s predictions for H\textsubscript{2} yield (equation 6.2) based on the furan levels present in the feed content (hydrolysate composition see Table F.2, Appendix F) that were fed to both LA-treated and untreated culture B (B-CS, B-RCS, BCS-L, B-RCS-L, Table 6.1) were compared to experimental findings of this study. For these samples, the model’s predictions did correlate with the observed experimental results seen in Figure 6.1b, except for cultures fed hydrolysed CS and treated with LA (i.e. B-CS-L), where large deviation between the outcomes predicted by the model and the experimental results were observed. These results suggest that in addition to furans present in the hydrolysate and LA, there exist interference of other compounds that were partially responsible for the difference in the H\textsubscript{2} yield. Studies by Mussatto and Roberto (2004), suggest that the
synergistic effect of furans and phenolic compounds, if present in the mixture, may be more toxic compared to the toxicity of these compounds applied.

An analysis of variance (ANOVA) was conducted to test the significance of fit for a linear fit. The ANOVA for \( \text{H}_2 \) production in culture A indicated that the F-value of 2.0 at a 95% confidence level (p-value = 0.226) implied that the model was insignificant (Table 6.4). For culture B, an F value of 7.072 at a 95% confidence interval (p-value = 0.030) was significant (Table 6.4). A coefficient of determination (R\(^2\)) close to 1 indicates a good correlation between the predicted and the observed values. In cultures A and B, the R\(^2\) values were 0.56 and 0.81, respectively (Figures 6.6a and b). Based on the F and p values, the models fit to the experimental data were adequate for culture B.

The residuals (model predicted – experimental observed) for the two cultures was tested for normality using the AD test (Figures 6.6c and d). The AD plots indicated normal distribution of the residuals. The AD values of 0.562 and 0.435 for culture A and B, respectively, were less than the critical value of 0.717 for a sample size of 9. The models were significant at a 5% level of confidence based on p values for both cultures which were larger than 0.05.

### 6.3.8 Microbial analysis

A cluster analysis was used to identify clusters among the mixed microbial cultures based on their similarity pattern. The cluster analysis is based on the Kulczynski similarity measure paired group algorithm. The analysis was conducted using the presence-absence of the T-RFs. The dendrogram is divided into 12 leaf nodes and 2 clusters or clades (Figure 6.7). The length of connecting lines reflects the degree of dissimilarity. For example, the similarity between A4 and B4 is greater than that between B1 and B2. The degree of similarity between A4, A5, B4 and B5 is approximately 54% to 80%. The goodness of fit of the T-RFs data set is supported by a cophenetic correlation coefficient of 0.87. Clade 1, with a similarity index range from 40 to 65%, showed the presence of \( \text{H}_2 \) producing organisms such as \textit{Clostridium} sp. and \textit{Flavobacterium} sp. In addition to \( \text{H}_2 \) producers in Clade 1, \textit{Methanococcus} sp. was detected in the control cultures fed furans (Expt. C-1). According to Belay et al. (1997) \textit{Methanococcus} sp. is capable of degrading furfural and HMF. A possible reason for the control cultures to cluster with the culture treated with LA may be due to the presence of the \( \text{H}_2 \) producers
belonging to the same genera. Clade 2, sharing a similarity index of 50% to 75%, were affiliated with the LA inhibited cultures A and B fed HMF ≥ 0.5 g L⁻¹ (Expt. # 3, 4 and 5).

![Clade Diagram]

**Figure 6.7 Kulczynski similarity index of the 16S rRNA gene T-RFs profiles**

**Notes:**
1. The numbers 1 to 5 and ‘C1’ represent the experimental conditions in Table 6.1 and the letters ‘A’ and ‘B’ denote the culture source
2. Samples with greater similarity in clades 1 and 2 are denoted with ○ and ●, in the cluster tree, respectively

Methane producers (*Methanococcus* sp.) in cultures treated with LA and fed 1 g L⁻¹ furans (Expt. # 1-5, Table 6.1) were inhibited while H₂ producers, *Clostridium* sp. and *Flavobacterium* sp., were unaffected. No methane was detected in cultures examined in experiments #1 to #5 (data not shown). According to Lopez et al. (2004), *Flavobacterium* sp. are capable of degrading furans. Furthermore, Akutsu et al. (2008) and Lu et al. (2009) have reported *Flavobacterium* sp. is capable of producing H₂ from complex substrate such as starch and corn stalk. The presence of *Flavobacterium* sp. in cultures fed furfural and HMF indicate they survived the chemical stress condition and likely degraded sugars in a liquid hydrolyzate. Data from this work contradicts reports by Quéméneur et al. (2012) where 1 g L⁻¹ furfural or 1 g L⁻¹ HMF inhibited H₂ production.
Notice increasing the furan level to \(2 \text{ g L}^{-1}\) (1 g L\(^{-1}\) furfural plus 1 g L\(^{-1}\) HMF) with 2 g L\(^{-1}\) LA likely inhibited *Clostridium* sp., *Flavobacterium* sp. in addition to *Methanococcus* sp.

Table 6.5 Experimental studies on inhibition caused by the furans on the fermentation process

<table>
<thead>
<tr>
<th>Culture</th>
<th>Inhibitor</th>
<th>Inhibitor Concentration</th>
<th>Inhibition Activity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. thermosaccharolyticum</em> W16</td>
<td>Furfural and HMF</td>
<td>0.8 g L(^{-1}) (each)</td>
<td>30% reduction in H(_2) production</td>
<td>(Cao et al., 2010)</td>
</tr>
<tr>
<td><em>Caldicellulosiruptor saccharolysis DSM 8903</em></td>
<td>Corn stalk hydrolysate</td>
<td>1.9 g of HMF and 1 g of furfural</td>
<td>11-48% reduction in H(_2) production</td>
<td>(Panagiotopoulos et al., 2011)</td>
</tr>
<tr>
<td><em>C. saccharolyticus and T. neapolitana</em></td>
<td>Furfural and HMF</td>
<td>1-2 g L(^{-1}) and 2-4 g L(^{-1})</td>
<td>50% reduction in H(_2) production</td>
<td>(de Vrije et al., 2009)</td>
</tr>
<tr>
<td><em>P. stipitis</em></td>
<td>Furfural HMF</td>
<td>0.5-1 g L(^{-1}) 1-4 g L(^{-1})</td>
<td>21-91% reduction in Ethanol productivity</td>
<td>(Delgenes et al., 1996)</td>
</tr>
<tr>
<td><em>C. guilliermondii</em></td>
<td>Furfural</td>
<td>1-2 g L(^{-1})</td>
<td>32-53% reduction in xylitol production</td>
<td>(Kelly et al., 2008)</td>
</tr>
</tbody>
</table>

Previous studies clearly indicate that furans are a potential threat to the process of fermentation (Saha, 2003). Evidence of the negative impact of furans on H\(_2\) fermentation is listed in Table 6.5. Therefore, removal of these inhibitors in the substrate prior to the addition of the medium as a feed for the fermentation process is necessary. The current study demonstrated that reducing furan levels from the lignocellulosic biomass improved the H\(_2\) yield by approximately 24% in comparison to the amount of H\(_2\) produced from the injection of liquid hydrolysate. Note, the culture fed with resin treated hydrolysate showing significant H\(_2\) production was enriched using LA. However, these parameters need to be optimized on a larger scale, along with other operational parameters such as HRT, substrate load and pH, to enhance the H\(_2\) yield.
6.4 Conclusions

In mixed anaerobic cultures treated with LA, fermentative H₂ production was affected by furans. Furan levels up to 1 g L⁻¹ in different furfural and HMF ratios were favorable to HPr or EtOH production in cultures A and B. Relative to the maximum yields, at higher furan levels with LA treatment, the H₂ yield decreased by 32±04% and 48±06% in cultures A and B, respectively. Addition of furans suppressed methane production and enhanced H₂ production, in both cultures A and B. In control cultures fed 1 g L⁻¹ of furfural and HMF at equal ratio, Methanococcus sp. and H₂ producers were presented.

Methanogenesis was not observed in LA treated cultures fed 1 and 2 g L⁻¹ furan. Clostridium sp. and Flavobacterium sp. were detected in both cultures A and B. Furans were converted to less toxic compounds in control and LA inhibited cultures.

The maximum H₂ yield observed in furan fed cultures was 1.89±0.27 mol mol⁻¹ glucose and 1.75±0.22 mol mol⁻¹ glucose in cultures A and B, respectively, for a feed containing, 0.75 g L⁻¹ furfural, 0.25 g L⁻¹ HMF and 5 g L⁻¹ glucose. The model demonstrated that the furfural or/and HMF concentrations ranging above 0.8 to 0.85 g L⁻¹ lowered the H₂ yield in cultures A and B fed LA. A PCA biplot revealed the metabolite distribution was dependent on the culture source. Alcohols and VFAs in liquid byproducts were associated with cultures A and B, respectively. A CCA based on the T-RFs and the fermentation metabolites revealed a weak interaction between the species composition and factors.

The synergistic effect of applying a methanogenic inhibitor (LA) with fermentation inhibitors present in the steam exploded hydrolysate of CS as a feed to the microflora revealed that, in order to maximize H₂ yield, optimization of the methanogenic inhibitor levels and other operational parameters for suppressing the H₂ consumers without compromising the activity of H₂ producers must be carried out.

6.5 References


Akutsu, Y., Li, Y.Y., Tandukar, M., Kubota, K., Harada, H. (2008). Effects of seed sludge on fermentative characteristics and microbial community structures in


CHAPTER 7: OPTIMIZATION OF HYDROGEN PRODUCTION FROM SWITCHGRASS DERIVED SUGARS USING A MIXED ANAEROBIC CULTURE IN AN UPFLOW ANAEROBIC SLUDGE BLANKET REACTOR – A STATISTICAL APPROACH

7.1 Introduction

Depleting fossil fuel supplies, energy security and global warming are factors driving the development of renewable energy supplies. Fermentative biological hydrogen (H\textsubscript{2}) production from renewable sources is an emerging technology, which can assist in alleviating these issues. However, logistic issues related to H\textsubscript{2} production, storage and distribution issues are yet to be resolved (Gupta et al., 2013; Jung et al., 2011). The current study is focused on biological H\textsubscript{2} production from low value biomass using mixed anaerobic communities. Hydrogen production using carbon neutral feedstock is considered sustainable when compared with its production from fossil fuel sources. Fermentative H\textsubscript{2} production from renewable substrate sources using mixed anaerobic communities is regarded to have significant potential among the different biological processes under consideration (Levin et al., 2004).

Lignocellulosics has been identified as a possible feedstock for full-scale biofuel production because it is available in large quantities (Vadas et al., 2008). Anaerobic fermentation of lignocellulosic biomass has gained widespread attention since it could be used to produce H\textsubscript{2} (Saratale et al., 2008). Switchgrass (SWG) and waste residues generated from corn and wheat processing are considered potential low-value lignocellulosic feedstocks for producing biofuels. Approximately 140 million tonnes of corn stover including waste from the corn stalks and leaves, 75 million tonnes of wheat waste and 250 million tonnes of SWG are produced annually in North America (AAFC; Kim and Dale, 2004; Walsh et al., 2003; Wood and Layzell, 2003). Among these feedstocks, SWG is a preferred bioenergy crop because of its high yield per acre and low nutrient requirements (Vadas et al., 2008).

Because lignocellulosic biomass are not utilized directly by fermentative microorganisms, pretreatment is necessary to produce soluble sugar monomers.
Among the pretreatment methods available, steam explosion has received considerable attention for producing hydrolysates containing feedstock chemicals (Taherzadeh and Karimi, 2008). According to Datar et al. (2007), steam explosion with acid catalysis yields a liquor containing high sugar levels. However, a major drawback to this method is the production of fermentation inhibitors such as furfural and 5-hydroxymethylfurfural (HMF). To achieve higher H$_2$ yields, these inhibitors must be removed from the liquor generated by steam explosion (Olsson and Hahn-Hagerdal, 1996).

Substrate pretreatment alone is unlikely to improve H$_2$ production and hence, increased efforts are focused on improving the fermentation process. Several researchers have investigated the possibility of H$_2$ production from a variety of substrates using mixed anaerobic cultures in sequencing batch and continuous flow bioreactors (Antonopoulou et al., 2010; Arreola-Vargas et al., 2013; Chaganti et al., 2013); however, a major challenge is sustained H$_2$ production. Sustained and stable H$_2$ production is not only dependent on operational parameters such as pH, hydraulic retention time (HRT), H$_2$ partial pressure but also on the reactor configuration as well as inoculum type and source (Abreu et al., 2009; Chaganti et al., 2012; Jung et al., 2011).

Mixed cultures are able to utilize a wide array of substrates and produce numerous byproducts because of their robustness and metabolic flexibility (Temudo et al., 2007). Theoretically, 1 mol of glucose can be converted into a maximum of 12 mol of H$_2$. If acetate is the only reduced carbon byproduct, the maximum yield is 4 mol of H$_2$ per mol glucose while 2 mol of H$_2$ per mol glucose is produced if butyrate is the only reduced carbon byproduct. However, the theoretical yield is difficult to achieve because metabolites such as ethanol, propionate and lactate are produced with acetate (Hawkes et al., 2002) and because of the syntrophic association between H$_2$ producers and consumers (Li and Fang, 2007). The cause for low H$_2$ yields is attributed to the presence of H$_2$ consumers such as methanogens, sulfate reducing bacteria and homoacetogens. Suppression of H$_2$ consumers to enhance H$_2$ production can be achieved by adjusting environmental (temperature, pH) and reactor operational (HRT) conditions as well as
adding chemical inhibitors or using a combination of these approaches (Chaganti et al., 2013; Karlsson et al., 2008; Terentiew and Bagley, 2003).

In a pH range from 5.2 to 6.7, \( \text{H}_2 \) production is favorable (Hawkes et al., 2002; Khanal et al., 2004). However, because methanogens and homoacetogens are active in this range (Leitao et al., 2006; Yang et al., 2013), it is necessary to add microbial inhibitors such as long-chain fatty acid (LCFAs) to inhibit these microorganisms. Linoleic acid (LA), a LCFA with 18 carbons and 2 unsaturated C=C bonds (C18:2), is a potent \( \text{H}_2 \) consumers’ inhibitor (Chowdhury et al., 2007). Studies by Rinzema et al. (1994) have also shown that aceticlastic methanogens is inhibited by approximately 1,200 to 1,600 mg L\(^{-1}\) capric acid (C10:0).

Adjusting the HRT can affect the \( \text{H}_2 \) yield by retaining or washing-out \( \text{H}_2 \) consuming methanogens (Chen et al., 2001). HRT values where maximum \( \text{H}_2 \) yields have been reported vary from 5 to 48 h for mixed cultures fed different substrates (Fan et al., 2006; Karlsson et al., 2008; Wang et al., 2013; Zhang et al., 2006). This large HRT range demonstrates that optimization is essential for different cultures and reactor systems fed various substrates.

Experimental design is essential in optimizing \( \text{H}_2 \) production because the outcome is influenced by many factors (Hawkes et al., 2002; Li and Fang, 2007). Data from past studies indicate that pH, HRT and culture treatment are among the most important factors controlling fermentative \( \text{H}_2 \) production (Chaganti et al., 2013; Lay, 2000). Interaction between these factors on \( \text{H}_2 \) producing cultures have been reported in several studies. According to Won and Lau (Won and Lau, 2011), the optimum pH for fermentative \( \text{H}_2 \) production is linked to the HRT. Factor interaction on \( \text{H}_2 \) production has also been reported by Ray et al. (2008). They showed that adjusting the pH and LA level are more effective than adjusting individual parameters. Based on these reports, a statistical design was used in this study to assess the relative contributions of different factors on optimizing conditions for enhancing the \( \text{H}_2 \) yield.

Response surface methodology (RSM) is used to optimize factors, which subsequently leads to a maximum response. RSM is a collection of mathematical and statistical techniques that are useful for modeling and analysis in applications where the
response of interest is influenced by several factors and the objective is to optimize the response (Montgomery, 2005). Among the various RSM methods, Box-Behnken design (BBD) has many broad application because of its simplicity; fewer experiments are required to be performed and the method allows efficient estimation of first- and second-order coefficients as well as interaction coefficients (Box and Behnken, 1960). The use of SWG as a model biomass has been demonstrated for bio-ethanol production by Vadas et al. (2008). However, there are no published reports of bio-\( \text{H}_2 \) production from SWG-derived sugars using mixed anaerobic cultures in continuous reactor systems. Hence, one objective of this study was to evaluate the effect of pH, HRT and LA concentration on \( \text{H}_2 \) production from a liquor derived from steam exploded SWG using a statistical approach. Another objective was to examine the effects of the three factors on a \( \text{H}_2 \) producing mixed anaerobic microbial community.

7.2 Materials and methods

The UASBRs (R1 and R2) were seeded with initial VSS of 10 g L\(^{-1}\) (culture B) fed with mixtures of glucose and xylose (see section 3.3). The reactors were initially operated in sequential batch operation at 24 h HRT (prior to experimental HRT outlined in Table 7.1) and the desired pH defined in the experimental condition (see Table 7.1). See section 3.5 for UASBR operation. For experiments conducted with LA after batch LA treatment (see section 3.6), a continuous LA feed was added at low feed concentrations to maintain a relatively constant concentration in the reactor. The composition of the substrate (resin treated SWG hydrolysate) feed is outlined in Table F.2, Appendix F. The operational conditions for each experiment and the design of the experiment are described in section 7.2.1. All the chemical and analytical methods used in this study are outlined in sections 3.7.3, 3.8 and 3.9 respectively. The COD balance was conducted on the analytes to see the fermenting ability of the substrate at various operating conditions. The influent substrate concentration was adjusted to 5 g COD L\(^{-1}\), which were chosen to keep the OLRs range from 7.5 to 15 g L\(^{-1}\) d\(^{-1}\). The microbial characterization is carried out as in section 3.10. A principal component analysis (PCA) was used to study the differences in the fermentation pattern in different operating condition using lignocellulosic sugars. The multivariate cluster analysis was used to
cluster the conditions showing similar microbial pattern in the data set. All the statistical analysis is conducted according to the procedures outline in 3.1.12.

### 7.2.1 Optimization study

The experimental design was based on the $3^k$ factorial Box–Behnken design (BBD) (Box and Behnken, 1960). The model was used to optimize key process parameters (HRT, pH and LA) to enhance H$_2$ production. The factors, levels and experimental design are shown in Table 7.1. The response variables (H$_2$ or methane (CH$_4$) yield) and associated factors were HRT (8, 12, and 16 h), pH (5.0, 6.0 and 7.0) and LA concentration (0, 1 and 2 g L$^{-1}$). The range for pH chosen were based on the optimum range of pH reported for increased H2 production by Pakarinen (2011) and Valdez-Vazquez and Poggi-Varaldo (2009). The range of HRT chosen was based on the experimental outcome from Chapter 5 and the fact that growth of H$_2$ producing consortia responsible in maximizing the H$_2$ yields is greater at short HRTs in comparison to the longer HRTs (Valdez-Vazquez and Poggi-Varaldo, 2009). The LA concentrations chosen was based on the studies conducted using batch reactors were increasing LA concentrations is said to favor H$_2$ production (Ray et al., 2010). A total of 14 experiments were conducted with two center points replicates (Table 7.1).

The relationship between the coded and actual values is described by equation 7.1.

$\begin{align*}
x_i &= \frac{(X_i - X_i^*)}{\Delta X_i} \\
\end{align*}$

where $x_i$ is the coded value of the $i$th independent variable, $X_i$ is the uncoded value of the $i$th independent variable, $X_i^*$ is the uncoded value of the $i$th independent variable at the center point, and $\Delta X_i$ is the step change value.

The quadratic polynomial equations used to predict conditions for maximum H$_2$ production and a minimum methane production can be described using equation 7.2. In equation 7.2, the terms

$Y = \alpha_o + \sum_{i=1}^{3} \alpha_i X_i + \sum_{i=1}^{3} \alpha_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{i<j=2}^{3} \alpha_{ij} X_i X_j$  \hspace{1cm} (7.2)
are defined as follows: $X_i$’s are input variables which influence the response variable $Y$ (H$_2$ or CH$_4$), $\alpha_0$ is an offset term, $\alpha_i$ is the $i$th linear coefficient, $\alpha_{ii}$ is the quadratic coefficient, and $\alpha_{ij}$ is the $ij$th interaction coefficient. The input values of $X_1$, $X_2$ and $X_3$ in equation 7.2 correspond to the experimental factors HRT, pH and LA concentration (Table 7.1). The equation was solved by setting the partial derivative to zero (Box and Wilson, 1951).

The model was evaluated using Minitab 16 (Minitab Inc., State College, PA, USA). An analysis of variance (ANOVA) was conducted using the responses (H$_2$ and CH$_4$ production) observed at each condition shown in Table 7.1. The statistical significance of the experimental responses was examined using the ANOVA. The ANOVA was performed on experimental data to test the significance of fit for the reduced quadratic model. A multiple regression analysis was performed using the experimental response to determine the coefficient values for fitting the model.
Table 7.1 Design matrix for the experimental factors and responses at different factor levels

<table>
<thead>
<tr>
<th>Expt. #</th>
<th>Coded</th>
<th>Actual</th>
<th>Coded</th>
<th>Actual</th>
<th>Coded</th>
<th>Actual</th>
<th>Observed*</th>
<th>Predicted</th>
<th>Observed*</th>
<th>Predicted</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>16</td>
<td>0</td>
<td>6</td>
<td>-1</td>
<td>0</td>
<td>1.1±0.7</td>
<td>9.6</td>
<td>31.6±2.5</td>
<td>32.5</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>12</td>
<td>-1</td>
<td>5</td>
<td>-1</td>
<td>0</td>
<td>34.4±1.7</td>
<td>29.4</td>
<td>19.1±2.4</td>
<td>17.1</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>12</td>
<td>1</td>
<td>7</td>
<td>-1</td>
<td>0</td>
<td>0.9±0.4</td>
<td>3.2</td>
<td>29.0±3.0</td>
<td>28.5</td>
</tr>
<tr>
<td>4</td>
<td>-1</td>
<td>8</td>
<td>0</td>
<td>6</td>
<td>-1</td>
<td>0</td>
<td>18.6±0.7</td>
<td>12.7</td>
<td>16.7±1.6</td>
<td>18.3</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>16</td>
<td>-1</td>
<td>5</td>
<td>0</td>
<td>1</td>
<td>61.5±3.2</td>
<td>57.9</td>
<td>3.8±0.2</td>
<td>5</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>16</td>
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<td>7</td>
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<td>47.4±3.6</td>
<td>36.5</td>
<td>15.4±1.6</td>
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<tr>
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<td>12</td>
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<td>6</td>
<td>0</td>
<td>1</td>
<td>66.3±5.8</td>
<td>65.2</td>
<td>4.8±1.2</td>
<td>4.3</td>
</tr>
<tr>
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<td>12</td>
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<td>6</td>
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<td>1</td>
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<td>3.7±2.7</td>
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<td>8</td>
<td>1</td>
<td>7</td>
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<td>1</td>
<td>27.8±0.9</td>
<td>31.4</td>
<td>12.2±2.5</td>
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<td>2.7±1.1</td>
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<td>6</td>
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<td>2</td>
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<td>4.7±1.3</td>
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</tr>
<tr>
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<td>12</td>
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<td>5</td>
<td>1</td>
<td>2</td>
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<td>92.8</td>
<td>0.9±0.2</td>
<td>1.4</td>
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<tr>
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<td>0</td>
<td>12</td>
<td>1</td>
<td>7</td>
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<td>2</td>
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<td>52.7</td>
<td>5.9±1.5</td>
<td>8</td>
</tr>
<tr>
<td>14</td>
<td>-1</td>
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<td>0</td>
<td>6</td>
<td>1</td>
<td>2</td>
<td>81.3±2.5</td>
<td>72.7</td>
<td>12.3±2.7</td>
<td>11.4</td>
</tr>
</tbody>
</table>

* ± represents the mean and standard deviation calculated from n = 4

Notes:
1. Expt. # on each row represents one experimental replicate; every run was carried out in two reactors in parallel.
2. The H₂ and CH₄ yield is chosen as the desired response for the model.
3. 1 g of dry biomass (SWG) = 0.93±0.04 g of TVS and 1 g TVS = 0.33 g COD.
4. H₂ yield for example, 95.1±1.4 mL of H₂ g⁻¹ TVS = 88.4±1.3 mL of H₂ g⁻¹ dry weight = 288±04 mL of H₂ g⁻¹ COD = 2.47±0.04 mol mol⁻¹ hexose.
The analysis of residuals (difference between the predicted and observed values in the response) was checked for probability distribution. A normal distribution of the residuals indicates a good fit to the experimental data. The Anderson-Darling (AD) test was used to determine deviation of the residuals from a normal distribution (Stephens, 1974). The D-optimality analysis was performed using an algorithm in the Minitab statistical software. The D-optimality analysis was used to obtain an optimal level for the three factors used in the design (maximize the H₂ yield and minimize the CH₄ yield). This was achieved by minimizing the variance in the parameter estimation of the model (Ray et al., 2010).

7.3 Results and discussion

7.3.1 Material balance of lignocellulosic biomass

A material balance on a mass basis for different chemical constituents in the SWG is shown in Figure 7.1. The results reveal that approximately 83% of the hemicellulosic sugars were recovered in the steam exploded liquor. The water-soluble extract was analyzed for sugars, acetic acid and sugar-degradation products (furfural and HMF). From 1,000 g of dry material, approximately 620 g of pretreated solids was recovered after pretreatment. The total sugar yield after steam explosion was 28±2% (on a mass basis) on a dry basis of the untreated biomass. The quantity of fermentation inhibitors (furfural, HMF and phenolic compounds) removed after treatment with the XAD-4 resin was 68±7% (on a mass basis). After resin treatment, the total hexose recovered was 23±2% (on a mass basis) of the untreated dry biomass.
Figure 7.1 Material balance for switchgrass (before and after steam explosion)

Notes:
1. a±b represents the mean and standard deviation calculated from n=3
2. HMF- 5-hydroxymethyl furfural

7.3.2 Optimization of maximum hydrogen and minimum methane yield

The optimum level for each single factor and combining two different factors (i.e., interaction effects) were determined using a BBD model. Many studies have examined the effect of different factors on H₂ production using a single factor design or one-factor-at-a-time approach (Nath and Das, 2011). For example, the effects of initial pH, substrate concentration and inhibitor concentration on H₂ production in batch cultures have been reported in many studies (Chowdhury et al., 2007; Fan et al., 2004; Ray et al., 2008; Van Ginkel et al., 2001). Other studies have optimized H₂ production using a multiple-factor design (or factorial design) (Karlsson et al., 2008; Lay et al., 2005; Sekoai and Kano, 2013). In this study, optimization of HRT and pH along with the LA concentration was conducted to enhance H₂ production from SWG.

The design matrix of the factors together with the experimental design is shown in Table 7.1. The data shows the independent variables X₁, X₂ and X₃ had a significant
effect on the \( \text{H}_2 \) yield (Table 7.1). The response at the center point (pH 6.0, 12 h HRT, and 1.0 g L\(^{-1}\) of LA) was 65.2\(\pm\)5.4 mL \( \text{H}_2 \) g\(^{-1}\) TVS and 4.3\(\pm\)1.3 mL \( \text{CH}_4 \) g\(^{-1}\) TVS. However, a maximum \( \text{H}_2 \) yield of 95.1\(\pm\)1.4 mL g\(^{-1}\) TVS was observed at pH 5.0, a 12 h HRT and 2 g L\(^{-1}\) LA (Table 7.1). In contrast, a maximum \( \text{CH}_4 \) yield of 31.6\(\pm\)2.5 mL g\(^{-1}\) TVS was obtained at pH 6.0, an HRT of 16 h and with no LA added. This deviation in the \( \text{H}_2 \) and \( \text{CH}_4 \) yields is likely due to a decrease in pH coupled with increasing the LA concentration. The increasing \( \text{H}_2 \) yield with increasing LA concentrations is in agreement with the findings by Chowdhury et al. (2007). These authors reported an increase in the \( \text{H}_2 \) yield and a decrease in the \( \text{CH}_4 \) yield with increasing LA concentrations (500 to 2,000 mg L\(^{-1}\)) in batch reactors fed glucose at an initial pH of 7.6. According to Fang and Liu (2002), pH is an important factor affecting \( \text{H}_2 \) production because of its effects on the \( \text{H}_2 \) production rate, metabolic pathways and microbial community. This suggests that a decrease in pH results in an increased \( \text{H}_2 \) yield and decreased \( \text{CH}_4 \) yield. In comparison to data from this study, Fang and Liu (2002) reported a maximum \( \text{H}_2 \) yield of 2.1\(\pm\)0.1 mol mol\(^{-1}\) glucose at pH 5.5 with a 6 h HRT. In this study, a maximum \( \text{H}_2 \) yield of 2.5\(\pm\)0.1 mol mol\(^{-1}\) hexose was observed at pH 5.0 with a 12 h HRT and 2 g L\(^{-1}\) LA (Table 7.1).

The regression equation obtained using the coded variables show the \( \text{H}_2 \) yield and \( \text{CH}_4 \) yield as a function of pH, HRT and LA concentration. Linear, quadratic and interaction terms are included in the second-order polynomial equation regardless of their significance (Equations 7.3 and 7.4).

\[
\begin{align*}
\text{H}_2 \text{ yield} &= a_0 + a_1 \times \text{HRT} + a_2 \times \text{pH} + a_3 \times \text{LA} + a_4 \times \text{HRT} \times \text{pH} + a_5 \times \text{HRT} \times \text{LA} + a_6 \times \text{pH} \times \text{LA} \\
&+ a_7 \times \text{HRT}^2 + a_8 \times \text{pH}^2 + a_9 \times \text{LA}^2 \\ 
\text{CH}_4 \text{ yield} &= b_0 + b_1 \times \text{HRT} + b_2 \times \text{pH} + b_3 \times \text{LA} + b_4 \times \text{HRT} \times \text{pH} + b_5 \times \text{HRT} \times \text{LA} + b_6 \times \text{pH} \times \text{LA} \\
&+ b_7 \times \text{HRT}^2 + b_8 \times \text{pH}^2 + b_9 \times \text{LA}^2
\end{align*}
\] (7.3) (7.4)

### 7.3.3 Analysis of the experimental design

(i) Effect of factor variables on the hydrogen and methane yield

The effect of three factor variables (pH, HRT and LA concentration) on the response variables (\( \text{H}_2 \) and \( \text{CH}_4 \) yields) is shown in Figure 7.2. Reducing the pH was associated with increasing the \( \text{H}_2 \) yield. An opposite trend was observed for the \( \text{CH}_4 \) yield and the
pH. Cultures fed an inhibitory LA concentration (1.0 g L\(^{-1}\)) exhibited a significant increase in the H\(_2\) yield when compared with the control cultures fed no LA. A similar effect was also reported by Pendyala et al. (2013) in batch reactors fed a mixture of steam-pretreated food and paper-cardboard waste. However, the maximum H\(_2\) yield observed by these authors was 72±13 mL g\(^{-1}\) TVS for granular cultures fed 5 g COD L\(^{-1}\) of a steam exploded liquor and treated with 2 g L\(^{-1}\) LA at 37 °C and pH 5.0. The yield reported by Pendyala et al. (2013) is approximately 23% less than that obtained in this study. Variations in the HRT within the levels under consideration had negligible effect on the H\(_2\) or CH\(_4\) yield (Figure 7.2). However, note according to Zhang et al. (2006), the H\(_2\) yield is influenced significantly by the HRT. These researchers also concluded that a stable H\(_2\) producing microbial population was established by washing out propionate producers at a low HRT.

Figure 7.2 Main effects plot of the experimental factors for H\(_2\) and CH\(_4\) yields

Notes:
1. Average values are shown for the model
2. Continuous red line ( ) is the mean value (48 mL g\(^{-1}\) TVS) of the H\(_2\) yield, while continuous black line ( ) indicates ±4 mL g\(^{-1}\) TVS (standard error)
3. Dashed red line ( ) is the mean value (12 mL g\(^{-1}\) TVS) of the CH\(_4\) yield, while dashed black line ( ) indicates ±1.3 mL g\(^{-1}\) TVS (standard error)

(ii) Contour plots

To assess the effect of varying pH, HRT and LA levels on methanogenic activity, two responses, the H\(_2\) yield and CH\(_4\) yield at the defined operating conditions (Table 7.1), were used in developing the contour plots (Figures 7.3A-C). Predicting the optimum
factor range was conducted by overlaying the $H_2$ and $CH_4$ responses. Notice the interaction effects of the independent variables on the two different responses was identified using the overlay plots (Mason et al., 2003). The overlay plots (Figures 7.3A-C) was used to predict the responses ($H_2$ and $CH_4$ yields) given the pH, HRT and LA concentration.

![Figure 7.3 Contour plots illustrating the effect of factor levels on $H_2$ and $CH_4$ yields: (A) pH versus LA concentration (at constant HRT =12 h), (B) HRT versus pH (at constant LA concentration =1 g L$^{-1}$) and (C) HRT versus LA concentration (at constant pH =6.0)](image)

Notes:
1. The black contour lines represent the $H_2$ yield under the following conditions: a. the continuous dark line (——) corresponds to the $H_2$ yield at 45 mL g$^{-1}$ TVS and b. the dashed line (-----) corresponds to the $H_2$ yield at 73 mL g$^{-1}$ TVS.
2. The gray contour lines with open circles represent the $CH_4$ yield under the following conditions: a. the solid line (——) corresponds to the $CH_4$ yield at 2 mL g$^{-1}$ TVS and b. the dashed line (-----) corresponds to the $CH_4$ yield at 13 mL g$^{-1}$ TVS.

Hydrogen yields greater than 73 mL g$^{-1}$ TVS with low $CH_4$ levels ($\leq$ 2 mL g$^{-1}$ TVS) were observed at pH less than 5.8 at and a LA concentration greater than 0.9 g L$^{-1}$ (Figure 7.3A). A similar effect was observed at pH values below 5.5 and HRT values in the range of 9 to 13 h (Figure 7.3B). Several reports have demonstrated that pH is an important factor influencing acidogenic fermentation (Fang and Liu, 2002; Lay et al., 2012; Ueno et al., 1996). In this study, the data indicate optimum factor ranges for increasing the $H_2$ yield were as follows: pH 5.0-5.5, 0.9-2.0 g L$^{-1}$ LA and 8-13 h HRT. In comparison, Pendyala et al. (Pendyala et al., 2013) reported a low $H_2$ yield (0.75 mol
mol\(^{-1}\) glucose equivalent) for batch cultures maintained over a pH range from 5.5 to 6.0 at 37 °C and fed a 9.4 g COD L\(^{-1}\) food and paper-cardboard hydrolysate plus 1.6 g L\(^{-1}\) LA. Supporting studies by Antonopoulou et al. (2010) and Fang and Liu (2002) have provided evidence of optimum H\(_2\) production at low pH.

The operating pH for optimum H\(_2\) production is variable and depends on factors such as reactor type, the type of substrate and source of inoculum. Reported pH for optimum H\(_2\) production in batch reactors are primarily for initial pH conditions. Note over the duration of batch studies the expected pH change is due to metabolite production. In continuous flow bioreactors, the pH range for enhanced H\(_2\) production is reported between pH 5.0-7.0 (Antonopoulou et al., 2010; Chang and Lin, 2004; Hawkes et al., 2002; Horiuchi et al., 2002; Kim et al., 2006). In comparison, in this study, between a pH range of 5.0 to 5.7 for HRT values between 8 to 13 h and in presence of 0.9 to 2.0 g L\(^{-1}\) LA, the H\(_2\) yield was ≥73 mL g\(^{-1}\) TVS (Figures 7.3A and B). However, with a pH range 5.0 to 7.0 and in the presence of 0.0 to 0.7 g L\(^{-1}\) of LA, the CH\(_4\) yield reached ≥13 mL g\(^{-1}\) TVS with HRT values between 8 to 16 h (Figures 7.3A and C). In controls, the CH\(_4\) yield attained a maximum of 31.6±2.5 mL g\(^{-1}\) TVS (Expt#1, Table 7.1). Low CH\(_4\) yields observed were attributed to a combination of factors, which includes low HRTs, low pH and a threshold LA concentration (Table 7.1). According to Chandra et al. (2012), a long HRT is required for establishing methanogenic conditions. Decreasing the HRT coupled with reducing the pH or an increase in LA concentration was effective in enhancing the H\(_2\) yield (Figures 7.3B and C). The combined effect of LA at a lower pH with a reduction in HRT has been reported to suppress methanogenesis and enhance the H\(_2\) yield (Chaganti et al., 2013). Studies by Liu et al. (2008) have shown the effect of varying pH at a constant HRT and also changing HRT at a fixed pH on H\(_2\) and CH\(_4\) production from a kitchen waste. Liu et al. (2008) also provided evidence showing that methanogenesis was suppressed at low HRTs and H\(_2\) production was not stable while operating at pH 7.0. They also showed that when the pH was maintained at 5.5 with a 3 d HRT, H\(_2\) production was stable at a level of 21±2 mL g\(^{-1}\) TVS.

The combined effect of HRT and LA concentration revealed that HRT values greater than 14 h or LA concentrations below 0.5 g L\(^{-1}\) were associated with high CH\(_4\) levels and
low H$_2$ yields (Figures 7.3C). In comparison, studies by Chowdhury et al. (2007) have shown methanogenesis is only suppressed in batch cultures at a threshold LA level of 2.0 g L$^{-1}$ for a pH at 7.6. Work demonstrating the impact of HRT on H$_2$ production by Chen et al. (2001) has shown that HRTs ranging from 6 to 13.3 h was required for stable H$_2$ production with simultaneous suppression of methanogenesis. A comparison of H$_2$ production from this study and data reported in the literature using a variety of substrates under different operating conditions is shown in Table 7.2.
Table 7.2 Comparison of H₂ production efficiencies by mesophilic dark fermentation process

<table>
<thead>
<tr>
<th>Reactor configuration</th>
<th>Inoculum; pre-treatment</th>
<th>Substrate; concentration</th>
<th>HRT (h)</th>
<th>pH</th>
<th>H₂ yield</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASBR</td>
<td>Anaerobic granular sludge; thermal treatment</td>
<td>Oat straw hydrolysate; 5 g COD L⁻¹</td>
<td>8</td>
<td>4.5</td>
<td>0.81 mol mol⁻¹ hexose</td>
<td>(Arreola-Vargas et al., 2013)</td>
</tr>
<tr>
<td>CSTR</td>
<td>Anaerobic digested sludge; combined heat and acid aeration</td>
<td>Glucose; 10 g L⁻¹</td>
<td>10</td>
<td>5.5</td>
<td>1.95±0.03 mol mol⁻¹ hexose</td>
<td>(Zhang et al., 2006)</td>
</tr>
<tr>
<td>CSTR</td>
<td>Anaerobic sludge; aeration</td>
<td>Molasses; 8 g COD L⁻¹</td>
<td>5</td>
<td>4.35ᵇ</td>
<td>1.5ᵃ mol mol⁻¹ hexose</td>
<td>(Wang et al., 2013)</td>
</tr>
<tr>
<td>Modified bioreactors</td>
<td>Anaerobic sludge; heat treatment</td>
<td>Agro and municipal waste; 40.5 g L⁻¹</td>
<td>86.3</td>
<td>7.9</td>
<td>58.62 ml g⁻¹ TVS</td>
<td>(Sekoai and Kano, 2013)</td>
</tr>
<tr>
<td>CSTR</td>
<td>Mixed culture; no pretreatment</td>
<td>Glucose; 7 g L⁻¹</td>
<td>6</td>
<td>5.5</td>
<td>2.1 mol mol⁻¹ hexose</td>
<td>(Fang and Liu, 2002)</td>
</tr>
<tr>
<td>Batch</td>
<td>Anaerobic granular sludge; linoleic acid</td>
<td>Food and paper-cardboard waste; 5 g COD L⁻¹</td>
<td>-</td>
<td>5.5</td>
<td>0.75±0.02ᵃ mol mol⁻¹ hexose</td>
<td>(Pendyala et al., 2013)</td>
</tr>
<tr>
<td>Batch</td>
<td>Anaerobic grass compost; heat shock</td>
<td>Food waste; NR</td>
<td>-</td>
<td>7.0</td>
<td>77±3 mL g⁻¹ TVS</td>
<td>(Lay et al., 2012)</td>
</tr>
<tr>
<td>UASBR</td>
<td>Anaerobic granular sludge; linoleic acid</td>
<td>Switchgrass hydrolysate; 5 g COD L⁻¹</td>
<td>10</td>
<td>5.0</td>
<td>99.9 ± 5.6 ml g⁻¹ TVS</td>
<td>This study</td>
</tr>
</tbody>
</table>

CSTR: continuous stirred tank reactor; ASBR: anaerobic sequencing batch reactor; UASBR: upflow-anaerobic sludge blanket reactor and NR: not reported

ᵃ calculated from given data
ᵇ mean pH
ᶜ 2.59 ± 0.15 mol mol⁻¹ hexose equivalents
7.3.4 Validating the response surface model

(i) Analysis of variance

A statistical analysis of the BBD response surface model was conducted by comparing the fit of a second-order polynomial equation with the experimental data using ANOVA (Table 7.3). The ANOVA was used to determine which of the effects in the model are statistically significant (equations 7.5 and 7.6). The ANOVA revealed that the quadratic models were significant for both $H_2$ and $CH_4$ production. Model terms with values of ‘Prob of F’ less than 0.05 are considered significant, whereas values greater than 0.05 are insignificant. The $F$ test values for many of the model terms had low probabilities ($p<0.005$). The $F$-statistic values of 76.8 and 105.4 (Table 7.3) for $H_2$ and $CH_4$, respectively, were greater than the $F$-critical value of 2.01 at $\alpha = 0.05$. These results indicate that the variance is not due to random chance but rather to the influence of the factors and their levels.

Table 7.3 ANOVA results for $H_2$ and $CH_4$ yields at different factor levels

<table>
<thead>
<tr>
<th>Source</th>
<th>$H_2$ Sum of Squares (SS)</th>
<th>$CH_4$ Sum of Squares (SS)</th>
<th>Degrees of freedom</th>
<th>$H_2$ Mean Square (MS)</th>
<th>$CH_4$ Mean Square (MS)</th>
<th>$H_2$ F-Value</th>
<th>$CH_4$ F-Value</th>
<th>$H_2$ p-value</th>
<th>$CH_4$ p-value</th>
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</thead>
<tbody>
<tr>
<td>Model</td>
<td>39278.6</td>
<td>4881.6</td>
<td>9</td>
<td>4364.4</td>
<td>542.4</td>
<td>76.8</td>
<td>105.4</td>
<td>0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>$X_1$</td>
<td>348.6</td>
<td>67.0</td>
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<td>348.6</td>
<td>67.0</td>
<td>6.1</td>
<td>13.0</td>
<td>0.017</td>
<td>0.001</td>
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<tr>
<td>$X_1^2$</td>
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<td>147.8</td>
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<td>1252.6</td>
<td>147.8</td>
<td>22.0</td>
<td>28.7</td>
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<td>0.000</td>
</tr>
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<td>$X_2$</td>
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<td>643.5</td>
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<td>8790.7</td>
<td>643.5</td>
<td>154.7</td>
<td>125.0</td>
<td>0.000</td>
<td>0.000</td>
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<td>$X_2^2$</td>
<td>291.6</td>
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<td>291.6</td>
<td>9.0</td>
<td>5.1</td>
<td>1.8</td>
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<td>$X_3$</td>
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<td>3222.1</td>
<td>949.4</td>
<td>56.7</td>
<td>184.5</td>
<td>0.000</td>
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<td>$X_1*X_2$</td>
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<td>4.9</td>
<td>1</td>
<td>552.0</td>
<td>4.9</td>
<td>9.7</td>
<td>1.0</td>
<td>0.003</td>
<td>0.336</td>
</tr>
<tr>
<td>$X_1*X_3$</td>
<td>49.7</td>
<td>505.9</td>
<td>1</td>
<td>49.7</td>
<td>505.9</td>
<td>0.9</td>
<td>98.3</td>
<td>0.355</td>
<td>0.000</td>
</tr>
<tr>
<td>$X_2*X_3$</td>
<td>192.2</td>
<td>23.5</td>
<td>1</td>
<td>192.2</td>
<td>23.5</td>
<td>3.4</td>
<td>4.6</td>
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<td>0.038</td>
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<td>Error</td>
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<td>46</td>
<td>56.82</td>
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<td></td>
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</tr>
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<td>Total</td>
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<td>5118.4</td>
<td>55</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Notes:
1. $X_1 = HRT; X_2 = pH$ and $X_3 = LA$.
2. Level of significance ($p > 0.05$ is considered statistically insignificant).
3. The values in bold are statistically insignificant at the 5% level of significance.
The model was evaluated based on the correlation coefficient (R). A high value indicates a good degree of fit. The R values of 0.925 and 0.945 for the H\textsubscript{2} and CH\textsubscript{4} models, respectively, suggest good correlation between the predicted and observed response values (Box et al., 1978). The coefficient of determination (squared multiple-correlation coefficient, R\textsuperscript{2}) values of 0.937 and 0.953 for H\textsubscript{2} and CH\textsubscript{4}, respectively, indicate that the model accounted for more than 93% and 95% of the total variance. Note the model predictions fit well with experimental observations. The interactions (Table 7.3) between HRT and LA (F = 0.8741, p = 0.354) and pH and LA (F = 3.3832, p = 0.07) were insignificant for the H\textsubscript{2} yield. For the CH\textsubscript{4} model, the interaction term (HRT x pH) and quadratic term (pH\textsuperscript{2}) were insignificant with p values > 0.05.

(ii) Response surface model verification

A multiple regression analysis of the experimental data was used to estimate the regression coefficients for the factors included in the model. The relationship between the response variables and the experimental factors based on the coded variables are shown as equations 7.5 and 7.6.

\[
\begin{align*}
\text{H}_2 \text{ yield} &= 65.183 - 3.300\times \text{HRT} - 16.574\times \text{pH} + 28.239\times \text{LA} + 5.874\times \text{HRT}\times \text{pH} \\
&\quad - 1.762\times \text{HRT}\times \text{LA} - 3.466\times \text{pH}\times \text{LA} - 9.892\times \text{HRT}^2 - 4.773\times \text{pH}^2 - 15.866\times \text{LA}^2  \\
\end{align*}
\]

\[
\begin{align*}
\text{CH}_4 \text{ yield} &= 4.304 + 1.447\times \text{HRT} + 4.484\times \text{pH} - 9.068\times \text{LA} + 0.551\times \text{HRT}\times \text{pH} \\
&\quad - 5.623\times \text{HRT}\times \text{LA} - 1.211\times \text{pH}\times \text{LA} + 3.398\times \text{HRT}^2 + 0.840\times \text{pH}^2 + 8.612\times \text{LA}^2  \\
\end{align*}
\]

The computed regression coefficients with their corresponding significance value (P) are shown in Table 7.4. Regression coefficients with P values < 0.05 indicate significant differences between the model and experimental observations.

The independent variables were examined to determine their significance in the model equation. Among the independent variables and their interactions that were analyzed, only the two terms for each H\textsubscript{2} (pH x LA and LA x HRT) and CH\textsubscript{4} (pH x HRT and pH x pH) were insignificant (P > 0.05) (Table 7.4) based on the ANOVA. Negative coefficients suggest that the factor or interaction showed an unfavorable effect on the H\textsubscript{2} or CH\textsubscript{4} yield. The negative coefficient for the independent variable pH (Equation 7.5) indicates an unfavorable effect on the H\textsubscript{2} yield with increasing pH. In comparison, the
positive coefficient for the pH term (Equation 7.6) indicates a favorable effect on the CH₄ yield with increasing pH. Notice the interaction between pH and HRT suggest a favorable effect on the H₂ yield.

Table 7.4 Regression coefficients for the response surface model

<table>
<thead>
<tr>
<th>Coefficient Term</th>
<th>Regression Coefficient</th>
<th>T</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td>65.183</td>
<td>24.458</td>
<td>5.366</td>
</tr>
<tr>
<td>HRT</td>
<td>-3.3</td>
<td>-2.477</td>
<td>3.607</td>
</tr>
<tr>
<td>pH</td>
<td>-16.574</td>
<td>-12.438</td>
<td>11.182</td>
</tr>
<tr>
<td>LA</td>
<td>28.239</td>
<td>21.192</td>
<td>-22.612</td>
</tr>
<tr>
<td>HRT × pH</td>
<td>5.874</td>
<td>3.117</td>
<td>5.359</td>
</tr>
<tr>
<td>HRT × LA</td>
<td>-1.762</td>
<td>-0.935</td>
<td>1.325</td>
</tr>
<tr>
<td>pH × LA</td>
<td>-3.466</td>
<td>-1.839</td>
<td>13.582</td>
</tr>
<tr>
<td>HRT²</td>
<td>-9.892</td>
<td>-4.695</td>
<td>0.972</td>
</tr>
<tr>
<td>pH²</td>
<td>-4.773</td>
<td>-2.265</td>
<td>-9.915</td>
</tr>
<tr>
<td>LA²</td>
<td>-15.866</td>
<td>-7.53</td>
<td>-2.136</td>
</tr>
</tbody>
</table>

Notes:
1. Regression coefficients were determined using coded values.
2. ‘T’ refers to the t-statistic value.
3. The values in bold are statistically insignificant at the 5% level.

Comparing the models responses and the experimental data was performed to assess the adequacy of the model. The responses computed from the model (using Equations 7.5 and 7.6) correlated well with the experimental data (Figures 7.4A and B). The regression coefficient for the H₂ and CH₄ model was 0.94 and 0.95, respectively.

The residuals were also examined using the AD test to evaluate the normal distribution of the residuals. The AD statistic was 0.58 for H₂ and 0.63 for CH₄. Values less than the critical value of 0.752 for a sample size of 56 at a 5% level of significance suggests the residuals satisfied the normal distribution requirement (P>0.05; Figure 7.5A). This confirmed the models fit with the experimental data over the defined parameter levels.
Figure 7.4 Assessment of the response surface model: (A) Model predicted H\textsubscript{2} yield versus the experimental H\textsubscript{2} yield; (B) Model predicted CH\textsubscript{4} yield versus the experimental CH\textsubscript{4} yield

Figure 7.5 (A) Anderson-Darling normality plot of residuals [AD = Anderson Darling statistic; P = confidence level]; (B) Optimality plot locating optimum factor levels for the desired response

The D-optimality plot was used to demonstrate minimization of the variance and to establish optimal levels of the independent variables to attain the desired response. A numerical optimization was performed by varying the D-optimal value between zero and one. The optimal levels of HRT, pH and LA for the maximum and minimum experimental response (H\textsubscript{2} and CH\textsubscript{4}, respectively) were identified at the largest D-
optimality value by varying the factor settings in the algorithm (Fedorov Exchange Algorithm). The optimality plot for the H$_2$ and CH$_4$ yield was obtained at a D-optimal value of 0.9986 (Figure 7.5B). The maximum H$_2$ and minimum CH$_4$ yields were 94.12 and 0.92 mL g$^{-1}$ TVS, respectively, at pH 5.0 with a 10.8 h HRT and 1.76 g L$^{-1}$ LA. Validation of the model performed at the nearest optimum condition revealed an experimental outcome of 99.9±5.6 mL H$_2$ g$^{-1}$ TVS and 0.5±0.1 mL CH$_4$ g$^{-1}$ TVS at pH 5.0, an HRT of 10 h and 1.75 g L$^{-1}$ LA. Note that the optimized conditions for maximum H$_2$ yield and minimum CH$_4$ yield from SWG that were obtained from the D-optimality analysis also fall within the optimum range depicted by the overlay contours shown in Figures 7.3A-C.

![Symmetrical diagram](image)

Figure 7.6 Schematic diagram illustrating the percent contribution of each model component on the response: (A) H$_2$ yield and (B) CH$_4$ yield

Notes:
1. HRT= $X_1$, pH= $X_2$ and LA= $X_3$
2. Table 7.3 presents the other components and their SS values, based on which of the percent contributions to the response was calculated
3. “First order” represents linear terms, and “quadratic” represents squared terms

(iii) Component contribution to responses

The percent contribution of each term in the model for the H$_2$ and CH$_4$ yield is shown in Figures 7.6A and B. The percent contribution is calculated based on the sum of squares (SS) obtained from the ANOVA (Table 7.3). The individual SS divided by the total SS is represented as a percent. The total percent contribution for the first-order,
quadratic and interaction terms was obtained by summing the contribution from each term (Figures 7.6A and B). The results indicate that the first-order component accounted for the major contribution to the H$_2$ or CH$_4$ yield. The percent contribution of the LA term ($X_3$) was significant in either case (63.4% and 52.8% for the H$_2$ and CH$_4$ yields, respectively). The interaction component ($X_1 \times X_2$, $X_2 \times X_3$ and $X_1 \times X_3$) for the predicted H$_2$ and CH$_4$ yield exhibited the lowest level of significance with a total contribution of approximately 2% and 11%, respectively (Figures 7.6A and B).

### 7.3.5 Metabolite production

A mass balance (on a COD basis) was used to examine the product distribution in the liquid and gas phases. The results of this analysis are provided in Table 7.5. The range of the COD mass balance from 83±13 to 111±09% validates the reliability of the data. The VFAs distribution profile is an indicator of the efficiency of the H$_2$ production.

Acetic acid (HAc) and butyric acid (HBu) were the major soluble metabolites detected under the different experimental conditions. Elevated HAc levels were observed in the control cultures at pH 5.0 and 6.0; however, in the LA treated cultures, the lower levels were observed except for the condition where maximum H$_2$ production was observed (Expt# 12, Table 7.1). In the presence of 2.0 g L$^{-1}$ LA, the HAc level increased and reached a maximum level of approximately 1,640±220 mg COD L$^{-1}$ with a corresponding maximum H$_2$ yield (Expt# 12, Tables 7.1 and 7.5) at pH 5.0. High H$_2$ yields are generally associated with elevated HAc and/or HBu levels (de Amorim et al., 2012; Lay et al., 2012). Notice the HAc concentration was low with increasing pH levels within the range of LA concentrations under consideration. Several studies have shown evidence of high HAc levels under low pH conditions (Chaganti et al., 2013; Datar et al., 2007; Khanal et al., 2004). A shift in the HRT did not have any effect on the HAc levels. The HBu concentration was maximized at the center point of the design with a concentration equivalent to 1,204±166 mg COD L$^{-1}$.
Table 7.5 Metabolites produced from switchgrass fermentation and COD balance

<table>
<thead>
<tr>
<th>Expt. #</th>
<th>COD equivalents in g L(^{-1})</th>
<th>Total COD (g L(^{-1}))</th>
<th>COD balance(^c) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H(_2)^a</td>
<td>CH(_4)^b</td>
<td>HL(_a)</td>
</tr>
<tr>
<td>1</td>
<td>0.01±0.003</td>
<td>1.40±0.11</td>
<td>0.16±0.03</td>
</tr>
<tr>
<td>2</td>
<td>0.43±0.040</td>
<td>0.84±0.09</td>
<td>0.11±0.04</td>
</tr>
<tr>
<td>3</td>
<td>0.01±0.01</td>
<td>1.30±0.12</td>
<td>0.23±0.04</td>
</tr>
<tr>
<td>4</td>
<td>0.22±0.02</td>
<td>0.73±0.08</td>
<td>0.12±0.03</td>
</tr>
<tr>
<td>5</td>
<td>0.75±0.05</td>
<td>0.18±0.03</td>
<td>0.32±0.07</td>
</tr>
<tr>
<td>6</td>
<td>0.61±0.05</td>
<td>0.80±0.07</td>
<td>0.08±0.01</td>
</tr>
<tr>
<td>7</td>
<td>0.75±0.08</td>
<td>0.25±0.03</td>
<td>0.20±0.03</td>
</tr>
<tr>
<td>8</td>
<td>0.77±0.09</td>
<td>0.18±0.03</td>
<td>0.11±0.03</td>
</tr>
<tr>
<td>9</td>
<td>0.36±0.02</td>
<td>0.52±0.05</td>
<td>0.13±0.02</td>
</tr>
<tr>
<td>10</td>
<td>0.82±0.07</td>
<td>0.15±0.02</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>11</td>
<td>0.68±0.05</td>
<td>0.28±0.03</td>
<td>0.39±0.05</td>
</tr>
<tr>
<td>12</td>
<td>1.14±0.05</td>
<td>0.07±0.01</td>
<td>0.29±0.04</td>
</tr>
<tr>
<td>13</td>
<td>0.59±0.02</td>
<td>0.30±0.04</td>
<td>0.08±0.02</td>
</tr>
<tr>
<td>14</td>
<td>0.97±0.11</td>
<td>0.35±0.04</td>
<td>0.07±0.02</td>
</tr>
</tbody>
</table>

\(a\): Based on 16 g COD mol\(^{-1}\) H\(_2\); \(b\): Based on 64 g COD mol\(^{-1}\) CH\(_4\); \(c\): COD mass balance (%) = (Total COD g L\(^{-1}\) / Influent COD (5.0 g L\(^{-1}\))) * 100

Notes:
1. Expt. # refers to the conditions described in Table 7.1
2. \(a±b\) represents the mean and standard deviation calculated from \(n = 4\)
3. COD equivalents towards biomass varied between 6 to 12% of the initial COD (data not shown)
4. HAc = acetic acid; HL\(_a\) = lactic acid; HPr = propionic acid; HBu = butyric acid; EtOH = ethanol and Res. Sugar = residual sugar
In this study, at any HRT condition, the high ethanol (EtOH) levels observed were associated with pH 6.0 and 7.0 in both LA-treated and untreated cultures (Table 7.5). Similar observations have been reported at high pH levels (6.0 to 6.8) for cultures fed sucrose operating at 8 h HRT (Lay et al., 2012). The high HPr levels detected were associated with low H₂ yields (Tables 7.1 and 7.5). This decrease in H₂ yield is explained by considering Equation 7.7 in which H₂ produced during acidogenesis is used for HPr production (Zhang et al., 2006).

\[ \text{C}_6\text{H}_{12}\text{O}_6 + 2\text{H}_2 \rightarrow 2\text{CH}_3\text{CH}_2\text{COOH} + 2\text{H}_2\text{O} \quad (7.7) \]

In addition to HAc, HPr and HBu, lactate (HLa) was also detected. The percent COD equivalents shifted to HLa were within the range of 2-8%. The presence of HLa followed by HPr was observed in mixed anaerobic cultures fed steam-exploded food and cardboard-paper waste blends in both control and LA-treated cultures (Pendyala et al., 2013). This suggests that the addition of LA or lower the pH does not prevent formation of reduced end products such as HLa and HPr.

### 7.3.6 Principal component analysis

The PCA biplot was used to demonstrate the effect of the different operating conditions employed on H₂ fermentation from SWG. The location of samples on the biplot illustrates the variability induced by different factors on the distribution of the fermentation pattern (Figure 7.7). The vector length for each metabolite shown in the PCA bi-plot represents the level of association of the metabolite with each of the principal components (PC 1 and PC 2). The strength of the relationship is indicated by the percent values on the ordinates of the bi-plot. Thus, the influence of these metabolites in grouping the operational conditions according to similarities in the relationships between correlated variables is depicted by the position of each operating condition in relation to the bi-plot's ordinates (PC 1 and PC 2), by its proximity to other operating conditions and metabolite loading vectors within the plane of the bi-plot.

In addition to PCA-biplot, PCA using log transformation of the variables is provided in the Figures G2a and b, Appendix G.
Figure 7.7 Principal component analysis plot of fermentation metabolites at different operational conditions with varying factor levels

Notes:
1. Only the first and second principal components are shown
2. The pH levels indicated as 5.0, 6.0 and 7.0 are adjacent to each label
3. The marker shapes represent the LA concentration: circles (0 g L\(^{-1}\)), triangles (1 g L\(^{-1}\)) and squares (2 g L\(^{-1}\))
4. HRT: 16 h (shaded), HRT: 12 h (open) and HRT: 8 h (black)
5. Control cultures (experiments with no LA) and high CH\(_4\) levels
6. Conditions with maximum H\(_2\) yields (Experiments #12 and 14; Table 7.1)
7. Low pH conditions with LA indicating increased H\(_2\) yield
8. HAc = acetic acid; HLa = lactic acid; HPr = propionic acid; HBu = butyric acid; EtOH = ethanol; H\(_2\) = hydrogen; CH\(_4\) = methane

The distribution of samples observed in the biplot is based on culture treatment i.e., addition of LA and/or pH (Figure 7.7). Clustering of the untreated cultures (0 g L\(^{-1}\) of LA) is an indication of their association with CH\(_4\) production in comparison to the LA fed culture (Expts. #1-4, Table 7.1). Conditions showing maximum H\(_2\) yields were grouped in a different cluster (Expts. #12 and 14, Table 7.1). Values in the brackets denote the loading value. Vectors for H\(_2\) (0.79), HBu (0.92) and EtOH (0.41) on the biplot were positioned in the positive direction. Methane was observed in the negative direction with loading values of both the first (0.93) and second components (0.27). The CH\(_4\) vector, which was oriented opposite to the H\(_2\) vector, indicated that high CH\(_4\) yields...
are associated with low H$_2$ yields. Similar observations for HAc, which had a loading (0.70) in the positive direction of the second component, and EtOH, which has a loading (0.83) in the negative direction, were observed. The orientation of variables (H$_2$ and EtOH) at opposite directions on the bi-plot is because lower yields of H$_2$ are associated with higher yields of EtOH. Low H$_2$ yields are associated with the production of reduced end products such as EtOH, HLa and HPr (Hawkes et al., 2002). In mixed anaerobic communities, analogous conclusions were reported based on the operating conditions and their association to experimental factor variables (Abreu et al., 2009; Chaganti et al., 2013; Pendyala et al., 2013). In the control cultures (no LA), methane production (17±2 to 32±3 mL g$^{-1}$ TVS) was observed under high pH conditions (Table 7.1), whereas the H$_2$ yield (62±3 to 95±1 mL g$^{-1}$ TVS) was associated with lower pH conditions (Table 7.1). Acetic acid production linked primarily with the untreated control conditions rather than with H$_2$-producing conditions was likely due to the acetogenic activity with untreated culture (Table 7.1). In this study, the elevated EtOH levels and associated lower H$_2$ yields observed at pH 6.0 and 7.0 is supported by work conducted by Cai et al. (2010). These authors reported high EtOH levels and reduced H$_2$ yields at pH 7.0 for Clostridium butyricum fed glucose.

7.3.7 Microbial profile of switchgrass fermented anaerobic consortia

A non-parametric multivariate cluster tree was developed from the T-RF profile for the SWG steam exploded liquor fed mixed microbial consortia. Similarity between different microbial species was determined using the Kulczynski similarity cluster index (Anderson et al., 2011). The right column (leaf nodes) of the dendrogram shows data for each individual condition and the nodes representing the clusters. The horizontal lines represent the similarity between the populations. The cluster tree was divided into three major clades (A-C) and one separate sample designated as D, which exhibited no significant similarity with the other clades (Figure 7.8).

High H$_2$ yields ($\geq$ 57±7 mL g$^{-1}$ TVS) at pH 5.0 and 6.0 and in the presence of LA was observed in the Clade A cultures with a similarity ranging from 10% to 25%. The reason for the low similarity among the clade A cultures might be due to differences in the microbial community at the species level. The maximum H$_2$ yield observed in cultures
maintained at pH 5, fed 2 g L\(^{-1}\) LA and at a 12 h HRT (Expt\# 12, Table 7.1) was observed in the clade A. Greater than 70% of the culture composition under this condition (Expt\# 12, Table 7.1) included *Clostridiaceae* (*Butyrivibrio crosstotus*, *C. botulinum*, *C. cochlearium*), and *Ruminococcaceae*. In addition to 2 g L\(^{-1}\) LA fed cultures, cultures fed with 1 g L\(^{-1}\) of LA and with a 16 h HRT and pH 5.0 was observed in clade A (Expt\# 5, Table 7.1). This discrepancy within the sample grouping could be due to similar pH levels, resulting in the enrichment of H\(_2\)-producing consortia which included *Clostridium beijerinckii*, *C. kainantoi*, *C. proteolyticum*, *C. oceanicum*, *Enterococcus saccharolyticus*, *C. cellulovorans* and *Eubacterium dolichum*.

**Figure 7.8 Multivariate cluster analysis of the terminal restriction fragments obtained from the Hae III enzyme digest**

**Notes:**
1. The first, second and third numbers of the sample labeling corresponds to the HRT (h), pH and LA concentration (g L\(^{-1}\)), respectively
2. Four clades (A-D) grouped based on their similarity in the cluster tree for each condition and clade E is separated from all other clades in the cluster tree
3. Maximum H\(_2\) and CH\(_4\) yields are represented as \(\bullet\) and \(\circ\), respectively
4. Cophenetic correlation coefficient (Coph. Corr)
Clade B contained microorganisms from cultures not fed LA (untreated control samples with dominant methane producing cultures) and cultures fed 1 g L\(^{-1}\) LA (Figure 7.7). Clade B shared a similarity index of 8% in the cluster tree; however, in the cultures within clade B similarity ranged from 8% to 42%. Cultures in this clade operated with low pH (5.0, 6.0 and a LA concentration (1 g L\(^{-1}\)) were observed in addition to LA fed culture operating at 16 h HRT and pH 7.0. Clade A cultures, which were associated with similar pH levels (5.0 and 6.0), were positioned away from clade B on the cluster tree and shared a 10% similarity. This variation might be due to the change in the HRT and/or LA concentrations. Although LA fed cultures in clades A and B and the sample designated as D shared a low similarity on the cluster tree (Figure 7.8), they produced similar H\(_2\) levels and exhibited a similar metabolic profile (Table 7.1, Figure 7.7). The low similarity between the clades containing samples from different fermenting conditions was likely due to differences in bands and their correspondingly related microbial species.

Clade C, which had the highest similarity index of approximately 62%, consisted of culture samples treated with LA, a pH level of 7.0 and lower HRTs (8 and 12 h). These conditions likely caused the separation of clade C cultures from those in clade B, which comprised cultures, not treated with LA. An exception to this was a culture in clade B which was treated with 1 g L\(^{-1}\) and operated under 16 h HRT at pH 7.0. The high cophenetic correlation coefficient of 0.91 supported using the T-RFLP data in the cluster analysis. A high cophenetic correlation closer to 1.0 indicates that more accuracy in clustering of the data based on the T-RFs.

In experiments performed without LA, irrespective of the change in the pH and HRT, showed the presence of different types of microbial communities (Actinobacteria, Bacteroidia, Betaproteobacteria, Clostridiaceae, Methanobacteria, Methanomicrobia, Methanococci, Ruminococcaceae and Synergistaceae) were detected with methanogens as the dominant group. Note the presence of Clostridium sp. and Ruminococcus sp. along with Synergistaceae under high pH conditions is likely the cause for the presence of EtOH and mixed acid metabolites. Similar fermentation patterns at high pH levels have been reported in studies using mixed anaerobic cultures (Ganesan et al., 2008; Liu et al., 2012).
Figure 7.9  Comparison of relative abundance of terminal restriction fragments by lowering the HRT and pH, detected using Hae III enzyme digest for (a) control cultures showing bacterial abundance (b) LA (2 g L\(^{-1}\)) fed cultures showing bacterial abundance (c) control cultures showing Archeal abundance (d) LA (2 g L\(^{-1}\)) fed cultures showing Archeal abundance.

According to Kong et al. (2010), *Ruminococcaceae, Proteobacteria, Bacteroidetes*, and *Actinobacteria* along with the archaea methanogens were detected in the rumen communities fed barley silage or grass hay diets with or without flaxseed. Lowering the HRT from 16 h to 8 h and changing the pH from 7.0 to 5.0 simultaneously reduced 50% of relative abundance of the T-RFs belonging to the methanogenic population and increased the relative abundance of T-RFs belonging to members of *Clostridiaceae* (Figure 7.9). Note, the expected increase in the H\(_2\) yield was not observed because of the possibly existence of methanogens and other H\(_2\) consumers. The present results are in contrast to work reported by Won and Lau (2011) and Liu et al. (2008). These authors observed that methanogens was suppressed by reducing the pH and lowering the HRT. Data from this study confirmed that lowering the pH from 7.0 to 5.0 and changing the HRT from 16 h to 8 h did not completely eliminate methanogenesis. In comparison, studies by Kim et al. (2004) have shown that hydrogenotrophic methanogens can tolerate...
acidic conditions under high HRT and low pH conditions. Similarly studies by Krakat et al. (2010) have shown an increase in the diversity of the methanogens under low HRT conditions.

In this study, decreasing the HRT from 16 h to 12 h and lowering the pH to 6.0 or 5.0 in the presence of LA (2 g L\(^{-1}\)), reduced the diversity of the microbial population. These conditions likely suppressed the activity of methanogenic and propionate producers. In addition, the relative abundance of the T-RFs belonging to the homoacetogenic population was reduced without affecting dominant H\(_2\) producers such as *Ruminococcaceae* and *Clostridiaceae* (Figure 7.9). Comparative studies by Zhang et al. (2006) have shown reducing the HRT to 6 h eliminates the propionate-producing population in a glucose fed heat and acid treated mixed culture maintained at pH 5.5 and at 37 °C. Wu et al. (2009) observed that H\(_2\) consumption by homoacetogenesis at longer HRTs using glucose as a substrate was suppressed under low HRT conditions. Supporting evidence by Chaganti et al. (2013) has shown that low pH coupled with LA treatment was able to decrease the homoacetogenic activity in a H\(_2\)-producing culture. Note in this study, reducing the HRT from 12 h to 8 h caused *Ruminococcaceae* to become more dominant than the *Clostridiaceae* population. Although *Ruminococcaceae* and *Clostridiaceae* are H\(_2\) producers, *Ruminococcaceae* can also produce ethanol; however, this route is dependent on the environmental conditions. According to Liu et al. (2012), in a UASB operating under a 12 h HRT and containing a mixed microbial consortium fed with glucose at pH 4.5, the major products produced included ethanol, H\(_2\), and HAc. These researchers also reported *Clostridiaceae* and *Ruminococcaceae* were the dominant microbial populations. In comparison to published data, a larger H\(_2\) yield was observed at a 12 h HRT (95±1 mL H\(_2\) g\(^{-1}\) TVS) when compared to a 8 h HRT (83±3 mL H\(_2\) g\(^{-1}\) TVS). Under both HRT conditions, the culture was maintained at a low pH (5.0 and 6.0) and fed LA (2 g L\(^{-1}\)).
7.4 Conclusions

In this study, a steam exploded SWG liquor was used as a feedstock for fermentative H₂ production. The factors considered in the study included HRT, pH and LA concentration. An optimization method based on the BBD model was used to optimize conditions for maximizing the H₂ yield. The data suggest that a steam exploded SWG hydrolysate is a potential substrate for biological H₂ production. Based on the range of experimental factors under examination, the conclusions of this study are as follows:

1) The BBD model was useful in optimizing the factor conditions to maximize the H₂ production and minimize the CH₄ production and is valid within factor levels under consideration.

2) All the factors under investigation influenced H₂ yield; however, pH and LA concentration had greater effects in comparison to the HRT.

3) The accuracy of the model, which was verified by the regression fit, indicated that the model prediction correlated well within the experimental data, while AD plot was used to confirm the normal distribution of the residuals.

4) The most appropriate fermentation conditions (derived from the D-optimality for maximum H₂ yield) produced a yield of 99.86±5.6 mL H₂ g⁻¹ TVS for cultures at pH 5.0, an HRT of 10 h and an LA concentration of 1.75 g L⁻¹.

5) When maximum H₂ production was observed, HAc and HBu were the dominant metabolites.

6) The PCA revealed that the clustering of the samples was based mainly on LA treatment and pH.

7) *Methanobacteria, Methanococci, and Methanomicrobia* as well as *Clostridiaceae, Ruminococcaceae, and Synergistaceae* were observed in the cultures operated with no LA addition.

8) Methanogens were suppressed with the addition of LA (1 g L⁻¹) at low HRTs and at a pH of 5.0. Maximum H₂ yield in cultures fed 2 g L⁻¹ LA, maintained at pH 5.0 and at a 12 h HRT was observed. Under these conditions, *Clostridiaceae* and *Ruminococcaceae* were the dominant microorganisms observed.
7.5 References


CHAPTER 8: EFFECT OF HYDRAULIC RETENTION TIME, NITROGEN SPARGING AND LINOLEIC ACID ON FERMENTATIVE HYDROGEN PRODUCTION FROM SWITCHGRASS USING A MIXED ANAEROBIC CULTURE

8.1 Introduction

Hydrogen (H\textsubscript{2}) is recognized as an alternative to fossil fuels because it is clean and renewable with a high energy yield capacity (Züttel et al., 2008). Hydrogen production via dark fermentation has attracted a significant amount of research interest because of high H\textsubscript{2} production rates (HPR) and the ability to utilize pretreatment liquors derived from underutilized agriculture residues (Levin et al., 2004; Ntaikou et al., 2010). Sustainable H\textsubscript{2} production via dark fermentation is dependent on future biorefineries utilizing low value woody and non-woody lignocellulosic biomass such as switchgrass (SWG).

Pure and mixed microbial populations have been used to produce H\textsubscript{2} (Rittmann and Herwig, 2012). Using mixed anaerobic cultures is advantageous because they are easily available and feed sterilization is not required when compared to pure cultures (Ntaikou et al., 2010). However, a major disadvantage associated with the use of mixed anaerobic cultures is H\textsubscript{2} losses which are linked to the syntrophic relationship between H\textsubscript{2} producers and H\textsubscript{2} consumers (Hawkes et al., 2002).

Decoupling the syntrophic association between H\textsubscript{2} consumers and H\textsubscript{2} producers is essential in increasing the H\textsubscript{2} yield. The H\textsubscript{2} yield is affected by various factors which include bioreactor operation, substrate type, sparging, pH, temperature and hydraulic retention time (HRT) (Buitron and Carvajal, 2010; Chaganti et al., 2011; Chu et al., 2008; Lee et al., 2012). The partial pressure of H\textsubscript{2} (pH\textsubscript{2}) is a key operating parameter which influences the H\textsubscript{2} yield. With increasing pH\textsubscript{2}, decreasing hydrogenase activity leads to unfavorable thermodynamic conditions (Valdez-Vazquez and Poggi-Varaldo, 2009). As the pH\textsubscript{2} increases, the formation of reduced byproducts such as lactic acid (HLa), ethanol (EtOH) and propionic acid (HPr) assist to alleviate the constraints imposed by unfavorable thermodynamic conditions (Levin et al., 2004).
### Table 8.1 Dark fermentative reactions involving various end products that govern hydrogen metabolism

<table>
<thead>
<tr>
<th>Reactions</th>
<th>Stoichiometry</th>
<th>Free energy ($\Delta G^\circ$) (kJ reaction$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate formation</td>
<td>$\text{C}<em>6\text{H}</em>{12}\text{O}_6 + 4\text{H}_2\text{O} \rightarrow 2\text{C}_2\text{H}_3\text{O}_2^- + 2\text{HCO}_3^- + 4\text{H}^+ + 4\text{H}_2$</td>
<td>-206.3 (8.1)</td>
</tr>
<tr>
<td>Butyrate formation</td>
<td>$\text{C}<em>6\text{H}</em>{12}\text{O}_6 + 2\text{H}_2\text{O} \rightarrow \text{C}_4\text{H}_7\text{O}_2^- + 2\text{HCO}_3^- + 3\text{H}^+ + 2\text{H}_2$</td>
<td>-254.8 (8.2)</td>
</tr>
<tr>
<td>Ethanol (EtOH) formation</td>
<td>$\text{C}<em>6\text{H}</em>{12}\text{O}_6 + 2\text{H}_2\text{O} \rightarrow 2\text{CH}_3\text{CH}_2\text{OH} + 2\text{HCO}_3^- + 2\text{H}^+$</td>
<td>-235.0 (8.3)</td>
</tr>
<tr>
<td>Propionate formation</td>
<td>$\text{C}<em>6\text{H}</em>{12}\text{O}_6 + 2\text{H}_2 \rightarrow 2\text{CH}_3\text{CH}_2\text{COO}^- + 2\text{H}_2\text{O} + 2\text{H}^+$</td>
<td>-359.2 (8.4)</td>
</tr>
<tr>
<td>Acetate and EtOH formation</td>
<td>$\text{C}<em>6\text{H}</em>{12}\text{O}_6 + 3\text{H}_2\text{O} \rightarrow \text{CH}_3\text{CH}_2\text{OH} + \text{CH}_3\text{COO}^- + 2\text{H}_2 + 2\text{HCO}_3^- + 3\text{H}^+$</td>
<td>-215.7 (8.5)</td>
</tr>
<tr>
<td>Homoacetogenesis (from H$_2$)</td>
<td>$4\text{H}_2 + 2\text{HCO}_3^- + \text{H}^+ \rightarrow \text{C}_2\text{H}_3\text{O}_2^- + 4\text{H}_2\text{O}$</td>
<td>-104.6 (8.6)</td>
</tr>
<tr>
<td>Methanogenesis</td>
<td>$4\text{H}_2 + \text{HCO}_3^- + \text{H}^+ \rightarrow \text{CH}_4 + 3\text{H}_2\text{O}$</td>
<td>-135.6 (8.7)</td>
</tr>
<tr>
<td>Acetogenesis</td>
<td>$\text{C}<em>6\text{H}</em>{12}\text{O}_6 \rightarrow 3\text{CH}_3\text{COO}^- + 3\text{H}^+$</td>
<td>-310.6 (8.8)</td>
</tr>
<tr>
<td>Acetate and EtOH fermentation</td>
<td>$\text{CH}_3\text{CH}_2\text{OH} + \text{H}_2\text{O} \leftrightarrow \text{CH}_3\text{COO}^- + \text{H}^+ + 2\text{H}_2$</td>
<td>+1.9 (8.9)</td>
</tr>
</tbody>
</table>

Decreasing the pH$_2$ to increase the H$_2$ yield can be accomplished by purging the bioreactor with inert gases or increasing the degree of mixing (Kim et al., 2006; Lay, 2000). Purging with inert gases reduces not only the pH$_2$ but also the CO$_2$ partial pressure. Since H$_2$ and CO$_2$ are the primary substrate for hydrogenotrophic methanogens and homoacetogens, decreasing the levels of dissolved H$_2$ and CO$_2$ may assist in increasing the H$_2$ yield by limiting the substrate availability for H$_2$ consumers (Hussy et al., 2003; Kraemer and Bagley, 2007; Massanet-Nicolau et al., 2010). Table 8.1 lists the dark fermentative reactions involved in H$_2$ production and consumption.

In addition to the pH$_2$ level, the pH as well as HRT has a major effect on H$_2$ production in continuous flow reactor systems. Operating at a pH range of 5.0 - 6.0 is preferred for optimum H$_2$ production (Lay, 2000). Lowering the HRT increases the HPR and H$_2$ yield by eliminating H$_2$ consumers and non-H$_2$ producers such as methanogens and propionic acid producing bacteria (Chen et al., 2001; Zhang et al., 2006). Studies using continuous or semi-continuous reactors have described the impact of HRT,
substrate loading along with gas sparging on H₂ consumption (Kim et al., 2012; Kyazze et al., 2006; Massanet-Nicolau et al., 2010; Mizuno et al., 2000). However, controlling these factors alone is unable to completely suppress H₂ consumption. Other factors which have been considered include culture treatment and adding inhibitors to control the growth of H₂ consumers. Thermal pretreatment is able to selectively enhance the growth of H₂ producers and suppress the activity of H₂ consumers (Cai et al., 2004; Kim et al., 2006; Pendyala et al., 2012). In addition, adding long chain fatty acids (LCFAs) such as linoleic acid (LA) has shown to control the growth of H₂ consumers and subsequently, enhance the H₂ yield (Chowdhury et al., 2007; Pendyala et al., 2012). Very few studies have assessed the impact of various factors on H₂ production from low value biomass in continuous flow reactors. Liu et al. (2013) reported a H₂ production rate (HPR) of 10 L L⁻¹ d⁻¹ using a mixture of food industry waste water and sulfuric acid treated rice straw hydrolysate fed to a heat treated culture in a continuous stirred reactor operating at 37°C and a pH of 5.5. In another study by El-Bery et al. (2013), a HPR equivalent to 0.4 L L⁻¹ d⁻¹ was reported for alkali treated rice straw hydrolysate using thermally pretreated activated sludge. Note both of these studies were conducting by varying the OLR and maintaining a constant HRT.

The objective of this study was to assess the effects of N₂ sparging, HRT and adding LA on fermentative H₂ production using a hydrolysate liquor derived from SWG steam explosion.

### 8.2 Materials and methods

Experiments were conducted in duplicate using two UASBRs (designated as reactor R1 and R2) to examine different operating conditions. The first experiment varied the HRT ranging from 6 to 12 h and the second experiment was run in parallel with the first to examine the effect of LA administered under similar operating conditions as used in the first experiment. In the third experiment, the effect of N₂ sparging of the bioreactor on H₂ yield was examined using nitrogen gas (99.999%, Praxair, ON) at a flow rate of 100 mL min⁻¹. The fourth experiment was run under comparable conditions to the third experiment and was conducted to examine the effect of N₂ sparging on LA-treated culture with varying HRTs from 6 to 12 h. All of the experiments were conducted using culture
B as the inoculum source with an initial VSS concentration of 10 g L\(^{-1}\) (section 3.3 for culture source and reactor maintenance). The experiments were conducted at 37 °C with an operating pH of 5.0 (See section 3.5 for UASBR operation). The summary of fermentation conditions applied in this series of experiments is outlined in Table 8.2.

<table>
<thead>
<tr>
<th>Culture treatment</th>
<th>Bioreactor sparging with (N_2)</th>
<th>HRT (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (Expt. #1; C-WN)</td>
<td>(x)</td>
<td>12, 8 and 6</td>
</tr>
<tr>
<td>LA (Expt. #2; LA-WN)</td>
<td>(x)</td>
<td>12, 8 and 6</td>
</tr>
<tr>
<td>Control (Expt. #3; C-N)</td>
<td>(\checkmark)</td>
<td>12, 8 and 6</td>
</tr>
<tr>
<td>LA (Expt. #4; LA-N)</td>
<td>(\checkmark)</td>
<td>12, 8 and 6</td>
</tr>
</tbody>
</table>

Table 8.2 Experimental details of fermentation conditions

Resin treated SWG hydrolysate was used as the feed for the fermentation experiments conducted in this study. The composition of the feed is outlined in Table F.2, Appendix F. The concentration of LA (a methanogenic inhibitor) used in the study (experiments 2 and 4) was the optimized concentration (1.75 g L\(^{-1}\)) determined previously (section 7.3.4). Similarly, the range of HRTs chosen for this study was based on the condition (i.e., HRT of 10.8 h) that resulted in maximum H\(_2\) and also minimized methane (section 7.3.4). Thus, the HRT range chosen for this study included the application of shorter (6 and 8 h) and longer (12 h) HRTs that overlapped the optimal HRT of 10.8 h. The schematic operation of the reactors is shown in section 3.5 (Figure 3.1). Note, influent substrate concentration used for this study is 6 g COD L\(^{-1}\). At each HRT condition shown in Table 8.1, experiments were repeated 6 times until H\(_2\) yields were similar in reactors R1 and R2. All of the chemical, analytical and enzymatic methods used in this study are described in detail in sections 3.7.3, 3.8, and 3.9, respectively. The microbial methods (terminal restriction fragment length polymorphism (TRFLP)) were carried out as described in section 3.10. The microbial data (relative abundance) were correlated with the H\(_2\) yield in a 3D plot based on the principal components. The flux balance analysis (FBA) was conducted to quantify the effects of the operational parameters on the H\(_2\) consumption in the metabolic pathway (section 3.11). The statistical analysis used to determine whether the observed effects of the operational parameters on the H\(_2\) yield
were significant ($P<0.05$) was conducted using Tukey’s test as described in sections 3.12.

8.3 Results and discussion

8.3.1 Evaluation of different parameters on hydrogen production

The effects of HRT, pretreating the inoculum with LA and reactor sparging on H$_2$ production were examined by applying various combinations of the different parameters (Table 8.3). Hydrogen was the major component in the biogas under the different fermentation conditions (Table 8.3 and Figure 8.1). The results for experiment #1 (control cultures, without LA or N$_2$ sparging) revealed that lowering the HRT had a significant effect on the H$_2$ yield. In control studies, the highest H$_2$ yield of 1.54±0.14 mol mol$^{-1}$ hexose was obtained at a 6 h HRT (Table 8.3, Expt. #1). The HPR for reactors operating at a 6 h HRT with control unsparged cultures reached 4.6±0.4 L L$^{-1}$ d$^{-1}$ (Figure 8.1a).

Table 8.3 Hydrogen yield under different fermentation conditions

<table>
<thead>
<tr>
<th>HRT (h)</th>
<th>C-WN (Expt#1)</th>
<th>LA-WN (Expt#2)</th>
<th>C-N (Expt#3)</th>
<th>LA-N (Expt#4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>0.61±0.06$^{a,A}$</td>
<td>1.34±0.09$^{b,A}$</td>
<td>1.22±0.10$^{b,A}$</td>
<td>2.38±0.18$^{c,A}$</td>
</tr>
<tr>
<td>8</td>
<td>1.05±0.08$^{a,B}$</td>
<td>2.08±0.12$^{c,B}$</td>
<td>1.61±0.12$^{b,B}$</td>
<td>2.41±0.12$^{d,A}$</td>
</tr>
<tr>
<td>6</td>
<td>1.54±0.14$^{a,C}$</td>
<td>2.12±0.17$^{b,B}$</td>
<td>2.26±0.11$^{b,C}$</td>
<td>2.56±0.10$^{c,A}$</td>
</tr>
</tbody>
</table>

Notes:
1. The Tukey’s test was conducted to determine the differences in the H$_2$ yield between each treatment condition (indicated by $^{a, b, c}$ and $^{d}$) and variation in HRT (indicated by $^{A, B}$ and $^{C}$). Values with same letter indicate no significant difference at $p<0.05$.
2. $^{a, b, c}$ and $^{d}$ indicate statistically different means in the same row.
3. $^{A, B}$ and $^{C}$ indicate statistically different means in the same column.
4. The average and standard deviation are for $n=12$. Two reactors (R1 and R2) operating under the same condition and each condition repeated 6 times.
5. The notations representing the fermentation condition are as follows: C = control; LA = linoleic acid addition; N = nitrogen sparging; WN = without nitrogen sparging.
Figure 8.1 Variations in (a) operating parameters and hydrogen and methane production rates under sparged and non-sparged conditions (b) control cultures (c) LA treated cultures

Notes:
1. HRT = hydraulic retention time; OLR = organic loading rate; HPR = H₂ production rate and MPR = methane production rate
2. C = Control cultures without addition of linoleic acid; LA = linoleic acid fed culture; WN = without nitrogen sparging and N = nitrogen sparged cultures
3. The production rates plotted shows average values for duplicate reactors R1 and R2
Studies by Arooj et al. (2008) have demonstrated that decreasing HRTs caused the H\textsubscript{2} yield to increase using a starch feed; however, when decreasing the HRT to a threshold level below 12 h they observed decreasing H\textsubscript{2} yields. In this study, increasing H\textsubscript{2} yields were observed as the HRT was reduced to 6 h for all conditions except for cultures fed LA and sparged with N\textsubscript{2} where change in H\textsubscript{2} yield with respect to HRT was insignificant (Table 8.3, Expt. #4). Reduced methanogenic activity and low methane production rates were caused by lowering the HRT (Figure 8.1b). Evidence in similar studies by Chen et al. (2001) showed decreasing methanogenic activity and increasing H\textsubscript{2} productivity with decreasing HRTs for a sucrose fed culture.

The impact of LA on impairing H\textsubscript{2} consumption in mixed anaerobic communities has been reported for cultures operating under batch conditions (Chaganti et al., 2011; Pendyala et al., 2012). However, not many studies have provided evidence demonstrating the effect of adding an inhibitor such as LA on microbial cultures in UASBRs. In this study, improved H\textsubscript{2} production was observed in LA treated cultures (Table 8.3, Expt. #2) and at all the HRTs examined, the H\textsubscript{2} yield was greater when compared to the control cultures. Under the same HRT condition, the H\textsubscript{2} yield for the LA treatment condition was statistically different when compared to the yield for the control cultures (Table 8.3; Expt. #1 (C-WN) versus Expt. #2 (LA-WN); Tukey’s test, \( p < 0.05 \)). Note, within the group of LA-treated cultures (Table 8.3, Expt. #2), the H\textsubscript{2} yield drastically increased when HRT was reduced from 12 h to 8 h; however, with further reduction in HRT to 6 h, no statistical change was detected. The HPRs observed in the LA treated cultures correspond to \( 4.6\pm0.3 \) L L\textsuperscript{-1} d\textsuperscript{-1} at an 8 h HRT and \( 6.3\pm0.5 \) L L\textsuperscript{-1} d\textsuperscript{-1} at a 6 h HRT (Figure 8.1c). The HPR observed for the LA treated cultures operating at 8 and 6 h HRT was approximately 37\% greater than the HPRs observed in the corresponding untreated control cultures (C-WN). Note the HPR observed for the LA treated cultures was approximately 8-fold greater than those reported for glucose and xylose fed cultures in sequencing batch reactors operating at 37 °C (Chaganti et al., 2013). Similar work by Liu et al. (2013) reported a HPR of \( 22\pm3.3 \) L L\textsuperscript{-1} d\textsuperscript{-1} over 15 day duration for a food industry waste-water fed to heat treated cultures in a continuous stirred tank reactor operating with a 4 h HRT, at 37°C and a pH of 5.5. The HPR observed by these authors then declined to
10.0±1.2 L L\(^{-1}\) d\(^{-1}\) when the feed was changed to a mixture of food industry wastewater plus rice straw hydrolysate. Although, Liu et al. (2013) reported relatively high HPRs for a food industry wastewater, their data is based not based on long-term steady-state data. Using a wheat straw hydrolysate, Kongjan and Angelidaki (2010) reported HPRs of 0.243 and 0.8214 L L\(^{-1}\) d\(^{-1}\) in continuously stirred tank reactors (CSTRs) and UASBRs, respectively, containing heat treated thermophilic cultures. In the current study LA treated cultures showed HPR up to 6.3 ± 0.5 L L\(^{-1}\) d\(^{-1}\) (unsparged cultures) and 7.8 ± 0.5 L L\(^{-1}\) d\(^{-1}\) (sparged cultures) which lasted for approximately for 10 days.

The impact of N\(_2\) sparging was examined as a means of increasing H\(_2\) production. The effect of N\(_2\) sparging is clearly shown when comparing sparged and non-sparging conditions for the untreated inoculum. Notice for the untreated culture sparged with N\(_2\), the H\(_2\) yield increase observed ranged from 47 to 100% for the range of HRTs under consideration (Table 8.3, Expt. #1 versus Expt. #3). Similar increases in H\(_2\) yields with gas purging was reported by Mizuno et al. (2000) and Tanisho et al. (1998) for continuous and batch cultures fed glucose and molasses, respectively. The elevated H\(_2\) levels produced with N\(_2\) sparging are comparatively higher than those reported for cultures fed complex substrates and purged with N\(_2\) gas (Massanet-Nicolau et al., 2010; Tanisho et al., 1998). Note a maximum H\(_2\) yield of 2.26±0.11 mol mol\(^{-1}\) hexose equivalent was obtained with N\(_2\) sparging at a flow rate of 100 mL min\(^{-1}\) (Table 8.3, Expt. #3). In comparison to unsparged control cultures, sparging N\(_2\) in untreated culture operating at a 6 h HRT increased the HPR by approximately 47% which corresponded to a maximum of 6.8±0.6 L L\(^{-1}\) d\(^{-1}\) (Figure 8.1b).

A combination of N\(_2\) sparging and LA treatment was used to evaluate the interactive effects on H\(_2\) production at the different HRT conditions (Table 8.3, Expt. #4). The results suggest that combining LA treatment with N\(_2\) purging is the most effective method to increase H\(_2\) production during continuous operation. At any given HRT condition, the H\(_2\) yields from the LA-treated inoculum and the controls were statistically different (Table 8.3; (Expts. #1 and 2) and (Expts. #3 and 4)). The mean H\(_2\) yield observed from N\(_2\) sparging and LA treatment was 2.45±0.13 mol mol\(^{-1}\) hexose (Table
8.3, Expt. #4). Note there was no significant statistical change in the H$_2$ yield for cultures fed LA and purged with N$_2$ and operating over the range of different HRTs conditions.

8.3.2 Effect of different fermentation conditions on soluble metabolite distribution

Liquid samples from different fermentation conditions were analyzed for VFAs and alcohols. The percent distribution of gaseous and soluble metabolites under different conditions is shown in Figure 8.2. In each experimental condition, acetic (HAc) and butyric acids (HBu) were dominant and in general, higher levels of HAc were observed in LA treated cultures, whereas HBu levels were greater in controls without LA (Figure 8.2). Elevated HAc levels ranging from 0.25 to 1.1 g COD L$^{-1}$ coupled with a maximum H$_2$ yield of 1.2 mol mol$^{-1}$ hexose has been reported by Liu and Fang (2003). Kongjan and Angelidaki (2010) reported HAc as the major end product in experiments conducted with wheat hydrolysate using heat treated thermophilic cultures. These authors reported a H$_2$ yield of 1.8 mol mol$^{-1}$ hexose with a HAc concentration of 25.9 mM. In the current study, a HAc concentration of 31±4 mM (data not shown) and a H$_2$ yield of 2.1±0.2 mol mol$^{-1}$ hexose was obtained with LA treated culture operating at a 6 h HRT.

In N$_2$ sparging studies conducted by Kyazze et al. (2006), a maximum H$_2$ yield of 1.15 mol mol$^{-1}$ hexose coupled with HAc and HBu were detected in mixed cultures fed sucrose and maintained at 35 °C and at pH 5.2 in a CSTR. These researchers also observed decreasing HAc and HBu levels with increased loading, whereas in this current study, in untreated N$_2$ sparged cultures, increasing HAc levels were linked to increasing loading rates, while the major carbon sink was HBu (Figure 8.2). Although, Kyazze et al. (2006) sparged mixed cultures with N$_2$, the 2.1±0.2 mol mol$^{-1}$ hexose H$_2$ yield obtained in the current study is approximately 2-fold greater than the yield reported by these researchers.
Figure 8.2 COD distribution of fermentation products at various fermenting conditions

**Notes:**
1. The labeling in x-axis corresponds to the fermentation conditions, the #s represent the HRT (h); LA: linoleic acid; C: control; WN: without nitrogen purging and N: nitrogen purging.
2. HAc = acetic acid; HPr = propionic acid; HBu = butyric acid; EtOH = ethanol; i-PrOH = iso-propanol and Res. hexose = residual hexose.
3. Initial COD concentration is equal to 6 g COD L⁻¹.

Except for cultures not treated with LA and sparged with N₂, decreasing propionic acid (HPr) levels was observed in all conditions with decreasing HRT (Figure 8.2). Supporting studies by Zhang et al. (2006) have shown decreasing HPr production with decreasing HRT for a glucose feed. In addition to VFAs, ethanol (EtOH) and iso-propanol (i-PrOH) was also detected under all conditions. EtOH levels varied between 0.6 to 0.8 g COD L⁻¹ in LA treated cultures operating at 8 h and 6 h HRTs (Figure 8.2). The control cultures without N₂ purging showed decreasing ethanol levels with decreasing HRTs although the percent decrease was insignificant. The presence of i-PrOH in addition to ethanol and other VFAs indicate mixed acid and alcohol fermentation. Similarly, Kim et al. (2012) demonstrated that under CO₂ sparging conditions in addition to the mixed VFAs, i-PrOH and EtOH levels were approximately 18% and 3% of the initial COD, respectively. In this study, i-PrOH and EtOH levels in
untreated cultures sparged with N\textsubscript{2} constituted approximately 9 to 13\% of the initial COD (Figure 8.2).

### 8.3.3 Flux balance analysis

In order to assess the impact of N\textsubscript{2} sparging (both in the presence and absence of LA) on the H\textsubscript{2} yield, a FBA was conducted to examine the H\textsubscript{2} consumption and production activities. The analysis of the predicted and observed H\textsubscript{2} production under the experimental conditions examined, are presented in Figures 8.3a and b for untreated and LA-treated cultures, respectively.

The results indicates that the decreasing the HRT lowered the consumption of H\textsubscript{2} and was able to divert more of the carbon flux towards H\textsubscript{2} production. For all of the fermentation conditions, the decrease in H\textsubscript{2} consumption with HRT was $\geq 35\%$, except for the cultures that did not receive any treatment (i.e., addition of LA or sparging with nitrogen). Treatment with LA inhibited H\textsubscript{2} consumption by 25 $\pm$ 2 to 65 $\pm$ 6\% (C-WN versus LA-WN, Figures 8.3a and b). Many researchers have reported that sparging with N\textsubscript{2} may reduce H\textsubscript{2} consumption by organisms such as homoacetogens (Hawkes et al., 2007; Hussy et al., 2003). In the current study, nitrogen sparging alone was able to reduce H\textsubscript{2} consumption by 40 $\pm$ 3\% at 12 h HRT, but combining the reduction in HRT with N\textsubscript{2} sparging reduced the H\textsubscript{2} consumption by 80 $\pm$ 7\%; however, the observed increase in H\textsubscript{2} yield was in the range of 32 $\pm$ 2 to 46 $\pm$ 4\% (C-WN versus C-N, Figure 8.3a).
Figure 8.3 Hydrogen-related flux analysis for (a) untreated and (b) LA-treated culture in UASBR operating at different fermentation conditions

Notes:
1. The letter on the labeling of the x-axis represents the fermentation condition outlined in Table 8.2 and 8.3.
2. #s 12, 8 and 6 in the x-axis represent HRT in hours.
3. The predicted yield is taken from the model’s predicted output for R12 in Table 3.4 (section 3.11); observed yield is from R14 and hydrogen consumed corresponds to the sum of homoacetogenic (R17), propanol formation (R21) and hydrogenotrophic methanogen (R29) reactions.

The combined effects of LA-treatment and N₂ sparging were able to limit H₂ consumption by 85±6 to 97±6%, but the increases observed in H₂ yield ranged from 17±1 to 44 ± 3% (LA-WN versus LA-N, Figure 8.3b), which suggests that treatment...
with LA alone was not sufficient to reduce the decline in H₂ production (Figure 8.3b). Note that, H₂ consumption due to homoacetogenesis was reduced by 86 ± 6% in cultures treated with LA and sparged with N₂ at 12 h HRT and with decreasing HRT no H₂ consumption due to homoacetogenesis was deducted, while treatment with LA alone was able to reduce homoacetogenesis by 62 ± 5% (R17 flux, data not shown).

In cultures without any treatment the consumption was mainly due to methanogenesis and acetogenesis, with at least ≥ 73±6% contributing from either methanogenesis or acetogenesis alone.

8.3.4 Hydrogenase activity under different fermentation conditions

The hydrogenase enzymatic assay was conducted for all of the fermentation conditions examined in this study. Both the evolution and the uptake specific activity (ESA and USA) of the microflora were characterized at each of the experimental conditions outlined in Table 8.2. Overall, the ESA of hydrogenase tended to increase with decreases in the HRT, except for the control cultures without N₂ sparging (C-WN), which showed similar ESA throughout the experiment (Figure 8.4a). The parameters that markedly influenced H₂ yield (Table 8.3) have the same impact on ESA at 12 h HRT. Maximum ESA of 32±3 µmol H₂ mg⁻¹ VSS h⁻¹ was observed in LA treated cultures sparged with N₂ operating at 6 h HRT. A similar level of activity (28±4 µmol H₂ mg⁻¹ VSS h⁻¹) was recorded at a 6 h HRT in the control cultures with N₂ sparging (C-N). ESA levels of 21±3 µmol H₂ mg⁻¹ VSS h⁻¹ were obtained from the LA-treated cultures without N₂ sparging (LA-WN) at 8 and 6 h HRTs, which is 10% (approximate) greater than those observed with control cultures without N₂ sparging. Pendyala et al. (2012) investigated both types of hydrogenase activity (ESA and USA) in mixed anaerobic communities in batch scale reactors under different pretreatment conditions. The results from their study revealed that treatment with LA improved the ESA, but was not able to suppress the USA of hydrogenase. In the current study, both increase in the ESA and suppression of the USA were achieved by the addition of LA and by lowering the HRT during continuous operation of UASBR (Figure 8.4a).
Figure 8.4 Impact of different operating conditions on the hydrogenase activity showing (a) evolution specific activity (ESA) and (b) uptake specific activity (USA)

Note: For legends description refer Expt#1-4 conditions outline in Tables 8.2

In contrast to the patterns observed between ESA and H₂ yield under the conditions tested, the patterns observed for USA do correspond to the H₂ consumption activity, so high USA would be correlated to low H₂ yields (Figures 8.3 and 8.4b). The maximum USA level of 40±4 µmol H₂ mg⁻¹ VSS h⁻¹ was observed at 12 h HRT in untreated control cultures without N₂ sparging (C-WN). The USA was much lower in the untreated control culture that was sparged with N₂ at 12h HRT and decreased further in both of the untreated control cultures (Expt #1 and 3) with reductions in the HRT to 8 h and 6 h (Figure 8.4b). Conditions with reduced pH₂ show increased hydrogenase activity (Kim et al., 2006; Mizuno et al., 2000). These researchers have substantiated that sparging helps
to reduce the pH$_2$ levels in the fermentation broth. The results from this study suggest that the hydrogenase in untreated control cultures showed increased ESA under N$_2$ sparging condition (Expt. #3, C-N vs Expt. #1, C-WN; Figure 8.4a). However, USA decreased markedly for sparging conditions that correlated with the reduced H$_2$ consumption observed in the bioreactors, i.e., improved H$_2$ yield (Figure 8.4b and Figure 8.3a).

In the LA-treated cultures (with and without N$_2$ purging), the decrease in the USA with decrease in HRT was about 50% on an average. Nearly 20-50% reduction in USA was observed in the LA treated cultures in comparison to the corresponding control cultures (Figure 8.4b).

Overall, the hydrogenase activity results suggest that decreasing the HRT along with N$_2$ sparging is effective in suppressing uptake activity of hydrogenase, which corresponds to the observed impact of N$_2$ sparging on H$_2$ yield i.e., suppression of H$_2$ consumption enhanced H$_2$ yield (Table 8.3). The ESA of hydrogenase showed a good correlation to their H$_2$ yield or HPRs (Table 8.3 and Figure 8.1).

8.3.5 Microbial association with fermentative hydrogen production

T-RFLP analysis with restriction enzyme Hae III revealed a total of 104 different T-RFs ranging from 41–348 bp (Figures 8.5a and b). Diversity in the fermentative microbial population was greater in the LA-treated samples when compared to untreated culture (without LA). However, in both LA-treated and untreated cultures, cultures sparged with N$_2$ showed less diversity in the microbial communities when compared to those without N$_2$ sparging.

The major microflora observed and belonging to the phylum Firmicutes included Clostridium sp., Eubacterium sp., Bacillus sp., in LA treated and control cultures, while Lactobacillus sp. and Alicyclobacillus sp. were observed in control cultures (Table 8.4). However, the presence of species belonging to the phylum Bacteroidetes, such as Flavobacterium sp., Bacteroides sp. were observed in LA treated cultures. In addition, Propionibacterium sp. was detected only in control cultures without LA treatment (Table 8.4).
Figure 8.5 Comparison of the different fermentation conditions in (a) control and (b) LA-treated cultures based on the terminal restriction fragments generated by Hae III enzyme digestion, showing the relative abundance of T-RFs

Notes: The legend represents the fermentation conditions described in Table 8.2

A PCA was applied to the T-RFLP data obtained from the Hae III data set. The data set considered in the analysis includes T-RFs band intensity of the samples with lengths greater than 50 bp. The first three principle components which are presented in a three-dimensional plane accounted for 57% of the total variation (Figure 8.6). The three-dimensional plot also presents the H₂ yield (mol mol⁻¹ hexose) obtained for each condition (represented by the color key shown on left side of the plot in Figure 8.6). The
three clusters observed based on the composition of the microbial population in the LA-treated samples and the control samples (without LA) are depicted in Figure 8.6. Note that clustering of the samples was closely associated with their H₂ yields and experimental conditions. The N₂ sparged cultures without LA treatment were clustered together while the control cultures without any treatment were clustered separately. Cultures treated with LA and able to produce high levels of H₂ were in close proximity to each other. The only LA-treated culture with a low H₂ yield (1.34±0.09 mol mol⁻¹ hexose) was linked to a 12 h HRT and without N₂ sparging was close to the LA treated cultures in a reactor operating at 8 and 6 h HRTs and sparged with N₂ (Figure 8.6).

![Figure 8.6 Principal component analysis of T-RFLP data showing three-dimensional localization of the samples from different fermentation conditions according to the similarity of the terminal restriction fragments generated by Hae III enzyme digestion](image)

**Notes:**
1. The letter represents the fermentation condition: C-control; LA-linoleic acid addition; N-nitrogen sparging, WN-without nitrogen sparging
2. #s 12, 8 and 6 in the plot represent the HRT in hours.
3. Clustering (indicated by ellipses) indicates high similarity of the microbial composition of samples within ellipse, whereas separation indicates more variation or differences in composition.
4. The coloring of the labels is coded with respect to their observed H₂ yield (mol mol⁻¹ hexose) (Table 8.3)
5. The color key for H₂ yield is presented on the right side of the plot.
### Table 8.4 Microbial composition observed under different experimental conditions

<table>
<thead>
<tr>
<th>Experimental condition</th>
<th>HRT (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Expt# 1, Control cultures without N₂ sparging</strong></td>
<td></td>
</tr>
<tr>
<td>Alicyclobacillus sp. and Bacillus sp.</td>
<td>12</td>
</tr>
<tr>
<td><em>Clostridium novyi</em> and Uncultured bacteria</td>
<td>6</td>
</tr>
<tr>
<td>Eubacterium sp., <em>Desulfobacterium</em> sp. and Propionibacterium sp.</td>
<td>12, 8</td>
</tr>
<tr>
<td><em>Clostridium cochlearium</em>, <em>C. botulinum</em>, <em>C. beijerinckii</em> and <em>C. sporogenes</em></td>
<td>12, 8</td>
</tr>
<tr>
<td>Bacteroides sp.</td>
<td>8, 6</td>
</tr>
<tr>
<td>Lactobacillus sp.</td>
<td>12, 8, 6</td>
</tr>
<tr>
<td><strong>Expt#2, LA treated cultures without N₂ sparging</strong></td>
<td></td>
</tr>
<tr>
<td><em>Clostridium novyi</em></td>
<td>12, 8</td>
</tr>
<tr>
<td>Bacteroides sp. and Flavobacterium sp.</td>
<td>12, 8</td>
</tr>
<tr>
<td>Eubacterium sp.</td>
<td>12, 8, 6</td>
</tr>
<tr>
<td>Thermosipho africanus and <em>Dethiosulfovibrio</em> sp.</td>
<td>8</td>
</tr>
<tr>
<td><em>Clostridium fallax</em></td>
<td>12</td>
</tr>
<tr>
<td><em>Clostridium beijerinckii</em>, <em>C. botulinum</em> and <em>C. sporogenes</em></td>
<td>6</td>
</tr>
<tr>
<td><strong>Expt# 3, Control cultures with N₂ sparging</strong></td>
<td></td>
</tr>
<tr>
<td>Eubacterium sp., Propionibacterium sp., Lactobacillus sp. and Alicyclobacillus sp.</td>
<td>12, 8</td>
</tr>
<tr>
<td><em>Clostridium novyi</em> and Uncultured bacteria</td>
<td>12, 8, 6</td>
</tr>
<tr>
<td>Flavobacterium sp.</td>
<td>12</td>
</tr>
<tr>
<td>Thermosipho africanus, <em>Clostridium beijerinckii</em>, <em>C. botulinum</em> and <em>C. sporogenes</em></td>
<td>8</td>
</tr>
<tr>
<td><em>Clostridium cochlearium</em>, <em>C. botulinum</em> and <em>C. septicum</em></td>
<td>12, 8, 6</td>
</tr>
<tr>
<td><strong>Expt# 4, LA treated cultures with N₂ sparging</strong></td>
<td></td>
</tr>
<tr>
<td>Alicyclobacillus sp.</td>
<td>12</td>
</tr>
<tr>
<td><em>Clostridium novyi</em> and Uncultured bacteria</td>
<td>12, 8, 6</td>
</tr>
<tr>
<td>Eubacterium sp.</td>
<td>12, 8, 6</td>
</tr>
<tr>
<td>Bacillus sp., <em>Clostridium beijerinckii</em>, <em>C. botulinum</em> and <em>C. sporogenes</em></td>
<td>8, 6</td>
</tr>
<tr>
<td>Bacteroides sp. and Flavobacterium sp.</td>
<td>12, 8</td>
</tr>
</tbody>
</table>

**Note:** Microorganism listed in table indicates T-RF bands with $\geq 5\%$ relative intensity observed under corresponding HRT.
Although lactate was not detected, *Lactobacillus* sp. were observed in control cultures without N₂ sparging and also in control reactors operating with a 12 and 8 h HRT and sparged with N₂ (Table 8.4). According to Kim et al. (2006), lactate production from a sucrose fed culture containing *Lactobacillus* sp. was observed in a reactor operating with a 12 h HRT and without N₂ sparging. One possible reason, for absence of HLa in control cultures under N₂ sparged and unsparged conditions might be conversion of HLa to HPr by *Propionibacterium* sp. According to Moat and Foster (2002), HPr production from HLa is mediated by microorganisms such as *Propionibacterium* sp. during anaerobic fermentation.

*Propionibacterium* sp. was detected in control cultures without sparging operating at 8 h and 12 h HRTs (Table 8.4, Expt. #1). In addition to *Propionibacterium* sp., *Clostridium* sp. was also detected in reactors operating with 8 h and 12 h HRTs while at a 6 h HRT, uncultured bacteria and *Clostridium* sp. were dominant (Table 8.4, Expt. #1). Studies conducted by Zhang et al. (2006) have shown that for a glucose fed culture, decreasing the HRT in a stepwise manner caused decreasing HPr producing bacteria levels and below 6 h the activity was diminished. In control reactors operating in sparging and non-sparging modes and at 8 h and 12 h HRT, the HPr levels reached approximately 0.5 g COD L⁻¹.

The high H₂ yield observed in the control cultures sparged with N₂ correlated well with the presence of *Clostridium* sp. (*Clostridium botulinum, C. cochlearium, C. septicum*) (Table 8.4, Expt. #3). In the LA treated cultures and in reactors operating at 12 h and 8 h HRTs, *Clostridium novyi, Bacteroides* sp. and *Flavobacterium* sp. were observed, while *Clostridium fallax* was observed only in LA treated cultures operating at 12 h HRT. Cultures operating at 6 h HRT and treated with LA contained an abundance of *Clostridium beijerinckii, C. botulinum* and *C. sporogenes*, while *Eubacterium* sp. was observed under all the HRTs conditions (Table 8.4, Expt. #2). In sparged pretreated inocula with high H₂ yields, different *Clostridium* sp. was reported by Kim et al. (2006) and Kim et al. (2012). These researchers detected a wide range of *Clostridium* sp. which included *Clostridium tyrobutyricum, Clostridium pasteurianum, Clostridium proteolyticum* and *Clostridium proteoclasticum*. In the current study, the presence of
different groups of \textit{Clostridium} sp. in cultures fed SWG derived sugars under different operating conditions show evidence of \textit{H}_2 producers responsible for \textit{H}_2 yields greater than 1.6 mol mol$^{-1}$ hexose (\textbf{Figure 8.6 and Tables 8.3 and 8.4}). The presence of \textit{Eubacterium} sp. in \textit{H}_2 producing cultures treated with LA was also reported for a \textit{H}_2 producing sewage sludge pretreated under alkaline conditions (Cai et al., 2004).

\textit{Flavobacterium} sp., \textit{Bacillus} sp. and \textit{Bacteroides} sp. were detected in LA treated cultures in reactors operating with 8 h and 12 h HRTs. \textit{Flavobacterium} sp. were able to ferment furan containing feed and able to produce \textit{H}_2, studies by Lopez et al. (2004), confirmed the presence of \textit{Flavobacterium} sp. in the cultures fed lignocellulosic hydrolysate under \textit{H}_2 producing conditions. Note, \textit{Flavobacterium} sp. were observed in batch LA treated cultures fed furans plus glucose (\textbf{section 6.3.8}). In acidogenic continuous flow reactors containing a \textit{H}_2 producing culture, Ren et al. (2007) observed \textit{Bacteroides} sp. plus \textit{Clostridium} sp., which showed HAc and EtOH type fermentation under \textit{H}_2 producing conditions. Chaganti et al. (2013), observed \textit{Clostridium} sp., \textit{Bacteroides} sp. and \textit{Eubacterium} sp. in LA treated cultures fed with lignocellulosic sugars (glucose and xylose) and operated under sequencing batch conditions. These authors observed \textit{H}_2, HAc and i-PrOH as major end products in their fermentation byproducts, with nearly 1-6\% of produced \textit{H}_2 diverted to homoacetogenic activity. In the current study feeding sugars derived from SWG lowered the alcohol production with various treatment condition applied and the major byproducts include \textit{H}_2, HAc and HBu as their end products. The presence of \textit{Bacteroides} sp. in control and LA treated cultures without sparging also confirmed the presence of homoacetogenic activity. Note, the \textit{H}_2 consumed via homoacetogenic activity under these conditions vary from 0.20 ± 0.02 to 0.60 ± 0.05 mol \textit{H}_2 consumed mol$^{-1}$ hexose (data derived from R17 flux for \textit{H}_2 consumption via homoacetogenesis, \textbf{Figure 8.3}).

The high \textit{H}_2 yield (2.45±0.13 mol mol$^{-1}$ hexose) associated with LA treated cultures under N$_2$ sparging condition is associated with the presence of \textit{Clostridium} sp., \textit{Bacillus} sp. and \textit{Eubacterium} sp. (\textbf{Figure 8.6, Tables 8.3 and 8.4, Expt. #4}). \textit{Clostridium} sp. accounted for approximately 50\% of relative abundance of the T-RFs in cultures sparged with N$_2$ and treated with LA (\textbf{Figure 8.5b, Table 8.4}). Note, \textit{Clostridium} sp. and
*Eubacterium* sp. were observed in a LA (1 g L\(^{-1}\)) treated H\(_2\) producing culture fed a SWG steam exploded hydrolysate in the reactors operating at a 16 h HRT and pH 5.0 (section 7.3.7). Similarly, in studies conducted by Abreu et al. (2012), *Bacillus* sp. was detected in H\(_2\) producing cultures fed lignocellulosic sugars and maintained at pH 5.5.

### 8.4 Conclusions

In this study, the effects of N\(_2\) sparging, LA treatment and changing HRT conditions on H\(_2\) production from a steam exploded SWG liquor was examined using mixed anaerobic cultures. Reduction in HRT alone though was able to show increased H\(_2\) yield, suppression of the H\(_2\) consumption was not evident with decrease in HRT. Sparging with N\(_2\) or the addition of LA improved the H\(_2\) yield with an associated reduction in H\(_2\) consumption, for which sparging greatly reduced the level of homoacetogenic activity. Microbial characterization studies showed the populations were affected by the different experimental factors. A combination of N\(_2\) sparging with LA-treatment proved to be more beneficial in increasing the H\(_2\) yield when compared to a single factor. The results showed that N\(_2\) sparging together with LA pretreatment of the culture increased the H\(_2\) yield to a maximum of 2.56±0.10 mol mol\(^{-1}\) of hexose when the reactor was operated at a 6 h HRT. The presence of *Clostridium* sp. was dominant in cultures associated with high H\(_2\) yields. A wide range of microorganisms which included *Propionibacterium* sp., *Bacteroides* sp., *Eubacterium* sp. and *Clostridium* sp. were observed in unsparged cultures.

### 8.5 References


CHAPTER 9.0 USING STEAM EXPLODED CORN STOVER HYDROLYSATE FOR SUSTAINABLE BIOHYDROGEN PRODUCTION: IMPACT OF ORGANIC LOADING RATE

9.1 Introduction

Hydrogen (H$_2$) has been gaining widespread importance in the energy sector as an alternative to depleting fossil fuel reserves. Hydrogen is an ideal energy carrier because it can be produced from renewable resources and its combustion byproduct is carbon neutral. Hydrogen is preferred over methane (produced during the anaerobic digestion) because of its high energy yield (143 kJ g$^{-1}$) which is 2.75 times greater than that of methane (Das and Veziroglu, 2001; Levin et al., 2004). Generation of H$_2$ from readily available lignocellulosic materials (about 220 billion tonnes of lignocellulosic per year, globally is produced) via dark-fermentation is of great significance with the increasing energy demand (Chong et al., 2009; Ren et al., 2009).

Among the available agricultural residue feedstocks in Canada, corn stover (CS) is preferred for its use in bioenergy generation for the following reasons: 1) Higher productivity (yield) of CS per acre of cultivated land in Canada (about 75 million tonnes per year of harvestable CS), 2) High energy content (19 MJ kg$^{-1}$) of CS residues and 3) Utilization of CS residues for H$_2$ production results in cleaner emissions when compared to direct burning of the biomass in the field (AAFC, 2013; Wright et al., 2009).

Biomass pretreatment of agriculture residues is essential in order to extract sugars. Utilizing raw biomass (untreated biomass) results in low H$_2$ yields, due to the fact that fermentative bacteria show less fermentability with complex substrates (Demribas, 2008). Steam explosion process in the presence of dilute acid addition offers great potential for implementation into the full-scale facilities. This process also offers several advantages such as low environmental impact and less hazardous chemicals discharge (Alvira et al., 2010). However, steam explosion has some disadvantages in addition to using dilute acid. The major disadvantage is the generation of fermentation inhibitors, such as furfural, hydroxyl methyl furfural (HMF) and phenolic compounds, in addition to the acetic acid (Palmqvist and Hahn-Hagerdal, 2000b).
Most H₂ production studies have been carried out with simple sugars or with complex sugars, such as sucrose and starch (Arooj et al., 2008; Spagni et al., 2010; Zhang et al., 2006). Only a few studies have examined using lignocellulosic derived sugars for bio-H₂ production. For example in studies conducted by de Vrije et al. (2009), and Antonopoulou et al. (2007), pretreated lignocellulosic biomass were used to produce H₂ using pure cultures. However, the major drawbacks associated with pure cultures include the maintenance of sterile feed and operating conditions. Several reports have noted the advantages of using mixed culture when using lignocellulosic-derived sugars as a substrate, not only because the operating conditions can be non-aseptic, but also for the ability of mixed culture to feed on a wide variety of substrates and to adapt to or tolerate the presence of inhibitors such as furans and phenolic compounds during the fermentation process (Horn et al., 2011; Quemeneur et al., 2012). Datar et al. (2007) reported H₂ yields greater than 2.2 mol mol⁻¹ hexose from steam exploded CS hydrolysate using mixed anaerobic cultures. However, their studies were conducted using batch process and there is a lack of data for full-scale continuously operated reactors.

The main impediment in employing mixed anaerobic cultures for bio-H₂ production in continuous flow systems is the need to suppress H₂ consumers such as methanogens and homoacetogens, as well as other non-H₂ producers, such as lactic acid-producing bacteria (LAB) and propionic acid-producing bacteria (PAB) (Abreu et al., 2011; Ren et al., 2007b; Zhang et al., 2006). Hence, pre-treatment of the inoculum with heat, acid or base, or chemicals, such as 2-bromoethanesulfonate (BES) or long chain fatty acids, along with changes in operational parameters, such as hydraulic retention time (HRT) or organic loading rate (OLR) and pH, is essential in optimizing bio-H₂ production (Abreu et al., 2011; Chaganti et al., 2013; Hafez et al., 2011). Changes in operating conditions primarily controlling the acidogenesis step which produces fatty acids such as acetic acid (HAc), lactic acid (HLa), propionic acid (HPr) and butyric acid (HBu). The various fermentation patterns observed includes HAc-type, HBu-type, and ethanol (EtOH)-type fermentation (Table 8.1, Equations 8.1, 8.2, 8.3 and 8.5). The HPr and HLa fermentation routes are non-H₂ producing in which H₂ is either consumed (Table 8.1, Equation 8.4). Alternatively, H₂ production is lowered due to NADH consumption (Equation 2.5) which is required for the H₂ evolution (Equation 2.4) (Li et al., 2009; Liu
et al., 2011). Researchers have reported changes in fermentation type based on the type with simple sugars (Liu et al., 2011; Ren et al., 2007a).

Investigating the fermentation type using lignocellulosic CS hydrolysate is important for maximizing the H$_2$ yields. Since these hydrolysates contain mixed sugars and potential inhibitors (such as furfural and HMF), which could alter the metabolic pathway towards different byproducts depending on the inoculum source (Veeravalli et al., 2013), a proper understanding of the fermentation pattern is beneficial. Furthermore, furans, phenols and acetic acid present in the hydrolysate increases the lag time of the fermentation process because of the toxicity imposed by inhibitors on microbial cultures. Acclimation of microbial cultures is required to avoid the lag phase and increase the substrate conversion efficiency of hydrolysates containing inhibitors (Palmqvist and Hahn-Hagerdal, 2000a; Weber et al., 2010). Furthermore, the inability of microorganisms in continuous full-scale operations not to degrade furans and phenolic compounds results in their discharge into receiving water bodies. For example, furfural accounts for nearly 10% of the COD in food processing waste (Park and Jung, 2003) and if untreated they will be discharged in effluents. In other cases, high levels of furfural and phenolic compounds have been reported in waste-water flow (sulfite evaporator condensate) from the wood-pulp industry (Environment Canada, 1997; IPCS, 2000). A summary of the environmental dangers posed by these toxins, especially their threat to aquatic life was reported by ACS (2011). Anaerobic treatment of furfural waste to obtain less toxic compounds has been reported in many studies (Benjamin et al., 1984; Boopathy, 2009). Many research articles have described the effects of these inhibitors on the fermentation type and metabolism during fermentative H$_2$ production (Palmqvist and Hahn-Hagerdal, 2000a; Quemeneur et al., 2012; Qureshi et al., 2012). However, the majority of this research has been reported in batch reactors. Furthermore, studies on biological H$_2$ production from lignocellulose or food-processing and pulp waste have examined key factors such as biomass pretreatment, H$_2$ productivity, type of fermentation and composition of the microbial community. Very little work has been reported on the impact of inhibitor removal during the fermentation process (Kadar et al., 2004; Pendyala et al., 2013). Consequently, the development of practical applications for generating energy in addition to treating these complex waste materials rich in sugars/carbohydrates
and contain fermentation inhibitors such as phenols or furans will be of great significance.

Therefore the objective of this study is to examine the potential of using lignocellulosic CS-derived sugars containing fermentation inhibitors as a substrate for biological H₂ production in a continuously fed upflow anaerobic sludge blanket reactor (UASBR). More specifically, one objective was to examine the effects of OLR on H₂ productivity and microbial dynamics using CS hydrolysate as a feed. Another objective was to examine the effect of fermentation inhibitors in the CS hydrolysate on mixed anaerobic fermentation.

9.2 Materials and methods

Dark fermentative H₂ production from the CS hydrolysate was performed using control (cultures fed no linoleic acid (LA)) and LA treated granular culture (Culture B) with an approximate VSS concentration of 12 g L⁻¹. Details regarding the source of inoculum and reactor maintenance is described in section 3.3. The study was conducted using CS hydrolysate as feedstock (containing sugars, furans and phenolic compounds) to assess the feasibility of using the feed (non resin treated) directly for fermentation. The substrate pretreatment (steam explosion) conditions and processing of the CS hydrolysate is outlined in section 3.2. The composition of the CS feed is outlined in Table F.2, Appendix F. The concentration of LA (a methanogenic inhibitor) selected for this study (1 g L⁻¹) was based on the experimental outcomes from Chapter 6 using CS hydrolysate in batch reactors. In the study described in Chapter 6, 2 g L⁻¹ of LA along with furans was shown to impose increasing inhibition on the fermentative H₂ production. The OLR was varied between 3.0 g COD L⁻¹ d⁻¹ (at 12 h HRT) to 50 g COD L⁻¹ d⁻¹ (at 6 h HRT) for the control cultures (i.e., without the addition of LA) and 3.0 g COD L⁻¹ d⁻¹ (at 12 h HRT) to 36 g COD L⁻¹ d⁻¹ (at 8 h HRT) for LA treated cultures (see section 3.5 for the UASBR operation). The loading rates selected were applied from low to high levels, so as to study the effects of these parameters on the biogas composition, the liquid byproducts and microbial composition. Experiments were conducted in duplicate using two UASBRs (designated as reactor R1 and R2). At each OLR condition shown in Table 9.1, experiments were repeated at least 10 times using reactors R1 and R2 (n=10 × 2). Note, prior to feeding 100% CS hydrolysate in stage 3 (Table 9.1), the reactors were
acclimatized to a feed contained a mixture of glucose, xylose and CS hydrolysate (1:1:1). This step was carried out to acclimatize the cultures in incremental increasing levels of the feed containing sugars, furans and phenols.

All the chemical and analytical methods used in this study are outlined in sections 3.7.3 and 3.8, respectively. The microbial characterization was performed using the methods described in section 3.10. The statistical analysis, such as canonical correspondence analysis (CCA) was conducted to elucidate the association of the fermentation pattern with the dynamic shifts in the microbial population observed in the corresponding operating conditions. Tukey's post-hoc statistical test was used to evaluate the significance of differences between two means. All the statistical methods used in this study were in accordance with the procedures outlined in section 3.12. The experiment was conducted at 37 °C at an operating pH of 5.0. The summary of the fermentation conditions applied in this experiment is outlined in Table 9.1.

### Table 9.1 Experimental progress of UASBR operation

<table>
<thead>
<tr>
<th>Stage</th>
<th>HRT (h)</th>
<th>OLR&lt;sup&gt;a&lt;/sup&gt; (g sugar L&lt;sup&gt;-1&lt;/sup&gt; d&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>OLR&lt;sup&gt;b&lt;/sup&gt; (g inhibitors L&lt;sup&gt;-1&lt;/sup&gt; d&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>OLR&lt;sup&gt;c&lt;/sup&gt; (g COD L&lt;sup&gt;-1&lt;/sup&gt; d&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Duration (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>24</td>
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<td>12</td>
<td>10.0</td>
<td>1.5</td>
<td>12</td>
<td>41-50</td>
</tr>
<tr>
<td>VII</td>
<td>12</td>
<td>15.0</td>
<td>2.2</td>
<td>18</td>
<td>51-60</td>
</tr>
<tr>
<td>VIII</td>
<td>12</td>
<td>20.0</td>
<td>2.9</td>
<td>24</td>
<td>61-70</td>
</tr>
<tr>
<td>IX</td>
<td>8</td>
<td>30.0</td>
<td>4.4</td>
<td>36</td>
<td>71-75</td>
</tr>
<tr>
<td>X</td>
<td>6</td>
<td>40.0</td>
<td>5.9</td>
<td>50</td>
<td>75-80</td>
</tr>
</tbody>
</table>

<sup>a</sup>represents the OLR based on sugar content (hexose equivalents) present in the feed (CS hydrolysate)

<sup>b</sup>represents the OLR based on furans and phenols present in the feed (CS hydrolysate)

<sup>c</sup>represents OLR based on the COD content of the feed (CS hydrolysate) containing sugar, furans, acetic acid and phenol (see Table F.2, Appendix F for concentrations)

**Notes:**
1. Operating conditions at different stages are applicable to both control and LA treated cultures
2. Stages 1 and 2 were fed with mixture of glucose, xylose and CS hydrolysate (1:1:1)
3. Experiment with LA lasted for a period of 75 days only (i.e., stage IX)
9.3 Results and discussion

9.3.1 Material balance of the sugars derived from corn stover

The material balance (Figure 9.1) reveals that approximately 32% (w/w) of the CS biomass was recovered as the sugars in the CS hydrolysate obtained via acid-impregnated steam explosion process. The sugar recovery efficiency was approximately 6% greater than that reported by Tucker et al. (2003). Tucker et al. (2003) used a similar pretreatment condition for extracting sugars from CS. Approximately, 5% (w/w) of the biomass obtained from CS was in the form of furfural and HMF (fermentation inhibitors derived from the dehydration of pentoses and hexoses contained in the biological material). The inhibitors also included phenolic acids derived from the acid-soluble lignin which comprised approximately 1.3% (w/w) of the biomass. Acetic acid in the liquor comprised approximately 2.3% (w/w) of the total dry biomass content. On average, 42% (w/w) of the CS solid biomass was lost in the production of the steam exploded liquid hydrolysate.

![Figure 9.1 Mass balance showing the composition of the corn stover before and after pretreatment](image-url)

Overall yield of total sugars = 325±10 g hexose per 1000 g dry biomass (32±1%)  
Overall yield of total sugars = 346±10 g hexose per 1000 g TVS
9.3.2 Biological hydrogen and methane production potential from corn stover

The H₂ and methane yields which were produced from CS using the continuous operation reactor system are presented in Table 9.2. The H₂ yields obtained from the CS hydrolysate ranged from 10.8±3.4 mL g⁻¹ TVS at 3 g COD L⁻¹ d⁻¹ to 104.4±7.6 mL g⁻¹ TVS at 18 g COD L⁻¹ d⁻¹ in the untreated control cultures. However, with LA treated cultures treated, the H₂ yields increased (ranging from 74.0±10.9 mL g⁻¹ TVS at 3 g COD L⁻¹ d⁻¹ to 102.6±14.6 mL g⁻¹ TVS at 9 g COD L⁻¹ d⁻¹) and then decreased to 0.7±0.1 mL g⁻¹ TVS at 36 g COD L⁻¹ d⁻¹.

<table>
<thead>
<tr>
<th>Stage</th>
<th>OLR (g COD L⁻¹ d⁻¹)</th>
<th>Biogas production in control reactors (mL g⁻¹ TVS)</th>
<th>Biogas production in LA treated reactors (mL g⁻¹ TVS)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>H₂</td>
<td>CH₄</td>
</tr>
<tr>
<td>III</td>
<td>3</td>
<td>10.8±3.4&lt;sup&gt;d&lt;/sup&gt;</td>
<td>73.5±7.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>IV</td>
<td>6</td>
<td>34.1±6.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>68.4±8.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>V</td>
<td>9</td>
<td>40.1±5.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>52.7±7.9&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>VI</td>
<td>12</td>
<td>76.7±7.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>21.9±5.8&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>VII</td>
<td>18</td>
<td>104.4±7.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.5±1.0&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>VIII</td>
<td>24</td>
<td>99.2±5.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.6±0.3&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>IX</td>
<td>36</td>
<td>76.1±5.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.2±0.1&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>X</td>
<td>50</td>
<td>65.1±5.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.04±0.01&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Notes:
1. ND- not detected
2. ‘a±b’ represents the mean ± standard deviation for n≥8
3. a, b, c, and d represent statistically different means within the same column (Tukey’s test, α=0.05)
4. The average and standard deviation are for n = 20. Two reactors (R1 and R2) operating under the same condition and each condition repeated 10 times.

The methane yields in untreated cultures ranged from 4.5±1.0 mL g⁻¹ TVS to 73.5±7.9 mL g⁻¹ TVS at 18.0 g COD L⁻¹ d⁻¹ and 3 g COD L⁻¹ d⁻¹, respectively. In comparison, due to the inhibition imposed by LA together with the toxic furans and phenolic compounds, low methane yields (ranging from 1.4±0.4 mL g⁻¹ TVS to 28.0±3.9 mL g⁻¹ TVS at loading rates below 12 g COD L⁻¹ d⁻¹) were detected in the LA treated cultures. The results obtained in this study were 10% greater than the H₂ yield (90.5 mL g⁻¹ TVS) and methane yield (64.9 mL g⁻¹ TVS) predicted by the model developed by Monlau et al. (2012) for lignocellulosic biomass based on the biochemical composition of
the lignocellulosic biomass (Appendix F, Table F.2). Work by Guo (2012) suggest that there exist a high correlation between fermentable carbohydrates produced from the hydrolysis of solid waste and H₂ yield. In the current study, for control cultures (non LA fed cultures), an increase in OLR up to 24 g COD L⁻¹ d⁻¹ increased the H₂ yield to 100±5 ml g⁻¹ TVS; however, with further increasing the OLR, the H₂ yield decreased to 65±6 mL g⁻¹ TVS. Experiments conducted by Pattra et al. (2008) using a hydrolysate from sugarcane bagasse, suggest that there is a strong correlation between the H₂ yield and the relative amounts of inhibitor to sugar concentrations derived from acid hydrolysis of the biomass. Note, these authors used a ratio of sugars to fermentation inhibitors (E ratio) to correlate with H₂ yields and concluded that a greater E ratio supports H₂ fermentation. In the current study, the H₂ yield dropped after 24 g COD L⁻¹ d⁻¹ owing to the high loading concentrations of the fermentation inhibitors in the CS hydrolysate (Tables 9.1 and 9.2).

The maximum H₂ yield obtained from the control and LA treated cultures at 18.0 and 7.5 g COD L⁻¹ d⁻¹, respectively, corresponds to approximately 2.4 mol mol⁻¹ hexose (Figures 9.2 and 9.3). This yield is close to the average feasible yield (2.5 mol mol⁻¹ hexose) proposed by Hawkes et al. (2007) for mixed anaerobic culture. The lower H₂ yields observed in both the control and LA treated cultures at higher OLRs indicate that H₂ production is not favorable for the non-resin treated CS hydrolysate and when feeding high inhibitor concentrations. According to Tai et al. (2010), increased inhibition with increasing concentration of the phenol containing medium was observed during fermentative H₂ production by Clostridium butyricum in the batch reactors.

The CH₄ yields from the CS hydrolysate in both control and LA treated cultures were comparatively low to those reported in the literature. This may be due to the presence of inhibitors such as furans and lignin-derived phenolic compounds (Alvarez and Lettinga, 1991; Lacour, 2011). Furthermore, treatment with LA under low pH conditions is known to inhibit methanogenesis by up to 90% (Chaganti et al., 2013). The high CH₄ yields reported in other studies (e.g. 396 mL g⁻¹ TVS) could be due to the use of batch operations or differences in the operating parameters, such as pH or temperature (Kaparaju et al., 2009). The high substrate loading resulting in increased distribution of the soluble metabolites such as HAc, HBu and EtOH might have also caused inhibition of H₂ production (Liu et al., 2008; Van Ginkel and Logan, 2005).
9.3.3 Effects of organic loading rate on hydrogen and methane production

Initially, the reactors were fed with pure sugars and CS hydrolysate in a mixture to examine the H\textsubscript{2} and CH\textsubscript{4} production from mixture of pure and CS hydrolysate in both control and LA treated cultures before changing to a feed containing 100% CS hydrolysate (Table 9.1). On day 11, the feed was switched to steam exploded CS hydrolysate with an OLR of 3.0 g COD L\textsuperscript{-1} d\textsuperscript{-1} (Table 9.1). During this stage, CH\textsubscript{4} production was dominant in both control and LA treated cultures (Figures 9.2 and 9.3). However, the addition of LA suppressed CH\textsubscript{4} yields in comparison to the control cultures (Figures 9.2 and 9.3). Note in the control reactors, CH\textsubscript{4} production continued to be dominant until day 40 at a loading of 9 g COD L\textsuperscript{-1} d\textsuperscript{-1} (Figure 9.2).
Figure 9.2 Performance of UASBR for cultures fed with corn stover hydrolysate showing the hydrogen and methane yields and their production rates

Notes:
1. The H$_2$ and CH$_4$ yield are calculated from the sugar content of the hydrolysate
2. HRT: hydraulic retention time; OLR: organic loading rate; HPR: hydrogen production rate and MPR: methane production rate
3. The production rates and yields plotted shows average values for duplicate reactors R1 and R2.
4. 467 mLH$_2$ g$^{-1}$ COD = 4.0 mol H$_2$ mol$^{-1}$ hexose
Figure 9.3 Performance of UASBR for LA-treated cultures fed with corn stover hydrolysate showing the H$_2$ and CH$_4$ yields and production rates

Notes:
1. The H$_2$ and CH$_4$ yields are calculated according to the sugar content of the CS hydrolysate
2. HRT: hydraulic retention time; OLR: organic loading rate; HPR: hydrogen production rate and MPR: methane production rate
3. The production rates and yields plotted shows average values for duplicate reactors R1 and R2.
4. 467 mL H$_2$ g$^{-1}$ COD = 4.0 mol H$_2$ mol$^{-1}$ hexose

The effects of OLR on H$_2$ and CH$_4$ production are clearly evident in the control (no LA added) reactors (Figure 9.2). The initial CH$_4$ production (mL g$^{-1}$ COD) during the
early stages of operation with OLRs of 6 and 9 g COD L\(^{-1}\) d\(^{-1}\) were observed to be 202±15 and 144±19, respectively. However, with increasing the OLR, the CH\(_4\) yields decreased by 60±8% and at OLRs of 36 to 50 g COD L\(^{-1}\) d\(^{-1}\), the decrease reached approximately 90%. Similarly, the H\(_2\) yields (mL g\(^{-1}\) COD) increased from 103±06 on days 21-40 (i.e., at OLRs of 6 g COD L\(^{-1}\) d\(^{-1}\) and 9 g COD L\(^{-1}\) d\(^{-1}\)) to 274±40 on days 53-70 (i.e., at OLRs of 18 g COD L\(^{-1}\) d\(^{-1}\) and 24 g COD L\(^{-1}\) d\(^{-1}\)) (Figure 9.2, Table 9.1). However, with further increasing the OLR to 50 g COD L\(^{-1}\) d\(^{-1}\), the H\(_2\) yield decreased by 30%. The H\(_2\) production rate (HPR) increased with increases in OLR and the maximum HPR obtained for the untreated control culture was 7.5±0.7 L L\(^{-1}\) d\(^{-1}\) at 50 g COD L\(^{-1}\) d\(^{-1}\), while LA treated cultures showed maximum HPR of 2.3±0.2 L L\(^{-1}\) d\(^{-1}\) at 7.5 g COD L\(^{-1}\) d\(^{-1}\). The HPRs obtained in this study using the control cultures is comparable to that reported by Zhang et al. (2013b) and Arooj et al. (2008), in which glucose (60 g L\(^{-1}\) d\(^{-1}\)) or starch (32 g COD L\(^{-1}\) d\(^{-1}\)) were used as the substrates. Kaparaju et al. (2009) conducted studies with wheat straw hydrolysate at thermophilic temperatures and obtained a maximum H\(_2\) yield of 178 mL g\(^{-1}\) hexose. This yield is approximately 40% lower than the maximum yield obtained in this study. The H\(_2\) yields and HPRs obtained by Ren et al. (2006) from molasses at an OLR range of 13 g COD L\(^{-1}\) d\(^{-1}\) to 65 g COD l\(^{-1}\) d\(^{-1}\) using mixed anaerobic cultures were in accordance with the results obtained in this study. These authors observed a decline in H\(_2\) production performance with an increase in OLR to 68 g COD L\(^{-1}\) d\(^{-1}\) similar to that observed in this study, where a 39% decrease in H\(_2\) production was observed when the OLR was increased from 24 g COD L\(^{-1}\) d\(^{-1}\) to 50 g COD L\(^{-1}\) d\(^{-1}\) (Figure 9.2).

The H\(_2\) yields obtained from the LA treated cultures were greater than the yields obtained from the control cultures at low OLRs of up to 9 g COD L\(^{-1}\) d\(^{-1}\) (Figures 9.2 and 9.3). Inhibition imposed by LA on the methanogenic environment is evident in the work of Chaganti et al. (2013), in which both glucose and xylose were used as substrate in an anaerobic sequential batch system. However, with an increase in OLR, the H\(_2\) production (mL g\(^{-1}\) COD) performance gradually decreased from 281±31 to 180±23 at 9 and 12 g COD L\(^{-1}\) d\(^{-1}\), respectively. A further increase in OLR to 18 g COD L\(^{-1}\) d\(^{-1}\), reduced the H\(_2\) production performance by 60±8%. The synergistic effects of the microbial inhibitors in the hydrolysate together with the methanogenic inhibitory effects
of LA addition might account for the decrease in H₂ production with increased organic load. Palmqvist et al. (1999) studied the individual and interaction effects of furans, fatty acids and phenolic compounds on EtOH-producing systems, which are applicable to H₂ production systems as well. Toxicity inhibition on the methanogens and the biodegradability of phenols and LCFAs (linoleic, oleic and stearic acid) contained in paper waste and forestry waste by anaerobic sludge was studied by Sierra-Alvarez (1990). The results from their study showed that increased toxicity reduced biogas production and the biodegradability of the hydrolysate obtained from the waste.

The reason for the increase in biological H₂ production observed in control cultures for OLRs between 12-36 g COD L⁻¹ d⁻¹ might be due to the effect of microbial inhibitors (furans) on H₂ consumers. Suppressed methanogenesis and increased HPRs were observed by Pakarinen et al. (2011) using grass silage, where the authors observed in addition to the OLR and decrease in HRT, the composition of the feed induced the H₂ production in the methanogenic reactors. Similarly low concentrations of the fermentation inhibitors enhanced the substrate utilization and EtOH production observed in yeast strains (Keating et al., 2006).

9.3.4 Soluble metabolite product distribution for control cultures

The distribution of the soluble metabolite product (SMP) from the feed and the degradation products of furfural are tabulated in Table 9.3 for control cultures. The reactor performance at various operating stages can be described by the metabolite distribution. The major soluble metabolites produced include HAc, HBu, EtOH and iso-propanol (i-PrOH). The percent COD reduction varied between 10% to 40% (approximately). The HAc and HBu levels increased from 0.25±0.03 g L⁻¹ to 4.6±0.5 g L⁻¹ and 0.25±0.03 g L⁻¹ to 5.0±0.4 g L⁻¹, respectively with the increase in OLR from 3 g COD L⁻¹ to 50 g COD L⁻¹ (Table 9.3). The HBu/HAc ratio has been used as a positive indicator for H₂ production (Kim et al., 2006). The results obtained in the current study show that increased levels of HBu and HAc was accompanied by higher H₂ yields until stage VII after which the yields dropped by approximately 32% (Table 9.2 and Figure 9.2).

The H₂ yield reported by Arooj et al. (2008) was 20% relatively low in comparison to the maximum yield obtained in this study. These authors owed this difference in H₂
yields due to the presence of reduced end products, such as lactate, valerate and caproate in the carbohydrate fed mixed anaerobic culture. Note none of these end products were detected in the current study. A decrease in the HRT lowered H$_2$ yield, and shifted the metabolic pathway to solventogenesis (EtOH and i-PrOH production) (Figure 9.2 and Table 9.3). Han et al. (2011) observed that increased EtOH production was associated with increasing the HPR at a high OLR in an immobilized reactor system fed glucose. These researchers found a high correlation ($R^2 = 0.97$) between ethanol and H$_2$ productivity. Similarly, a shift towards solventogenic fermentation was observed at high OLRs by Guo et al. (2008), in which molasses was used as a substrate in high rate systems. Studies by Ren et al. (2007b) suggest that H$_2$-producing ethanoligens in mixed anaerobic cultures could produce H$_2$ yields in the range of 1.5-2.2 mol mol$^{-1}$ hexose. The results obtained from the current study also indicate that the H$_2$ yields in untreated control cultures vary between 180 mL g$^{-1}$ COD to 300 mL g$^{-1}$ COD (1.5-2.4 mol mol$^{-1}$ glucose) with an EtOH concentration ranging from 0.35 g L$^{-1}$ to 2.5 g L$^{-1}$ and 0.25 g L$^{-1}$ to 1.6 g L$^{-1}$ of PrOH (Figure 9.2 and Table 9.3).

Apart from these major metabolites, other products, such as HPr and i-PrOH, were observed in the soluble metabolites. The HPr concentration observed ranged from 0.5 to 0.7 g L$^{-1}$ in stages VII to X. Studies by Zhang et al. (2006) using anaerobic digested sludge fed glucose reported a decrease in HRT caused an increase in HBu levels and a decrease in HPr levels was associated with increasing the H$_2$ yield. i-PrOH was observed primarily at OLRs between 18 g COD L$^{-1}$ d$^{-1}$ to 50 g COD L$^{-1}$ d$^{-1}$ (stage VII to X) and in all of these cases, the amount of i-PrOH constituted less than 6% of the initial COD (Table 9.3). Note in current study, the decrease in H$_2$ yields which was observed from stage VIII to X (Figure 9.2) indicated that presence of HPr and i-PrOH in addition to increase in solvent levels such as EtOH and PrOH was responsible the low H$_2$ yields (< 2 mol mol$^{-1}$ glucose) and hence, low productivity. According to Ren et al. (1997), the presence of reduced end products is evidence for the observed low H$_2$ yields.
Table 9.3 Summary of the product distribution and the COD mass balance of the influent feed in the control cultures

<table>
<thead>
<tr>
<th>Parameters</th>
<th>III</th>
<th>IV</th>
<th>V</th>
<th>VI</th>
<th>VII</th>
<th>VIII</th>
<th>IX</th>
<th>X</th>
</tr>
</thead>
<tbody>
<tr>
<td>COD influent (g L&lt;sup&gt;-1&lt;/sup&gt; d&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>3</td>
<td>6</td>
<td>9</td>
<td>12</td>
<td>18</td>
<td>24</td>
<td>36</td>
<td>50</td>
</tr>
<tr>
<td>Acetic acid (g L&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>0.25±0.03</td>
<td>0.47±0.07</td>
<td>1.1±0.1</td>
<td>1.4±0.1</td>
<td>2.3±0.3</td>
<td>3.6±0.3</td>
<td>3.4±0.5</td>
<td>4.6±0.5</td>
</tr>
<tr>
<td>Propionic acid (g L&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>0.3±0.03</td>
<td>0.25±0.03</td>
<td>0.6±0.05</td>
<td>0.3±0.03</td>
<td>0.7±0.05</td>
<td>0.5±0.04</td>
<td>0.7±0.07</td>
<td>0.7±0.08</td>
</tr>
<tr>
<td>Butyric acid (g L&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>0.25±0.03</td>
<td>0.42±0.04</td>
<td>0.9±0.1</td>
<td>2.1±0.2</td>
<td>3.3±0.5</td>
<td>4.1±0.2</td>
<td>5.0±0.4</td>
<td>4.1±0.4</td>
</tr>
<tr>
<td>i-Propanol (g L&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>0±0</td>
<td>0±0</td>
<td>0.06±0.01</td>
<td>0.14±0.02</td>
<td>0.3±0.1</td>
<td>0.5±0.16</td>
<td>0.48±0.06</td>
<td>0.4±0.08</td>
</tr>
<tr>
<td>Ethanol (g L&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
<td>0.34±0.07</td>
<td>0.75±0.08</td>
<td>0.93±0.13</td>
<td>2.2±0.3</td>
<td>2.6±0.4</td>
</tr>
<tr>
<td>Propanol (g L&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>0±0</td>
<td>0.14±0.01</td>
<td>0±0</td>
<td>0.28±0.03</td>
<td>0.56±0.09</td>
<td>0.92±0.12</td>
<td>1.0±0.15</td>
<td>1.6±0.2</td>
</tr>
<tr>
<td>Furoic acid (g L&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>0.03±0.0</td>
<td>0.1±0.01</td>
<td>0.18±0.02</td>
<td>0.2±0.03</td>
<td>0.35±0.03</td>
<td>0.46±0.06</td>
<td>0.57±0.12</td>
<td>0.52±0.03</td>
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<tr>
<td>Furfuryl alcohol (g L&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>0.09±0.02</td>
<td>0.15±0.02</td>
<td>0.22±0.03</td>
<td>0.47±0.06</td>
<td>0.47±0.06</td>
<td>0.58±0.22</td>
<td>1.0±0.12</td>
<td>1.2±0.4</td>
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<tr>
<td>Residual Sugar (g L&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
<td>2.7±0.7</td>
<td>5.1±0.6</td>
</tr>
<tr>
<td>Residual furfural (g L&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>0.01±0.0</td>
<td>0.06±0.01</td>
<td>0.08±0.01</td>
<td>0.12±0.01</td>
<td>0.38±0.04</td>
<td>0.48±0.04</td>
<td>0.86±0.02</td>
<td>1.4±0.1</td>
</tr>
<tr>
<td>Residual HMF (g L&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>0±0</td>
<td>0.02±0.0</td>
<td>0.03±0.0</td>
<td>0.04±0.0</td>
<td>0.09±0.01</td>
<td>0.11±0.02</td>
<td>0.17±0.01</td>
<td>0.24±0.01</td>
</tr>
<tr>
<td>Biomass (g COD L&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>0.15</td>
<td>0.3</td>
<td>0.45</td>
<td>0.6</td>
<td>0.9</td>
<td>1.2</td>
<td>1.2</td>
<td>1.25</td>
</tr>
<tr>
<td>SMP (g COD L&lt;sup&gt;-1&lt;/sup&gt;)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.5±0.18</td>
<td>2.7±0.3</td>
<td>4.7±0.6</td>
<td>8.7±1.1</td>
<td>15.7±2.2</td>
<td>23.1±2.9</td>
<td>32.5±3.7</td>
<td>40.3±4.9</td>
</tr>
<tr>
<td>SMP (g COD d&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>12.3±1.5</td>
<td>22.8±2.7</td>
<td>40.1±5.2</td>
<td>74.1±9.4</td>
<td>133.7±18.5</td>
<td>196±24</td>
<td>276±32</td>
<td>342±41</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt; (L L&lt;sup&gt;-1&lt;/sup&gt; d&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>0.08±0.05</td>
<td>0.49±0.09</td>
<td>0.87±0.12</td>
<td>2.22±0.21</td>
<td>4.53±0.33</td>
<td>5.73±0.31</td>
<td>6.60±0.51</td>
<td>7.53±0.69</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.5±0.3</td>
<td>3.0±0.5</td>
<td>5.3±0.7</td>
<td>13.6±1.3</td>
<td>27.8±2.0</td>
<td>35.1±1.9</td>
<td>40.4±3.1</td>
<td>46.1±4.2</td>
</tr>
<tr>
<td>CH&lt;sub&gt;4&lt;/sub&gt; (L L&lt;sup&gt;-1&lt;/sup&gt; d&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>0.53±0.06</td>
<td>0.99±0.13</td>
<td>1.14±0.17</td>
<td>0.63±0.17</td>
<td>0.19±0.09</td>
<td>0.09±0.02</td>
<td>0.02±0.00</td>
<td>0.01±0.00</td>
</tr>
<tr>
<td>CH&lt;sub&gt;4&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.9±1.3</td>
<td>22.2±2.9</td>
<td>25.7±3.9</td>
<td>14.2±3.8</td>
<td>4.3±2.1</td>
<td>2.1±0.4</td>
<td>0.42±0.1</td>
<td>0.13±0.0</td>
</tr>
<tr>
<td>COD balance (%)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>94±11</td>
<td>91±12</td>
<td>92±13</td>
<td>98±14</td>
<td>106±15</td>
<td>112±13</td>
<td>100±11</td>
<td>87±10</td>
</tr>
</tbody>
</table>

<sup>a</sup> Soluble metabolite products (SMP) calculated based on the respective COD equivalents. For example, for acetic acid, it is 1.07 g COD g<sup>-1</sup> acetic acid.

<sup>b</sup> Based on 8 g COD g<sup>-1</sup> H<sub>2</sub> and 4 g COD g<sup>-1</sup> CH<sub>4</sub>.

<sup>c</sup> COD balance (%) = (∑(SMP, H<sub>2</sub> and CH<sub>4</sub> COD)*100)/ Influent COD

Notes: 1. a ± b indicate mean ± standard deviation for n = 10; 2. 10% of the initial COD is diverted to biomass is assumed.
In addition to the glucose degradation products, other byproducts of furans degradation were observed in the fermentation broth. The degradation products included furoic acid and furfuryl alcohol (Table 9.3). Interestingly, the level of furoic acid stabilized at the level of 0.5±0.06 g L\(^{-1}\) in the effluent. However, the concentration of furfuryl alcohol, which is considered to be a more reduced end product of the furfuraldehyde, increased in the effluent from 0.1±0.02 to 1.2±0.4 g L\(^{-1}\), over the period of operation. The residual furan (furural plus HMF) concentrations in the effluent were 1.0±0.03 g L\(^{-1}\) and 1.6±0.1 g L\(^{-1}\), at OLRs 36 g COD L\(^{-1}\) d\(^{-1}\) and 50 g COD L\(^{-1}\) d\(^{-1}\). Several studies have reported that the inhibition imposed by the various furans in combination caused increased inhibition of H\(_2\) productivity for furan concentrations greater than 1 g L\(^{-1}\) (Cao et al., 2010; Panagiotopoulos et al., 2011).

Approximately 12±04% to 18±02% of the sugars in the hydrolysate were unfermented (remained as residual sugars in the effluent) at OLRs ≥ 24 g COD L\(^{-1}\) d\(^{-1}\). The COD balance, including the biomass and gas products, accounted for ≥ 92±11% at all of the loading rates examined.

### 9.3.5 Soluble metabolite product distribution for LA-treated cultures

The SMP distribution and a COD mass balance for the LA-treated cultures on the metabolites (gas and liquid) is presented in Table 9.4. HAc and HBu were the major metabolites produced in stages III to VI with HAc and HBu contributing 18±2 to 28±3% and 20±2 to 34±7% of the influent COD, respectively (calculated From Table 9.4). The distribution of HAc and HBu which were reduced with increasing the OLR resulted in decreasing the H\(_2\) yields. The HPr concentration varied from 0.14±0.01 to 1.1±0.1 g L\(^{-1}\) (Table 9.4) was greater than the HBu levels observed during stages VII to IX.

The concentrations of the alcohols during the stages VII to IX increased from 1.6±0.2 to 4.7±0.6 g L\(^{-1}\) with EtOH and PrOH as major components. However, more than 26% of the sugars remained unfermented in the effluent (Table 9.4). Studies by Qureshi et al. (2012), using wheat straw hydrolysate supplemented with furfural and HMF concentrations ranging from 0 to 3 g L\(^{-1}\) revealed that increases in the concentration of furans in the hydrolysate caused decreases in the HAc and HBu levels without affecting solvent production.
Increasing the substrate loading in the LA treated cultures caused a decrease in both H₂ and VFA productivity, along with an increase in alcohol production. Similar results were reported by de Amorim et al. (2012) in anaerobic fluidized bed reactors operated with a heat-treated inoculum fed glucose at 8 h HRT. These researchers reported less than 0.5 mol H₂ mol⁻¹ glucose at an OLR of 25 g glucose L⁻¹ d⁻¹ with a maximum VFA concentration of 2.5 g L⁻¹ and an EtOH concentration of 2.0 g L⁻¹. The HAc and total alcohol concentrations observed at similar loading levels (24 g COD L⁻¹ d⁻¹) in the current study, with LA-treated cultures were 1.9±0.1 g L⁻¹ and 2.76±0.32 g L⁻¹, respectively. The H₂ yield obtained at this experimental condition was 15±3 mL g⁻¹ COD (approximately 0.13 mol mol⁻¹ glucose) (Figure 9.3 and Table 9.4).

The low VFA and H₂ productivity observed in the LA treated cultures might be due to the presence of fermentation inhibitors, such as furans and phenols, in addition to the LA treatment. The antagonistic effects observed on the fermentation of CS hydrolysate is likely caused by the interactions between various factors, such as a combination of microbial inhibitors, inhibitory effects plus substrate loading or the effects of inhibitors plus low pH conditions. Furthermore, the degradation byproducts of the LA treatment when present are considered to be more inhibitory in combination than alone, even at certain threshold levels (Salvador et al., 2011).
Table 9.4 Summary of the product distribution and the COD mass balance of the influent feed in the LA-treated cultures

<table>
<thead>
<tr>
<th>Parameters</th>
<th>III</th>
<th>IV</th>
<th>V</th>
<th>VI</th>
<th>VII</th>
<th>VIII</th>
<th>IX</th>
</tr>
</thead>
<tbody>
<tr>
<td>COD influent (g L(^{-1}) d(^{-1}))</td>
<td>3</td>
<td>6</td>
<td>9</td>
<td>12</td>
<td>18</td>
<td>24</td>
<td>36</td>
</tr>
<tr>
<td>Acetic acid (g L(^{-1}))</td>
<td>0.8±0.09</td>
<td>1.2±0.26</td>
<td>1.6±0.15</td>
<td>2.05±0.15</td>
<td>1.9±0.13</td>
<td>1.9±0.09</td>
<td>2.7±0.16</td>
</tr>
<tr>
<td>Propionic acid (g L(^{-1}))</td>
<td>0.14±0.01</td>
<td>0.53±0.1</td>
<td>0.53±0.03</td>
<td>0.7±0.05</td>
<td>0.7±0.05</td>
<td>0.77±0.03</td>
<td>1.1±0.1</td>
</tr>
<tr>
<td>Butyric acid (g L(^{-1}))</td>
<td>0.6±0.1</td>
<td>0.75±0.07</td>
<td>0.95±0.12</td>
<td>1.3±0.1</td>
<td>1.1±0.1</td>
<td>0.8±0.07</td>
<td>0.68±0.09</td>
</tr>
<tr>
<td>i-Propanol (g L(^{-1}))</td>
<td>0±0</td>
<td>0.02±0.0</td>
<td>0.1±0.01</td>
<td>0.14±0.02</td>
<td>0.24±0.02</td>
<td>0.48±0.07</td>
<td>0.7±0.09</td>
</tr>
<tr>
<td>Ethanol (g L(^{-1}))</td>
<td>0±0</td>
<td>0±0</td>
<td>0.18±0.02</td>
<td>0.29±0.03</td>
<td>0.5±0.05</td>
<td>0.88±0.11</td>
<td>2.1±0.3</td>
</tr>
<tr>
<td>Propanol (g L(^{-1}))</td>
<td>0±0</td>
<td>0.08±0.01</td>
<td>0.15±0.01</td>
<td>0.32±0.03</td>
<td>0.9±0.1</td>
<td>1.4±0.14</td>
<td>1.9±0.2</td>
</tr>
<tr>
<td>Furoic acid (g L(^{-1}))</td>
<td>0.06±0.01</td>
<td>0.08±0.01</td>
<td>0.12±0.01</td>
<td>0.2±0.01</td>
<td>0.15±0.02</td>
<td>0.24±0.02</td>
<td>0.18±0.01</td>
</tr>
<tr>
<td>Furfuryl alcohol (g L(^{-1}))</td>
<td>0.13±0.01</td>
<td>0.25±0.03</td>
<td>0.35±0.05</td>
<td>0.4±0.04</td>
<td>0.5±0.06</td>
<td>0.5±0.02</td>
<td>0.75±0.06</td>
</tr>
<tr>
<td>Residual Sugar (g L(^{-1}))</td>
<td>0±0</td>
<td>0.27±0.06</td>
<td>0.82±0.11</td>
<td>1.7±0.15</td>
<td>4.8±0.2</td>
<td>5.9±0.2</td>
<td>12.5±1.0</td>
</tr>
<tr>
<td>Residual Furfural (g L(^{-1}))</td>
<td>0.02±0.0</td>
<td>0.07±0.01</td>
<td>0.1±0.01</td>
<td>0.16±0.02</td>
<td>0.55±0.07</td>
<td>0.8±0.08</td>
<td>1.4±0.1</td>
</tr>
<tr>
<td>Residual HMF (g L(^{-1}))</td>
<td>0.01±0.0</td>
<td>0.03±0.0</td>
<td>0.03±0.0</td>
<td>0.07±0.01</td>
<td>0.17±0.02</td>
<td>0.28±0.02</td>
<td>0.53±0.04</td>
</tr>
<tr>
<td>Biomass (g COD L(^{-1}))</td>
<td>0.15</td>
<td>0.3</td>
<td>0.45</td>
<td>0.6</td>
<td>0.9</td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td>SMP (g COD L(^{-1}))(^a)</td>
<td>2.6±0.4</td>
<td>4.9±0.8</td>
<td>7.5±0.8</td>
<td>10.9±0.9</td>
<td>17.2±1.3</td>
<td>21.4±1.5</td>
<td>36±3</td>
</tr>
<tr>
<td>SMP (g d(^{-1}))</td>
<td>22.2±3.1</td>
<td>42.0±6.6</td>
<td>63.4±7.0</td>
<td>92.7±7.9</td>
<td>146.5±10.8</td>
<td>182±13</td>
<td>306±27</td>
</tr>
<tr>
<td>H(_2)</td>
<td>0.53±0.08</td>
<td>1.37±0.11</td>
<td>2.23±0.32</td>
<td>2.01±0.22</td>
<td>0.63±10</td>
<td>0.29±0.11</td>
<td>0.06±01</td>
</tr>
<tr>
<td>H(_2)(^b)</td>
<td>3.3±0.5</td>
<td>8.4±0.7</td>
<td>13.6±1.9</td>
<td>12.3±1.3</td>
<td>3.9±0.6</td>
<td>1.7±0.6</td>
<td>0.4±0.06</td>
</tr>
<tr>
<td>CH(_4)</td>
<td>0.20±0.03</td>
<td>0.12±0.01</td>
<td>0.04±0.01</td>
<td>0.04±0.01</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>CH(_4)(^b)</td>
<td>4.6±0.6</td>
<td>2.7±0.27</td>
<td>0.9±0.2</td>
<td>0.9±0.2</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>COD balance (%)(^c)</td>
<td>112±16</td>
<td>99±13</td>
<td>98±11</td>
<td>100±9</td>
<td>95±7</td>
<td>86±7</td>
<td>95±9</td>
</tr>
</tbody>
</table>

\(^a\) Soluble metabolite products (SMP) calculated based on the respective COD equivalents. For example, for acetic acid, it is 1.07 g COD g\(^{-1}\) acetic acid.

\(^b\) Based on 8 g COD g\(^{-1}\) H\(_2\) and 4 g COD g\(^{-1}\) CH\(_4\).

\(^c\) COD balance (%) = \((\sum\text{SMP, H}_2\text{ and CH}_4\text{ COD}) \times 100)/\text{Influent COD}

Notes: 1. a ± b indicate mean ± standard deviation for n = 10; 2. 10% of the initial COD is diverted to biomass is assumed.
9.3.6 Removal of furans and phenols via dark fermentation

Reduction in the levels of furfural, HMF and phenols contained in the hydrolysate was observed in both control and LA treated cultures. The reduction efficiencies observed in the current study imply that dark fermentation can be used not only for H₂ production, but can also be used for decreasing the furan and phenol levels present in wastewaters.

The conversion of the furans to less toxic compounds was primarily based on the influent inhibitor concentration in the hydrolysate. The effective contribution to the total removal of the fermentation inhibitors by each individual inhibitor during different experimental stages (Table 9.1) is presented in Figures 9.4a and b for the control and LA treated cultures, respectively. The percent reduction of these fermentation inhibitors observed in the control cultures ranged from 55±5% to 83±6%. The reduction of phenols in the hydrolysate contributed nearly 10±1% to 21±1% of the total reduction of the inhibitors present in the hydrolysate (Figure 9.4a). Hernandez and Edyvean (2004) studied the anaerobic treatment of wastewater containing phenols in a two-stage reactor and reported that a major reduction in phenol was observed in the acidogenic phase, but increased phenol concentration caused greater inhibition of methanogens during the second phase. This suggests that the increase in H₂ yield observed with control cultures from stages VI to VIII in the current study might be due to the presence of threshold levels of these inhibitors which are able to suppress methanogenesis and diverted electrons from methanogens to H₂ producers.
Figure 9.4 Reduction of the fermentative inhibitors, including furfural, HMF and phenol contained in the hydrolysate by: (a) control (b) LA-treated cultures at different operating stages

Notes:
1. The inhibitor loading on the x-axis refers to the sum of the influent furfural, HMF and phenol concentrations fed to the reactor per day.
2. The stage at which the reactor is operating is given below the corresponding toxicity loading outline in Table 9.1.
4. The percent reduction of furans and phenols is calculated from the influent and effluent furan concentrations.
5. The error bars represent standard deviation for n = 3
Borole et al. (2009) used microbial fuel cells to control the accumulation of inhibitors, such as furfural, HMF and lignin degradation products, in biorefinery cycle water. The level of the inhibitors accumulation reported by these authors was ≤ 20 mM. Borole et al. (2009) also reported reduction efficiencies of furfural and or HMF were close to 100%. In the current study, the furans in the aldehyde form were converted to less toxic chemicals such as acids and alcohols (e.g. furoic acid and furfuryl alcohol) (Tables 9.3 and 9.4). On an average, reduction of furfural and HMF constituted 70±9% of the total reduction (Figures 9.4a and b). Biological transformation of furans to less toxic compounds by enteric bacterial strains was observed under anaerobic conditions (Boopathy et al., 1993). In the current study, the reduction in phenolic compounds was 50±13% in control cultures fed the steam exploded hydrolysate operating at OLRs ranging from 3 g COD L\(^{-1}\) d\(^{-1}\) and 50 g COD L\(^{-1}\) d\(^{-1}\). In comparison, studies using pure culture such as Clostridium butyricum showed phenol degrading efficiency of 35% to 67% for dosage levels ≤ 0.4 g L\(^{-1}\) (Tai et al., 2010). In the current study, at a similar level of phenol concentration (0.4 g L\(^{-1}\)), 35±5% of the total phenols were reduced (stage VII to X, Figure 9.4 a). Note, Tai et al. (2010) used single inhibitor (phenol) to test the reduction efficiency, in the current study a combination of inhibitors were used in the influent.

In LA treated cultures, the percent reduction of the fermentation inhibitors varied from 37±3% to 80±7%. The reduction efficiencies during high OLR was observed to be comparatively lower than at low OLRs for the LA treated cultures. The results obtained in the current study are different from those reported by Zhang et al. (2013a), in which the reduction efficiencies of the furfural content present in the hydrolysate of oil palm fibrous wastes (i.e., empty fruit bunch) digested by Enterobacter sp. FDS8 were higher than those observed in the LA treated cultures for concentrations up to 4 g L\(^{-1}\).

The reduction of fermentation inhibitors suggests that anaerobic digestion (by dark fermentation) could be used to reduce the levels of toxic substances present in the wastewater effluent or in hydrolysates derived from the pulp and paper industry or food wastes (e.g. honey syrup processing or coffee manufacturing industries) (Hakulinen and Salkinojasalonen, 1982; Lakshmidevi and Muthukumar, 2010).
9.3.7 Microbiological analysis

The T-RFs obtained from the \textit{Hae} III digest were used to study variations in the microbial population in both control and LA treated cultures at different OLRs (6, 18 and 36 g COD L$^{-1}$ d$^{-1}$). A canonical correspondence analysis (CCA) was used in elucidating the relationships between the assemblages of microbial species and the environmental factors to which the microorganisms were exposed. In this analysis, the fermentation byproducts were selected as environmental factors.

The first three ordination axes accounted for 71% of the total variability within the original dataset (Figure 9.5a and b). The differences between the amount of variability explained by the first (26%) and second axis (25%) are trivial, while axis 3 accounted for 21% of the total variability. Therefore, in addition to the plot of axis 1 vs axis 2, axis 2 vs axis 3 is also plotted to illustrate patterns of variance within the original dataset.

The results of the CCA show that CH$_4$ and PrOH are associated with the first axis, whereas H$_2$ and HBu are correlated in the negative direction (Figure 9.5a). Likewise, HAc and HPr are correlated with axis 2 and FuAc is negatively correlated with axis 2 (Figure 9.5a). A species-environmental correlation of 0.3 to 0.8 (approximately) was observed for the dataset (Table 9.6). These correlations show that there is a quantitative association of the microbial species with the environmental factors selected for analysis. A high correlation indicates correlation strong relationship between the species populations and the factor variables represented by the closest ordinate or axis.
Figure 9.5 Canonical correspondence analysis (CCA): triplot of the association of fermentation byproducts with the microbial T-RFs under different fermentation conditions (a) axis 1 vs axis 2 (b) axis 2 vs axis 3

Notes:
1. The samples are labeled with red code (a number followed by treatment type). The # represents the COD loading (g L\(^{-1}\) d\(^{-1}\)) and the treatment conditions refer to the control (C), represented by closed triangles (▲), and linoleic acid (LA)-fed cultures, represented by open triangles (Δ).
2. Species are indicated by blue dots (●) with their corresponding T-RFs bandwidth # (base pairs).
3. Quantitative environmental variables are indicated by green lines which include the following: HAc = acetic acid; HPr = propionic acid; HBu = butyric acid; i-PrOH = iso-propanol; EtOH = ethanol; PrOH = propanol; FuAc = furoic acid; FuOH = furfuryl alcohol.
4. Only selected species that represents the T-RFs bands with more than 6% relative intensity are displayed (the corresponding species name is listed in Table 9.5).
Table 9.5 List of microorganisms corresponding to terminal restriction fragments determined by their 16S rRNA sequence

<table>
<thead>
<tr>
<th>T-RF (bp)</th>
<th>Organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>55</td>
<td>Geobacter metallireducens GS-15, Thioalkalivibrio sp. K90mix</td>
</tr>
<tr>
<td>56</td>
<td>Eubacterium tenue, Paracoccus denitrificans</td>
</tr>
<tr>
<td>59</td>
<td>Eubacterium tenue ATCC 25553</td>
</tr>
<tr>
<td>61</td>
<td>Clostridium cocoides ATCC 29236, Flavobacterium ferrugineum ATCC 13524,</td>
</tr>
<tr>
<td></td>
<td>Moorella thermoacetica ATCC 39073</td>
</tr>
<tr>
<td>65</td>
<td>Bacteroides distasonis, Clostridium cocoides, Flavobacterium ferrugineum</td>
</tr>
<tr>
<td>70</td>
<td>Bacteroides distasonis</td>
</tr>
<tr>
<td>71</td>
<td>Achromatium JD8.</td>
</tr>
<tr>
<td>72</td>
<td>Achromatium JD8. AF129550, Leptotrichia sp. AF189244</td>
</tr>
<tr>
<td>78</td>
<td>Methyllococcus capsulatus and Methylobacter sp.</td>
</tr>
<tr>
<td>81</td>
<td>Bacteroides fragilis NCTC 9343</td>
</tr>
<tr>
<td>88</td>
<td>Desulfomicrobium baculatum DSM 4028, Flavobacterium aquatile ATCC 11947</td>
</tr>
<tr>
<td>93</td>
<td>Flavobacterium columnare str. (JIP 49/87) ATCC 49513. AB023660</td>
</tr>
<tr>
<td>104</td>
<td>Bacteroides eggerthi</td>
</tr>
<tr>
<td>110</td>
<td>Sphingobacterium spiritivorum</td>
</tr>
<tr>
<td>208</td>
<td>Clostridium beijerinckii, Fusobacterium simiae</td>
</tr>
<tr>
<td>398</td>
<td>Butyrivibrio crossotus, Haloanaerobium acetoethylicum</td>
</tr>
</tbody>
</table>

The CCA ordinations represented by axis 1 and 2 shows that all of the H₂-producing conditions are grouped on the left side of the plot and that the major variables associated with these conditions include HAc, HBu, H₂ and EtOH. In comparison, on the right side of this plot non-H₂ producing culture conditions are located (LA treated cultures with loading rates of 18 g COD L⁻¹ d⁻¹ and 36 g COD L⁻¹ d⁻¹) in close proximity to the control cultures which were fed a lower loading rate (6 g COD L⁻¹ d⁻¹) and in which CH₄ production was dominant (Figure 9.5a). A closer examination of the plot reveals that Clostridium sp., Flavobacterium sp., Eubacterium sp., Bacteroides sp. and Sphingobacterium sp. were associated with the control cultures operating under 18 g COD L⁻¹ d⁻¹ and 36 g COD L⁻¹ d⁻¹ (Figure 9.5a, Table 9.5).
Table 9.6 Summary of canonical correspondence analysis ordination

<table>
<thead>
<tr>
<th>Axes</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>Total inertia</th>
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</thead>
<tbody>
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<td>0.9917</td>
<td>0.9353</td>
<td>0.7927</td>
<td>0.7403</td>
<td>0.3657</td>
<td>3.8258</td>
</tr>
<tr>
<td>Species-environment correlations</td>
<td>0.7327</td>
<td>0.3663</td>
<td>0.5743</td>
<td>0.3267</td>
<td>0.8119</td>
<td></td>
</tr>
<tr>
<td>Cumulative percentage variance of species data</td>
<td>25.92</td>
<td>50.37</td>
<td>71.09</td>
<td>90.44</td>
<td>99.998</td>
<td></td>
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</tbody>
</table>

Ren et al. (2007b) reported the dominance of *Clostridium* sp., *Bacteroides* sp. and *Ethanoligenens* sp. in mixed anaerobic communities under H$_2$-producing conditions. These authors studied EtOH-based H$_2$ production with cultures fed molasses under low pH conditions (4.5-6.0) and observed relatively high yields of H$_2$ of up to 0.45 L g$^{-1}$ COD with EtOH and HBu as the dominant byproducts. The results from the current study also show a similar association between the environmental factors (HBu, EtOH and H$_2$) with species associated with H$_2$-producing conditions (i.e., control cultures fed 18 g COD L$^{-1}$ d$^{-1}$ and 36 g COD L$^{-1}$ d$^{-1}$). The presence of *Flavobacterium* sp., along with the H$_2$-producing culture conditions and their closer association with furan derivative products, such as FuAc and FuOH, is evident in the CCA plot (Figure 9.5a, Table 9.5). Lopez et al. (2004) reported that *Flavobacterium* sp. is able to degrade the furans in a lignocellulosic hydrolysate and produce H$_2$ as well. Schroder et al. (1994) reported the breakdown of glucose to H$_2$ and HAc by anaerobically grown *Eubacterium* sp. under thermophilic conditions. *Eubacterium* sp., a homoacetogen studied by Tanner et al. (1981), is able to use H$_2$ and CO$_2$ as the potential substrate. Studies by Pendyala et al. (2013) and Saady et al. (2012) show the dominance of *Eubacterium* sp. in LA treated cultures fed glucose or a food and cardboard-paper waste blend, respectively. Both of these studies observed very little or no homoacetogenic activity under the conditions examined (i.e., in batch reactors, operating under mesophilic temperatures and low pH conditions (4.5 to 5.5)). In addition to the species described above, *Sphingobacterium* sp. was found grouped with species associated with control cultures under high loading rates (Figures 9.5a and b, Table 9.5). *Sphingobacterium kitahiroense* was observed as a dominant strain in the cultures fed steam exploded cornstalk hydrolysate which were able to produce H$_2$ in batch reactors with repeated cycles (Lu et al., 2009).
Eubacterium sp. and Bacteroides sp. were detected in LA treated H$_2$ producing cultures (6-LA, Figure 9.5a). According to Chaganti et al. (2013), both of these species were detected in granular cultures treated with LA and fed xylose under low pH conditions. These authors found that Bacteroides sp. belonging to the homoacetogenic group and Eubacterium sp. were able to exist in conditions producing maximum H$_2$ levels. However, they reported that approximately 5% of the H$_2$ produced was diverted towards the acetogenic reaction. In the current study, the major metabolite observed along with H$_2$ under this condition was HBu (Table 9.4).

The plot of the second and third axis (Figure 9.5b) explained 46% of the variability in the dataset and showed the distribution pattern for low loading conditions was scattered across the plane. In the current study, with LA treated cultures operating at 18 g COD L$^{-1}$ d$^{-1}$ and 36 g COD L$^{-1}$ d$^{-1}$ and control cultures at 36 g COD L$^{-1}$ d$^{-1}$, less assimilation of the sugars in the hydrolysate was observed. This is evident from the residual sugar content in the effluent (Table 9.3 and 9.4). Alcohol dominance under these conditions were associated with Thioalkalivibrio sp., Geobacter metallireducens, Bacteroides sp., Eubacterium sp. and Clostridium sp. in case of control cultures and Thioalkalivibrio sp., Methylococcus sp., Methylobacter sp., Flavobacterium sp., Butyrivibrio crosstos, and Clostridium sp. in case of LA treated cultures.

The presence of Flavobacterium sp. under high loading rates in the control reactors and LA fed reactors confirmed the reduction efficiency of the furans. The presence of Moorella thermoacetica and Butyrivibrio crosstos under LA fed conditions operating at 18 and 36 g COD L$^{-1}$ d$^{-1}$ is inconsistent with levels of HAc and HBu observed under this condition. Moat et al. (2002) reported the association between HBu production and Butyrivibrio sp. Similarly, Wirth et al. (2012) reported the presence of Moorella thermoacetica along with Clostridia that produce acetic acid in biogas-producing cultures. However, insignificant levels of VFAs or H$_2$ were observed in LA fed reactor operating under 36 g COD L$^{-1}$ d$^{-1}$, may be due to the antagonistic effects of inhibitors (furans plus LA). Similar synergistic or antagonistic effects were observed in wastewater containing toxins or inhibitors (Kugelman and Chin, 1971).

The presence of Thioalkalivibrio sp., Fusobacterium sp., Clostridium beijerinckii and Bacteroides sp. was coupled to high alcohol production among the fermentation
byproducts produced. Ren et al. (2007a) examined the fermentation type in acidogenic continuous flow reactors and noted the major presence of *Fusobacterium* sp. and *Bacteroides* sp., in addition to *Clostridium* sp. Ren et al. (2007a) observed that their fermentation pattern was closely associated to the byproducts produced during dark fermentation. Furthermore, their results suggest that both of *Bacteroides* and *Fusobacterium* species are capable of producing EtOH and other alcohols and in addition *Fusobacterium* sp. could also produce HBu depending on the fermentation conditions. Studies by Qureshi et al. (2012) also confirm that *Clostridium beijerinckii* produces high levels of alcohols, such as EtOH, acetone and butanol, at high substrate concentrations. Overall, the CCA explained the patterns and the variability in the data set, including the associations of the fermentation byproducts with the culture samples under different loading conditions.

## 9.4 Conclusions

This study confirms that the CS could be a potential substrate for biohydrogen production at suitable operational conditions. The following conclusions were drawn from the study.

1. CS appears to be a potential substrate for biological H$_2$ production in continuous reactor systems.
2. At low OLRs (3 g L$^{-1}$ d$^{-1}$ to 9 g L$^{-1}$ d$^{-1}$) in untreated control cultures, methane (CH$_4$) production was dominant. Any increase in the OLR suppressed the methanogenic activity by $\geq 60\pm 8\%$.
3. A maximum H$_2$ yield of 102±6 mL g$^{-1}$ TVS was obtained at OLRs operating at 18 g COD L$^{-1}$ d$^{-1}$ and 24 g COD L$^{-1}$ d$^{-1}$ in control cultures. A similar H$_2$ yield was obtained at a lower OLR of 10 g COD L$^{-1}$ d$^{-1}$ in LA treated cultures. The CH$_4$ yields (mL g$^{-1}$ TVS) varied between 22±6 in LA-treated cultures and 74±8 in control cultures at OLRs $\leq 12$ g COD L$^{-1}$ d$^{-1}$.
4. Addition of LA improved the H$_2$ yield at low OLRs. However, a negative effect was observed at high OLR due to the toxicity imposed by combination of inhibitors (furans and phenols) and LA.
5. High levels of HAc and HBu was observed under H\textsubscript{2}-producing conditions and solvent (EtOH and i-PrOH) production dominated at high OLRs in both control and LA treated cultures.

6. A CCA tri-plot using environmental factors and the species relative intensity levels (assessed using T-RFs) revealed a close association of the byproducts with the microflora.

7. The control cultures fed with high OLRs and the LA-treated cultures fed with low OLRs (associated with H\textsubscript{2}-producing conditions) were dominated by *Clostridium* sp., *Flavobacterium* sp., *Eubacterium* sp., and *Bacteroides* sp. In comparison, the other operating conditions were dominated by the presence of organisms related to *Butyribrio crosstotus*, *Moorella thermoacetica*, and *Methylobacter* sp were observed in addition to *Clostridium* sp., *Eubacterium* sp., and *Bacteroides* sp.

8. Alcohol production in the control cultures was primarily related to the presence of *Thioalkalivibrio* sp., *Fusobacterium* sp., *Clostridium beijerinckii* and *Bacteroides* sp. High alcohol productivity reduces the stress level imposed on the microflora by changing the metabolic pathway.

9. Toxic inhibitors (furans and phenols) at low concentrations contained in CS hydrolysate make this feedstock an ideal substrate for H\textsubscript{2} production by inhibiting methanogens. Reduction efficiencies of these toxic substances greater than 60% (approximately) were observed in the control cultures.

9.5 References


CHAPTER 10: CONCLUSIONS, RECOMMENDATIONS AND ENGINEERING SIGNIFICANCE

10.1 Summary and conclusions

Primarily, the major research focus on fermentative H$_2$ production was carried out using starch containing wastewaters, sucrose and pure sugars (Kothari et al., 2012; Lin et al., 2012). Studies using agricultural feed stock and waste materials for biohydrogen production have gained attention over the past decade (Hay et al., 2013; Liu et al., 2013). Using lignocellulosic hydrolysate for H$_2$ production is a more practical approach because of their renewable nature and availability (Chen et al., 2013; Kirtay, 2011). Fermentative H$_2$ production research in the past has focused on biomass pretreatment, operational parameters optimization such as pH, temperature, substrate concentration, inert gas sparging, inhibitor concentration and reactor operation (Gupta et al., 2013; Monlau et al., 2013; Nath and Das, 2011). A large amount of data have been reported for work conducted with batch, semi-continuous and continuous flow systems or by using pure cultures, co-cultures and mixed cultures (Chen et al., 2013; Liu et al., 2013; Rittmann and Herwig, 2012). Recent reports on fermentative H$_2$ production suggest that more research studies on continuous flow systems needs to be conducted at the laboratory-scale before scaling up operation to full-scale reactors (Dinamarca and Bakke, 2011; Khanna and Das, 2013).

The overall goal of the current research was to produce elevated H$_2$ yields from agricultural residues in a continuous process via dark fermentation. In the research presented in this thesis, biological H$_2$ production from pure and lignocellulosic sugars via dark fermentation was studied in continuous upflow anaerobic sludge blanket reactors (UASBRs) inoculated with mixed anaerobic cultures.

The objectives of the current research was accomplished by controlling the hydraulic retention time (HRT), organic loading rate (OLR), pH and culture pretreatment using linoleic acid (LA), a methanogenic inhibitor. Although, pH, HRT, OLR and LA have been extensively studied, the use of LA on continuous biohydrogen production using lignocellulosic biomass derived sugars were not assessed previously (Chaganti et al., 2013; Mohammadi et al., 2012; Saady et al., 2012).
In this research, efficient process performance with stable H\textsubscript{2} production rates (HPR) was obtained and characterized. The study also assisted to understand the relationships between fermentation metabolites and their distribution pattern under different operating conditions. In addition to the fermentation metabolism, rapid changes in the microbial community structure with changes in the operating conditions of the continuous process were revealed and linked to reactor performance. Several engineering techniques were employed to enhance the H\textsubscript{2} production potential using mixed anaerobic cultures fed substrates derived from agricultural residues such as switchgrass (SWG) and corn stover (CS). In addition, statistical tools were used to understand the impact of experimental factors (operational parameters) on the dependent response variables which included the fermentation byproducts and the microbial community structure.

Experimental studies were initiated with glucose to assess the continuous fermentative H\textsubscript{2} production using LA inhibited flocculated and granulated cultures in UASBRs (Chapter 4). The reactors containing flocculated and granulated cultures showed similar H\textsubscript{2} production rates (HPRs) when long HRTs were applied to the fermentation systems (Table 4.2). The untreated cultures produced methane (CH\textsubscript{4}) with yields ranging from 0.3 mol mol\textsuperscript{-1} glucose to 1.2 mol mol\textsuperscript{-1} glucose, even at conditions under lower pH (5.0) levels (Figure 4.1). Under these conditions \textit{Methanospirillum hungatei} and \textit{Methanobacterium palustre} were abundant. A H\textsubscript{2} yield of 2.65±0.45 mol mol\textsuperscript{-1} glucose and 2.46±0.10 mol mol\textsuperscript{-1} glucose (approximate) was observed in LA treated flocculated and granulated cultures, respectively operating at a 24 h HRT (Figure 4.1). These results are contradictory from those reported by Saady et al. (2012), where the authors reported relatively low H\textsubscript{2} yields in LA treated granulated cultures fed glucose in batch reactors.

Adding LA induced a shift in metabolic pathway towards H\textsubscript{2}-acetic acid (HAc)-butyric acid (HBu) type of fermentation from the CH\textsubscript{4} type of fermentation in control cultures (Figure 4.2 and Figure 4.3). In addition to H\textsubscript{2} production, the granular and flocculated cultures showed LA degradation in continuous scale reactors. The effects of LA on the fermentation type were confirmed by statistical analysis of metabolite data using principal component analysis (PCA). Separate clusters of control cultures associated with CH\textsubscript{4} and ethanol (EtOH) and the LA treated cultures associated with H\textsubscript{2}.
and HAc were observed in the bi-plot (Figure 4.5). Granular cultures both untreated and LA treated showed better retention of biomass granules (Figure 4.8). The granules were enriched with *Enterococcus* sp., *Clostridium* sp., *Bacteroides* sp. and *Eubacterium* sp.. Biomass wash-out was observed with flocculated cultures fed LA and operating at low HRT. Under these conditions, the relative abundance of *Clostridium* sp. and *Enterococcus* sp. increased. Studies by Ren et al. (2007) and Wirth et al. (2012) have indicated that species belonging to *Clostridium*, *Enterococcus* and *Bacteroides* sp. were dominant in H₂ producing communities operated in the full-scale reactors. In general, stable performance, high biomass retention and high diversity of the microflora are likely primarily responsible for selecting granular cultures over flocculated cultures (Figures 4.1, 4.6 and 4.8).

In Chapter 5, the effect of HRT and OLR on H₂ production and reactor performance was examined using mixed cultures fed glucose at pH 5.0±0.2. Studies with varying OLR at the same HRT (24 h) using untreated mixed consortia in stage I revealed that increasing the OLR decreased CH₄ production and increased the H₂ yield (Figure 5.1). This is in agreement with previously reported studies which showed suppressed methanogenesis with increasing substrate loading (Spagni et al., 2010). The maximum H₂ yield 1.70±0.05 mol mol⁻¹ glucose in stage I was observed at an OLR of 8 g L⁻¹ d⁻¹ (Table 5.2). The dynamic changes in the microbial profile were established by varying the OLR while maintaining a constant HRT (24 h). The analysis revealed ≥ 90% *Propionibacteriaceae* and *Synergistaceae* were suppressed while the increasing *Clostridiaceae* and *Ruminococcaceae* levels suggested that an increase in substrate loading caused an elimination of non-H₂ producers without affecting the existing H₂ producing population (Figure 5.9). Work presented by Liu et al. (2012) is consistent with data from the present study where increasing the OLR increased the relative abundance of *Clostridiaceae* and *Ruminococcaceae* in a carrier based H₂ producing consortia operating in an UASBR fed with glucose. According to Liu et al. (2012), the major fermentation metabolites produced by *Clostridiaceae* and *Ruminococcaceae* includes HAc and EtOH, while in the current study, HBu was observed in addition to HAc and EtOH (Figure 5.4a).
In stage II, reducing the HRT (with corresponding increases in OLR) was associated with increased H$_2$ productivity (Figure 5.2). Hydrogen yield ranging from 2.1 to 2.5 mol mol$^{-1}$ glucose was maintained during the HRTs ranging from 12 to 20 h with no appreciable CH$_4$ produced during HRTs shorter than 16 h. This suggests that the optimal HRT is within 12 to 16 h (Figure 5.2, Table 5.2). Note the high HPRs observed were associated with low HRTs (<5 h). A maximum HPR of $15.4 \pm 1.4$ L L$^{-1}$ d$^{-1}$ and a H$_2$ yield of $1.29 \pm 0.04$ mol mol$^{-1}$ were observed at a 1.5 h HRT and an OLR of 96 g L$^{-1}$ d$^{-1}$ (Figure 5.2, Table 5.2). These conditions suggest that operating at HRTs ranging from 12 to 20 h resulted in increasing the H$_2$ yield, while decreasing HRT to < 5 h showed a negative effect on the H$_2$ yield. The H$_2$ yields obtained from this study is greater than the yield obtained in continuous reactor systems operated with glucose as the substrate (Lin and Chang, 2004; Zhang et al., 2007).

The flux model indicates that the H$_2$ flux directed towards consumption (H$_2$-methanogenic flux and H$_2$-acetogenic flux) decreased with an increase the OLR during both stage I and II (Figure 5.5). Decreasing the HRT to 16 h with a gradual increase in the OLR induced a shift in the bacterial community. The dominant (relative abundance) microorganisms detected under these conditions and belonging to the class Clostridia included Ethanoligenens sp., Clostridium sp., Alkaliphilus sp., Butyrivibrio sp., Moorella thermoacetica and Parabacteroides sp., (Table 5.3). Mixed acid and alcohol fermentation together with H$_2$ production was observed in the presence of these microorganisms. A decrease in HRT to 5 h resulted in the elimination of Bacteroidetes along with an increase in Clostridia (comprising Clostridium sp., Ethanoligenens sp., Thermaanaerovibrio sp. and Alkaliphilus sp.) which was responsible for increasing the quantity of EtOH produced. Increasing EtOH production with increasing OLR was confirmed with increasing electron equivalents diverted to EtOH (Figure 5.4a and b).

The PCA revealed that changes in the fermentation pattern were linked with changes in OLR and HRT. Cultures operating under low and high OLRs were associated with CH$_4$ and HPr and H$_2$, HAc and EtOH, respectively, with cultures under the two operating conditions clustered into separate groups (Figure 5.7).

Adding LA to methanogenic granular cultures (stage III) fed glucose and operating at HRTs from 12 h to 6 h with OLRs corresponding to 16 g L$^{-1}$ d$^{-1}$ to 32 g L$^{-1}$ d$^{-1}$ resulted in
increasing H₂ production in comparison to the untreated control cultures operating a in similar range. In all of the LA treated conditions, a H₂ yield ≥ 2.0 mol mol⁻¹ glucose was observed, with HPRs reaching up to 9.2±1.4 L L⁻¹ d⁻¹ (Table 5.2 and Figure 5.3). The outcomes of this study also suggest that the inoculum pretreatment with a methanogenic inhibitor, such as LA, facilitated the diversion of electron fluxes to H₂ through HAc-HBu type fermentation (Table 5.2 and Figure 5.3). This strategy ultimately led to improving the H₂ yields to ≥ 2.5 mol mol⁻¹ glucose. Overall, the findings indicate that reactors operating at HRTs below 20 h and OLRs ≥ 10 g L⁻¹ d⁻¹ are preferred for enhanced H₂ production, while pretreatment of inoculum with LA is essential for complete suppression of H₂ consumption and H₂ yields ≥ 2.0 mol mol⁻¹ glucose.

The preliminary studies with batch reactors fed synthetic lignocellulosic compounds containing glucose plus furan (furfural and hydroxyl methylfurfural (HMF)) revealed that furans were able to suppress the methanogenesis to a limited extent (Chapter 6). However, note complete methanogenic suppression was accomplished by the addition of LA. Hydrogen yields observed in the LA treated (2 g L⁻¹) cultures fed substrate containing 1 g L⁻¹ of furan (comprising furfural and HMF) reached 1.82±0.25 mol mol⁻¹ glucose (Figure 6.1a). Relative to the maximum yield obtained with pure glucose and furans (1 g L⁻¹), increasing the furan levels to LA inhibited cultures decreased the H₂ yield by 40±5%. In the case of untreated cultures fed lignocellulosic hydrolysate (steam exploded corn stover (CS)), H₂ yields of up to 1.7±0.2 mol mol⁻¹ hexose were obtained (Figure 6.1b). However, the maximum H₂ yield of 2.25 ± 0.17 mol mol⁻¹ hexose equivalents was observed in LA treated cultures fed resin treated hydrolysate (i.e., hydrolysate treated with polymeric adsorbent resin to reduce the levels of microbial inhibitors, such as furans and phenolic compounds). Mussatto and Roberto (2004), emphasized the need for reducing furan levels in lignocellulosic hydrolysate to enhance the fermentation process. Overall, the results suggest that furan levels less than 1 g L⁻¹ are preferred in combination with the addition of LA in order to obtain H₂ yields greater than 2.0 mol mol⁻¹ glucose. The H₂ yields obtained in this study is greater than or equal to those obtained by other pretreated cultures fed acid hydrolysed substrates (Fangkum and Reungsang, 2011; Yang et al., 2010). The H₂ yield reported by Fangkum and Reungsang (2011) and Yang et al. (2010) were 1.5 and 2.05 mol mol⁻¹ glucose, respectively.
The PCA data revealed that the action of furans on fermentation shifted the metabolism towards solventogenesis in order to relieve the stress imposed on the microorganisms (Figure 6.3). The dominant microbial populations which included *Clostridium* sp. and *Flavobacterium* sp. were capable of degrading furans to less toxic compounds, and producing H$_2$ as well, under the operating conditions examined. The cluster analysis indicated that grouping of the clades was based on the initial concentration of the fermentation inhibitors in the fermenting media (Figure 6.7).

Optimization of process parameters using response surface methodology (RSM) for enhanced H$_2$ production from mixed sugar hydrolysate in continuous systems (UASBRs) revealed optimal parameter levels of 10.8 h HRT, pH 5.0 and LA concentration of 1.75 g L$^{-1}$. Under these conditions, for a resin treated hydrolysate of steam-exploded switchgrass (SWG) and an influent feed concentration of 5 g COD L$^{-1}$, the H$_2$ yield was 100±6.0 mL H$_2$ g$^{-1}$ TVS (303±20 mL g COD$^{-1}$ (approximately 65% of the theoretical maximum)), respectively (Figure 7.5b). Enhanced H$_2$ production in combination with increased suppression of methanogens (*Methanomicrobia* and *Methanococci*) was obtained by the application of the operating parameters that were closest to the optimum conditions determined by RSM optimization. The byproducts PCA revealed that clustering of low HRT (8 and 12 h) operating conditions were closely associated with the production of H$_2$, HBu and HAc. Methane production was associated with untreated control cultures with pH levels varying from 5.0 to 7.0 (Table 7.1, Figure 7.7). The multivariate cluster analysis based on the similarity of the microbial T-RFs showed high similarity levels between clustering of samples subjected to similar conditions with dominant H$_2$-producing populations of *Clostridiaceae* and *Ruminococcaceae* (Figure 7.8).

The effects of different operational strategies on the mixed consortia for enhancing the H$_2$ yield was examined using HRT, nitrogen sparging and LA treatment (Chapter 8). The results revealed that the application of a combination of the treatment conditions enabled the recovery of more H$_2$ in the gas phase. The different operational strategies used in this study to enhance the H$_2$ yield from resin treated SWG hydrolysate showed that sparging the bioreactor with nitrogen and using LA treated culture allowed stable and enhanced H$_2$ production with yields averaging 2.56±0.10 mol mol$^{-1}$ hexose (Table 8.3). Reducing HRT alone was able to reduce the CH$_4$ yield and improved H$_2$ yield up to 1.5
mol mol\(^{-1}\) hexose (Table 8.3, Figure E2, Appendix E). The yields obtained in the untreated cultures fed SWG hydrolysate was less than those obtained by untreated cultures fed glucose and operated under similar OLRs (Table 8.3 vs Table 5.2). The possible reason for this could be presence of the non-H\(_2\) producing species such as *Propionibacterium* sp. and *Lactobacillus* sp. in the control cultures in addition to *Clostridium* sp. (Table 8.4).

In comparison to the control cultures without sparging, sparging with N\(_2\) reduced H\(_2\) consumption by 60% on average, and increased H\(_2\) productivity by more than 32% with the H\(_2\) yield reaching 2.26±0.11 mol mol\(^{-1}\) hexose at a 6 h HRT (Table 8.3 and Figure 8.3). This increase in H\(_2\) yield was associated with increased hydrogenase evolution specific activity and relative abundance of *Clostridium* sp. (Figures 8.4, 8.6 and Table 8.4). The increase in H\(_2\) yields observed after LA treatment alone showing the dominance of *Clostridium* sp., *Eubacterium* sp. and *Bacteroides* sp., were 15% less than the yields obtained for cultures treated with LA and sparged with N\(_2\). These cultures also showed a 92% (average) reduction in H\(_2\) consumption (Figure 8.3 and Table 8.3). Kim et al. (2006) observed a H\(_2\) yield reaching 1.8 mol mol\(^{-1}\) hexose in CO\(_2\) sparged heat treated cultures which were dominant with *Clostridium* sp. Similar to the work by Kim et al. (2006) observations in this current study suggests that reducing H\(_2\) partial pressure by N\(_2\) sparging lead to increasing the hydrogenase flux and hence, the net H\(_2\) yield (Figure 8.3). In current study, the higher level of hydrogenase evolution specific activity and decreased hydrogenase uptake specific activity observed in LA treated culture sparged with N\(_2\) were correlated with decrease in H\(_2\) consumption flux (Figures 8.3 and 8.4). The dominant species observed under these condition included *Clostridium* sp. and *Bacillus* sp. (Table 8.4).

A close examination of the PCA associated with microbial T-RFs intensity and H\(_2\) yields revealed that LA treated cultures were clustered separately from the control cultures associated with low H\(_2\) yields (Figure 8.6). In addition, the N\(_2\) sparged cultures were grouped in a separate cluster from the control and LA treated cultures. Overall, inoculum pretreatment and lowering the HRT did not maximize the H\(_2\) yields nor suppressed H\(_2\) consumption completely. However, a combination of these factors
together with bioreactor sparging assisted in producing high and stable production rates from the reactor for a period of 30 days.

Long-term H₂ production from pretreated CS was demonstrated in continuous systems (UASBRs) (Chapter 9). Promising H₂ yields of 102±7 mL g⁻¹ TVS (274±40 mL g⁻¹ COD, 2.4 mol mol⁻¹ glucose) were obtained at OLRs ranging from 18 to 24 g COD L⁻¹ d⁻¹ with a 12 h HRT over 20 days (Table 9.2 and Figure 9.2). Upon comparing the outcomes from LA treated cultures, significant differences were noted in H₂ and CH₄ yields. A H₂ yield of 98±11 mL g⁻¹ TVS (281±31 mL g⁻¹ COD, 2.4 mol mol⁻¹ glucose) was obtained at 9 g COD L⁻¹ d⁻¹ after which the H₂ yield decreased with increasing OLRs (Table 9.2 and Figure 9.3). The results suggest that treatment of inoculum is essential at low OLRs for feed containing low inhibitor (furfural and HMF) concentrations. With high loading rates, a combination of LA, furans and phenols cause antagonistic effects, leaving major sugars in the hydrolysate unfermented and/or shifting the metabolic pathway to alcohol production in order to alleviate the stress levels caused by fermentation inhibitors (Table 9.4).

Control cultures not treated with LA were able to metabolize sugars as well as the furans by converting them into less toxic compounds at OLRs reaching 24 g COD L⁻¹ d⁻¹ and producing high H₂ yield. However, at higher OLRs, inhibition of the H₂ producers was observed, along with changes in the fermentation pathway to reduced end products, such as EtOH and propanol (PrOH) (Table 9.3). Note, H₂ yields ≥ 200 mL g⁻¹ COD was observed with control cultures operating at high OLRs (50 g COD L⁻¹) (Figure 9.2).

A canonical correspondence analysis (CCA) was used to examine the relationship between the fermentation byproducts and changes in composition and structure of the microbial community under different conditions (Figure 9.5). The results of the CCA revealed that H₂ production in mixed cultures fed hydrolysate was associated with Clostridium sp., Flavobacterium sp. and Sphingobacterium sp., as well as non-H₂ producing bacteria, such as Eubacterium tenue and Moorella thermoacetica. The high OLRs associated with alcohol production were primarily linked to Clostridium beijerinckii, Thioalkalivibrio sp., Bacteroides sp., and Fusobacterium sp.

Overall, the results of these studies suggest that the operation of reactors at OLRs ranging from 20 g COD L⁻¹ d⁻¹ to 30 g COD L⁻¹ d⁻¹ with HRTs ranging from 6 h to 12 h
is suitable for high substrate conversion efficiency with \( \text{H}_2 \) yields and production rates greater than 2.5 mol mol\(^{-1}\) hexose (glucose) and 9 L L\(^{-1}\) d\(^{-1}\), respectively. However, for low OLRs, pretreatment of the inoculum using biodegradable and renewable resources, such as long chain fatty acids (e.g. the LA used in the current research), is essential. Reduction of the furans in the hydrolysate before feeding the hydrolysate to the culture is essential for operating conditions with loading rates greater than 30 g COD L\(^{-1}\) d\(^{-1}\).

**10.2 Engineering significance and recommendations**

Depleting fossil fuels and increasing concerns over climatic change at the global level has presented \( \text{H}_2 \) as a potential energy source. Hydrogen is not only preferred for its clean and renewable source but it can be utilized by existing energy technologies such as fuel cells and combustion turbines (Demirbas, 2009). At present, the current demands on \( \text{H}_2 \) production are met through steam reforming of natural gas, oils, coal gasification and electrolysis of water (Hay et al., 2013). These technologies are energy intensive and are linked to major economic and environmental concerns. Hence, biological \( \text{H}_2 \) production using fermentative methods is preferred because the technology can utilize naturally occurring microbial cultures, cheap non-sterile agriculture residues and able to operate under relatively low temperature and pressure conditions (Gupta et al., 2013). Fermentative \( \text{H}_2 \) production is preferred because of its ability to feed on variety of feed stocks that is available in plenty such as agricultural residues. The main focus of the work is to use these available agricultural residues for \( \text{H}_2 \) generation. Using lignocellulosic hydrolysate as a substrate offers advantages over pure sugars such as more practical and cost effective to apply on a larger scale and so has a greater potential for commercial application. In Canada approximately 80 million tonnes of switchgrass (SWG) and 75 million tonnes of corn stover (CS) is been harvested on an annual basis (AAFC, 2013; Wright et al., 2009).

In current research biological \( \text{H}_2 \) production from lignocellulosic biomass via dark fermentation was carried out in continuous reactor systems using mixed anaerobic granular cultures. Conclusions from the research findings that have practical implications for future research and development of biofuel production are described in this section.

The initial studies conducted with pure glucose and LA inhibited cultures suggest that the \( \text{H}_2 \) production potential of granular cultures is similar to flocculated cultures. The
results indicate granular culture showed more stable performance and were able to degrade the LCFAs as well. This finding is important because, LA was able to inhibit methanogens in granular cultures as well as in flocculated cultures under the conditions examined. In addition, the ability to degrade LCFAs also suggests that using oleo wastes could be used to inhibit H\textsubscript{2} consumers such as methanogens. This strategy is important in full-scale applications because using oil/lipids waste is economically feasible when compared to using refined vegetable oils or pure LCFAs. Based on studies using LA, further work is required using waste vegetable oils from fried food processes.

Varying HRTs and OLRs suggest that applying low OLRs and low HRTs is beneficial in terms of enhancing the HPR and H\textsubscript{2} yield. Suppressing H\textsubscript{2} consumers at low OLRs could be accomplished by adding LA. Data from this study are significant for full-scale systems because the operating conditions which were identified leads to high H\textsubscript{2} yields and high HPRs. Further, studies are required to confirm the finding of the study using granular as well as flocculated cultures in pilot-scale continuous flow bioreactors.

Optimization of process parameters for H\textsubscript{2} production from lignocellulosic sugars derived from SWG revealed that pH as well as inoculum pretreatment with LA were able to increase H\textsubscript{2} production. Over the range of HRTs tested, the effect on the response variable (H\textsubscript{2} yield) suggests that the pH and LA concentration had a significant effect in comparison to the HRT. This suggest that closer to the optimum HRT the H\textsubscript{2} yield were dependent on the culture conditions such as operating pH and inoculum pretreatment.

Studies conducted to enhance fermentative H\textsubscript{2} production from SWG derived sugars using LA treatment and N\textsubscript{2} sparging reveal that a combined effect is more beneficial than an individual factor. The effects of internal biogas sparging needs to be examined on an intermittent basis as means to increase the removal of H\textsubscript{2} from the liquid phase and thereby increase the H\textsubscript{2} yields. Appropriate gas separation techniques such as membrane separation should be investigated to reduce the partial pressure of H\textsubscript{2} and thereby increase the H\textsubscript{2} productivity.

Data from this research study have shown that agricultural residues such as corn stover and SWG hydrolysate could be used as potential feedstock for H\textsubscript{2} production. Results from this study demonstrated that a corn stover hydrolysate containing threshold level of furans can be used to produce H\textsubscript{2} in a continuously fed reactor without any
pretreatment of the culture. The study also demonstrated that the degradation of inhibitors (furans plus phenols) is possible under H₂ producing conditions.

In addition, results on role of operational parameters for varying H₂ yields from this study will serve as significant base work for pilot-scale studies. The role of microbial dynamics in fermentative H₂ production investigated in this research would assist in selecting suitable operational conditions under which the H₂ yield and HPR are maximized.

The following recommendations should be consider for further developing this technology:

1) Understanding the microbial composition of the initial seed culture may assist in developing suitable approach to control the operational parameters in continuous reactor systems. In addition, simultaneously tracking the dynamic changes in the microflora at each stage of operation might assist in understanding the metabolic shifts associated with operational changes in the reactor system.

2) Combinations of operational strategies should be employed and studied for potential application to the elimination of HPr and lactic acid formed during the fermentation process.

3) Isolation of dominant species observed under different operating stages to study their metabolism under different environmental conditions (e.g. pH and other stress conditions) on a batch scale will likely provide further insight about the different influences on the metabolism of these organisms in mixed anaerobic fermentation.

4) In addition to H₂, alcohols, such as ethanol and butanol are also considered as potential sources for biofuels. Consequently, additional research on alcohol-oriented bioH₂ production should be considered.

5) Additional energy recovery from using the fermentation byproducts from photofermentation processes or microbial fuel cells should be examined.

6) Studies exploring using H₂ and CO₂ as a feed to hydrogenotrophic organisms to produce high molecular compounds such as alcohols and other biomolecule fuels through carbon fixation should be investigated.
10.3 References


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APPENDIX A: CALIBRATION CURVES

Figure A.1 Gas calibration curves based on moles

Figure A.2 Gas calibration curves based on percentage
Figure A.3 Volatile fatty acid calibration on HPLC

Figure A.4 Volatile fatty acid calibration on ion-exchange chromatograph
Figure A.5 Alcohol calibration

Figure A.6 Glucose calibration
Figure A.7 Mixed sugar calibration

Figure A.8 Furan compounds calibration
Figure A.9 Long chain fatty acids calibration curves

Figure A.10 Long chain fatty acids extraction recovery plot
Figure A.11 Cellulose and total sugar calibration using anthrone method

Figure A.12 Reducing sugar calibration using DNSA method
Figure A.13 Phenol calibration

Figure A.14 COD calibration
APPENDIX B: QA/QC RESULTS

B.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. The precision 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 was carried out in the lab by lowering the concentrations in the standard level. In this Appendix, tables or notes have been provided with precision, recovery and detection limits of each instrument.

\[
\text{RPD} = \left( \frac{|S_1 - S_2|}{S_a} \right) \times 100 \quad (B.1.1)
\]

where:

- \( S_1, S_2 \) = observed sample values
- \( S_a \) = mean of the observed sample values

\[
\%R = \left( \frac{S_p - S_a}{S_k} \right) \times 100 \quad (B.1.2)
\]

where:

- \( S_p \) = measured value (area) of spiked sample
- \( S_a \) = average of the observed sample values
- \( S_k \) = know value of the standard spiked

Notes:
1. A shift in calibration curve and detection limits was observed, with change in column or detector etc., of the instrument. For which, 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.
B.2 Elemental analysis-CHNS

The elemental analysis was conducted at Department of Chemistry and Bio-Chemistry, University of Windsor, Ontario. The instrument specifications list: accuracy ≤ 0.3% and precision ≤ 0.2% with Helium as carrier gas. The detection limits were less than 0.5% for CHNS with a sample volume of 2 mg.

B.3 Gas Chromatograph

The calibration curve or standard curve had less than 5% deviation over the period of research conducted. The detection limits were 0.0032 kPa (5 µL per 160 mL) for H₂ and 0.0064 kPa (10 µL per 160 mL) for CH₄.

The LCFA recovery is shown in Appendix A (Fig. A 10). The graph shows the extraction efficiency of each fatty acid. The detection limits for the LCFAs were 30 mg L⁻¹ for an injection volume of 2 µL.

B.4 High Performance liquid chromatograph

Table B.1 HPLC-VFA method-QA/QC results

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Spiked concentration (mg L⁻¹)</th>
<th>Percent Recovery (%R)</th>
<th>RPD (%)</th>
<th>DL (mg L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate</td>
<td>20</td>
<td>94.06</td>
<td>5.28</td>
<td>2.0</td>
</tr>
<tr>
<td>Acetate</td>
<td>20</td>
<td>85.76</td>
<td>6.64</td>
<td>2.0</td>
</tr>
<tr>
<td>Propionate</td>
<td>20</td>
<td>94.09</td>
<td>10.83</td>
<td>2.0</td>
</tr>
<tr>
<td>Formate</td>
<td>20</td>
<td>116.65</td>
<td>18.18</td>
<td>2.0</td>
</tr>
<tr>
<td>Butyrate</td>
<td>20</td>
<td>106.34</td>
<td>6.72</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Table B.2 HPLC-Furan method-QA/QC results

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Spiked concentration (mg L⁻¹)</th>
<th>Percent Recovery (%R)</th>
<th>RPD (%)</th>
<th>DL (mg L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Furfural</td>
<td>50</td>
<td>106.50</td>
<td>2.85</td>
<td>0.5</td>
</tr>
<tr>
<td>HMF</td>
<td>50</td>
<td>100.83</td>
<td>1.95</td>
<td>0.5</td>
</tr>
<tr>
<td>Furoic acid</td>
<td>50</td>
<td>92.58</td>
<td>3.59</td>
<td>1.0</td>
</tr>
<tr>
<td>Furyl alcohol</td>
<td>50</td>
<td>102.13</td>
<td>1.38</td>
<td>1.0</td>
</tr>
</tbody>
</table>
### B.5 Ion-exchange chromatograph

#### Table B.3 IC-Alcohol and glucose method-QA/QC results

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Spiked concentration (mg L(^{-1}))</th>
<th>Percent Recovery (%R)</th>
<th>RPD (%)</th>
<th>DL (mg L(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>i-Propanol</td>
<td>50</td>
<td>98.28</td>
<td>1.45</td>
<td>8</td>
</tr>
<tr>
<td>Ethanol</td>
<td>50</td>
<td>98.68</td>
<td>6.34</td>
<td>8</td>
</tr>
<tr>
<td>Propanol</td>
<td>50</td>
<td>109.65</td>
<td>14.78</td>
<td>8</td>
</tr>
<tr>
<td>i-Butanol</td>
<td>50</td>
<td>95.68</td>
<td>9.61</td>
<td>8</td>
</tr>
<tr>
<td>Butanol</td>
<td>50</td>
<td>93.56</td>
<td>10.65</td>
<td>8</td>
</tr>
<tr>
<td>Glucose</td>
<td>50</td>
<td>95.46</td>
<td>5.83</td>
<td>1</td>
</tr>
</tbody>
</table>

#### Table B.4 IC-Sugar method-QA/QC results

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Spiked concentration (mg L(^{-1}))</th>
<th>Percent Recovery (%R)</th>
<th>RPD (%)</th>
<th>DL (mg L(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>100</td>
<td>96.91</td>
<td>5.41</td>
<td>1</td>
</tr>
<tr>
<td>Xylose</td>
<td>100</td>
<td>99.62</td>
<td>7.96</td>
<td>1</td>
</tr>
</tbody>
</table>

### B.6 Chemical methods

#### Table B.5 Chemical methods-QA/QC results

<table>
<thead>
<tr>
<th>Method</th>
<th>Spiked Analyte</th>
<th>Spiked concentration (mg L(^{-1}))</th>
<th>Percent Recovery (%R)</th>
<th>RPD (%)</th>
<th>DL (mg L(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNSA (Reducing sugar)</td>
<td>Glucose (G)</td>
<td>50</td>
<td>96.35</td>
<td>5.40</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>Xylose (X)</td>
<td>86.09</td>
<td>92.72</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>G+X</td>
<td>92.72</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anthrone (Total sugar)</td>
<td>Glucose (G)</td>
<td>20</td>
<td>89.02</td>
<td>4.44</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Xylose (X)</td>
<td>84.07</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>G+X</td>
<td>91.58</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COD</td>
<td>Glucose</td>
<td>100</td>
<td>94.20</td>
<td>7.85</td>
<td>100</td>
</tr>
<tr>
<td>Phenol</td>
<td>Catechol</td>
<td>40</td>
<td>94.88</td>
<td>0.85</td>
<td>10</td>
</tr>
</tbody>
</table>
APPENDIX C: SAMPLE CALCULATIONS

C.1 Gas calculation

Batch studies

The calibration curve equation for H₂ is y=4E+11x.

where:
y= area under the peak
x= gas concentration (moles)

For an area under the curve = 56898 and 11.4 psi pressure in 160 mL bottle with 50 mL working volume, the H₂ yield from 5000 mg L⁻¹ of glucose is shown below.

\[
\text{moles of H}_2 \text{ detected} = \frac{56898}{4E + 11} = 1.422E - 07
\]

\[
\text{Pressure conversion} = \frac{(14.7 + 11.4) \text{ psi}}{14.7 \text{ psi/ atm}} = 1.78 \text{ atm}
\]

Note: 14.7 psi (or atmospheric pressure) was added to the pressure readings measured in the batch reactor, for the pressure meter is calibrated to zero at atmospheric pressure.

\[
\text{Head space correction} = \frac{(110)\text{mL}}{0.025\text{mL}} = 4400
\]

Note: Head pace correction is incorporated in the calculation to represent the actual mass (moles) produced in the batch system. 110 mL represents the head space volume ((160-50) mL) and 25 µL is the injection volume which is represented as mL.

\[
\text{mole of H}_2 \text{ produced in 160mL bottle} = (\text{moles of H}_2 \text{ detected}) \times \text{pressureconversion} \times \text{Head space correction}
\]

\[
\text{mole of H}_2 \text{ produced in 160mL bottle} = (1.422E - 07) \times (1.78) \times (4400) = 1.137E - 03 \text{molesbottle}^{-1}
\]

\[
\text{µmole of H}_2 \text{ produced in 160mL bottle} = (1.137E - 03 \text{molesbottle}^{-1}) \times 10E6 \frac{\text{µmole}}{\text{mole}} = 1137 \frac{\text{µmole}}{\text{bottle}}
\]
Yield calculation:

As stated earlier for 5000 mg L\(^{-1}\) of glucose, the H\(_2\) yield is calculated below:

\[
\text{amount of glucose present in bottle} = 5000 \frac{\text{mg}}{L} \times (50E - 3)L \times \left(\frac{1 \text{ mmol}}{180 \text{ mg}}\right) \times \left(\frac{1000 \mu\text{mol}}{1 \text{ mmol}}\right) = 1388.89 \frac{\mu\text{mol of glucose}}{\text{bottle}}
\]

\[
\text{H}_2 \text{ yield per mole of glucose} = \frac{1137 \frac{\mu\text{mol } H_2}{\text{bottle}}}{1388 \frac{\mu\text{mol glucose}}{\text{bottle}}} = 0.82 \frac{\text{mol } H_2}{\text{mol glucose}}
\]

Continuous studies

The calibration curve equation for H\(_2\) is \(y=3946.5x\).

where:
y= area under the peak
x= gas concentration (% basis)

Note: Gas calibrations were conducted by injecting a known volume of desired gas (H\(_2\) or CH\(_4\)) into a 160 ml serum bottle filled with nitrogen (N\(_2\)). The gas chromatography area count is a function of ml of gas per 160 ml bottle, which is converted to a percent basis.

For an area under the curve = 152341 and 1000 count in the gas counter, the H\(_2\) yield from 5000 mg L\(^{-1}\) of glucose; H\(_2\) production rate (HPR) at an HRT of 8 h is shown below:

\[
\% \text{ of } H_2 \text{ measured in GC} = \frac{152341}{3946.5} = 38.60\%
\]

\[
\text{mL of biogas produced in the system} = (1000*15) \text{ mL} = 15000 \text{ mL}
\]

where: 15 represents the mL per count in the gas counter (calibrated on a weekly basis)

\[
\text{Std. biogas volume} = \left(\frac{15000 \text{mL} \times 273.15 \text{ K} \times 1 \text{ atm}}{290.15 \text{K} \times 1 \text{ atm}}\right) = 14120 \text{ mL}
\]

where: 273.15 K represents the standard temperature, 1 atm is the pressure at standard condition, 290.15 K represents the temperature of the gas meter from which gas sampling is collected, 1 atm is the pressure inside the gas meter.

\[
\text{mL of } H_2 \text{ produced} = (14120 \times 38.06/100) = 5450 \text{ mL } H_2
\]
HPR = \left( \frac{5.45L}{8.5L} \right) \times \left( \frac{1}{8h} \right) \times \left( \frac{24h}{1day} \right) = 1.92 \text{ L/day}

(Note: 8 h corresponds to the HRT and 8.5 L correspond to working volume of the reactor)

moles of H_2 produced = \frac{5450}{22400} = 0.243 \text{ mol of H}_2

(Note: 22400 mL represents the standard volume occupied by any gas of 1 mole).

Glucose fed to reactor = \left( \frac{5000mg}{L} \right) \times 8.5L \times \left( \frac{1mmol}{180mg} \right) \times \left( \frac{1mol}{1000mmol} \right) = 0.236 \text{ mol glucose}

H_2 yield = \frac{0.243 \text{ mol H}_2}{0.236 \text{ mol glucose}} = 1.02 \text{ mol H}_2 \text{ mol}^{-1} \text{ glucose}

(Note: Same calculation is applicable for CH}_4\text{ yield and CH}_4\text{ production rate as well, with the corresponding slope of CH}_4\n
C.2 Analyte concentration

A sample calculation for an analyte (glucose) is shown here. The calculation is applicable for all analytes such as VFAs, alcohols, furans, sugars and phenols used in chemical or analytical methods.

For a glucose peak area of 52.654 nC.min, the concentration calculation is shown below:

The calibration curve equation y = 0.2114x

where: y (nC. min) = area under the peak

x = concentration in mg L\(^{-1}\)

\[
\text{Gluocse concentration} = \frac{\text{peak area}}{\text{slope of curve}} \times \text{dilution factor}
\]
\[
\text{Gluocse concentration} = \frac{52.654}{0.2114} \times 15 = 3736 \text{mg L}^{-1}
\]

**Note:** The dilution factor used for analyzing the sample in the instrument = 15.

### C.3 Electron balance

The electron balance takes into account the available electron in the feed solution (sugar solution) and the products produced in the fermentation system (both gaseous and liquid byproduct). The electron balance also assumes 10% present in the initial feed are synthesized in the biomass. The number of electron equivalents available for the fermentation is derived from the half reactions. **Table C.1** represents the electron balance performed from the experimental results obtained in **Chapter 5**.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Mol. wt. (g mmol(^{-1}))</th>
<th>Concentration of the analyte (mg L(^{-1}))</th>
<th>mmol</th>
<th>Electron equivalents per mol (e(^{-}) equiv mol(^{-1}))</th>
<th>e(^{-}) equiv output</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose in feed*</td>
<td>180</td>
<td>8000</td>
<td>377</td>
<td>24</td>
<td>9.05</td>
</tr>
<tr>
<td>(\text{H}_2)**</td>
<td>1.01</td>
<td>618.28</td>
<td>618.28</td>
<td>2</td>
<td>1.23</td>
</tr>
<tr>
<td>(\text{CH}_4)**</td>
<td>16.04</td>
<td>41.47</td>
<td>41.47</td>
<td>8</td>
<td>0.33</td>
</tr>
<tr>
<td>Lactate</td>
<td>89.07</td>
<td>185.50</td>
<td>17.77</td>
<td>12</td>
<td>0.25</td>
</tr>
<tr>
<td>Acetate</td>
<td>59.04</td>
<td>1280.05</td>
<td>184.42</td>
<td>8</td>
<td>1.73</td>
</tr>
<tr>
<td>Propionate</td>
<td>73.07</td>
<td>312.11</td>
<td>36.34</td>
<td>14</td>
<td>0.60</td>
</tr>
<tr>
<td>Butyrate</td>
<td>45.02</td>
<td>1020.93</td>
<td>99.75</td>
<td>20</td>
<td>1.99</td>
</tr>
<tr>
<td>i-Propanol</td>
<td>60.01</td>
<td>174.89</td>
<td>17.09</td>
<td>18</td>
<td>0.32</td>
</tr>
<tr>
<td>Ethanol</td>
<td>46.07</td>
<td>305.20</td>
<td>29.83</td>
<td>12</td>
<td>0.36</td>
</tr>
<tr>
<td>Residual glucose</td>
<td>180</td>
<td>680</td>
<td>27.24</td>
<td>24</td>
<td>0.77</td>
</tr>
<tr>
<td>Biomass</td>
<td></td>
<td></td>
<td></td>
<td>0.91</td>
<td></td>
</tr>
<tr>
<td>Sum</td>
<td></td>
<td></td>
<td></td>
<td>8.49</td>
<td></td>
</tr>
<tr>
<td>Percent balance</td>
<td></td>
<td>[\left(\frac{\text{Initial } e^{- \text{ equiv}}}{\text{final } e^{- \text{ equiv}}}\right)^*100 = \left(\frac{8.49 * 100}{9.05}\right) = 93.82%]</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(e^{-}\) equiv = electron equivalent

* \(e^{-}\) equiv in the feed

** \(\text{H}_2\) and \(\text{CH}_4\) are expressed on mmol
C.4 COD balance

The COD balance is performed similar to that of the electron balance, which is based on the COD equivalents. COD balance is performed in Chapters 6.3 and 8.0, where agricultural waste material is used as the feed, comprising a mixture of sugars, furan and acetate as the major carbon source. However, in Chapter 7.0 the data is calculated based on hexose equivalents, showing the electron balance. The Table C.2, shows a sample COD balance performed from the results obtained from Chapter 7.

Table C.2 Model calculation of the COD balance

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Bio-gas yield (L g⁻¹COD)</th>
<th>Concentration of the analyte (mg L⁻¹)</th>
<th>COD conversion factor</th>
<th>COD concentration (mg L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>COD in feed*</td>
<td>-</td>
<td>5000</td>
<td>1.00</td>
<td>5000</td>
</tr>
<tr>
<td>H₂ᵃ</td>
<td>0.17</td>
<td>76.25</td>
<td>8.00</td>
<td>610</td>
</tr>
<tr>
<td>CH₄ᵇ</td>
<td>0.06</td>
<td>237</td>
<td>4.00</td>
<td>948</td>
</tr>
<tr>
<td>Lactate</td>
<td>-</td>
<td>74.56</td>
<td>1.07</td>
<td>79.78</td>
</tr>
<tr>
<td>Acetate</td>
<td>-</td>
<td>829.61</td>
<td>1.07</td>
<td>887.69</td>
</tr>
<tr>
<td>Propionate</td>
<td>-</td>
<td>250.73</td>
<td>1.51</td>
<td>378.61</td>
</tr>
<tr>
<td>Butyrate</td>
<td>-</td>
<td>374.68</td>
<td>1.82</td>
<td>678.17</td>
</tr>
<tr>
<td>i-Propanol</td>
<td>-</td>
<td>ND</td>
<td>2.40</td>
<td>-</td>
</tr>
<tr>
<td>Ethanol</td>
<td>-</td>
<td>305.20</td>
<td>2.09</td>
<td>148.02</td>
</tr>
<tr>
<td>Residual glucose</td>
<td>-</td>
<td>709.63</td>
<td>1.07</td>
<td>752.52</td>
</tr>
<tr>
<td>Biomassᵇ</td>
<td>-</td>
<td>503.91</td>
<td>1.28</td>
<td>645.01</td>
</tr>
<tr>
<td>Sum</td>
<td></td>
<td></td>
<td></td>
<td>5127.51</td>
</tr>
</tbody>
</table>

Percent balance: \( \left( \frac{\text{final COD equiv}}{\text{initial COD equiv}} \right) \times 100 = \left( \frac{5128 \times 100}{5000} \right) = 102.56\% \)

* COD equiv in the feed
ᵃ Calculated value from oxidation equation of the reported biomass formula.
b ²H₂ and CH₄ COD equivalent calculation see Chapter 6.3
C.5 Standard Deviation

The standard deviation between the data sets was calculated using the following formula:

\[
\sigma = \sqrt{\frac{\sum (x - \bar{x})^2}{N - 1}}
\]

where:

\( \sigma \) = the standard deviation
\( x \) = each value in the data set
\( \bar{x} \) = mean value of the data set
\( N \) = number of values used for calculating standard deviation in the data set

For example, for the calculating concentration of acetate from the reactor operation at completion one HRT at a particular condition

Reactor 1 the values are= 858.61 and 892.63 mg L\(^{-1}\)
Reactor 2 the values are= 942.87 and 1025.68 mg L\(^{-1}\)

Here, we have 2 values from each reactor, summing to N=4.

\[
\bar{x} = \frac{858.61 + 892.63 + 942.87 + 1025.68}{4} = 929.94
\]

\[
\sigma = \sqrt{\frac{\sum (858.61 - 929.94)^2 + (892.63 - 929.94)^2 + (942.87 - 929.94)^2 + (1025.68 - 929.94)^2}{4 - 1}}
\]

\( \sigma = 72.60 \)
APPENDIX D: ADDITIONAL DATA

D.1 Solid retention time

The sludge or solid retention time (SRT) was calculated using the equation D.1.

\[ SRT = \frac{V_r \times X_r}{Q_e \times X_e} \]  \hspace{1cm} (D.1)

Where, \( V_r \) and \( X_r \) were defined as effective volume of reactor and microorganism concentration in the reactor. \( Q_e \) and \( X_e \) are flow rate and microorganism concentration in the effluent of the reactor.

Based on the effluent concentration of the biomass and HRT outlined in Chapter 4 and 9, the SRT was calculated and presented in Table D1 and D2, respectively.

<table>
<thead>
<tr>
<th>HRT (h)</th>
<th>Flocculated - control</th>
<th>Flocculated LA</th>
<th>Granulated - control</th>
<th>Granulated LA</th>
</tr>
</thead>
<tbody>
<tr>
<td>48</td>
<td>59±8</td>
<td>34±3</td>
<td>80±10</td>
<td>71±6</td>
</tr>
<tr>
<td>36</td>
<td>37±3</td>
<td>22±2</td>
<td>48±4</td>
<td>47±5</td>
</tr>
<tr>
<td>24</td>
<td>20±2</td>
<td>13±1</td>
<td>29±4</td>
<td>26±2</td>
</tr>
</tbody>
</table>

Notes:
1. LA: linoleic acid; HRT: hydraulic retention time and SRT: solid retention time
2. SRT was calculated based on the biomass washed out of reactors R1 and R2 using equation D.1. The SRT was calculated from triplicate values from each reactor
Table D.2 Solid retention time for the cultures fed with corn stover hydrolysate and operating in UASBRs

<table>
<thead>
<tr>
<th>Stage</th>
<th>HRT (h)</th>
<th>OLR (g COD L(^{-1}) d(^{-1}))</th>
<th>SRT (h)</th>
<th>Control</th>
<th>LA treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>24</td>
<td>5.4</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>II</td>
<td>12</td>
<td>2.7</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>III</td>
<td>12</td>
<td>3</td>
<td>618±75</td>
<td>527±61</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>12</td>
<td>6</td>
<td>621±56</td>
<td>463±69</td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>12</td>
<td>9</td>
<td>556±80</td>
<td>391±41</td>
<td></td>
</tr>
<tr>
<td>VI</td>
<td>12</td>
<td>12</td>
<td>485±60</td>
<td>431±60</td>
<td></td>
</tr>
<tr>
<td>VII</td>
<td>12</td>
<td>18</td>
<td>497±56</td>
<td>398±54</td>
<td></td>
</tr>
<tr>
<td>VIII</td>
<td>12</td>
<td>24</td>
<td>530±54</td>
<td>333±47</td>
<td></td>
</tr>
<tr>
<td>IX</td>
<td>8</td>
<td>36</td>
<td>315±40</td>
<td>212±27</td>
<td></td>
</tr>
<tr>
<td>X</td>
<td>6</td>
<td>50</td>
<td>215±26</td>
<td>NA</td>
<td></td>
</tr>
</tbody>
</table>

Notes:
1. Operating conditions at different stages are applicable to both control and LA treated cultures
2. Stages 1 and 2 were fed with mixture of glucose, xylose and corn stover (CS) hydrolysate (1:1:1) and from stage 3 100% CS was used up for the feed
3. Experiment with LA lasted for a period of 75 days only (i.e., stage IX)
4. LA: linoleic acid; HRT: hydraulic retention time; OLR: organic loading rate, SRT: solid retention time, NA: not applicable
5. SRT was calculated based on the biomass washed out of reactors R1 and R2 using equation D.1. The SRT was calculated from triplicate values from each reactor
Figure E.1 Operation parameters and soluble metabolite profiles in continuous H₂ production using glucose as substrate during stage 1 (increasing OLR at constant HRT (24 h)) and stage 2 (increasing OLR with decreasing HRT) in Chapter 5

Notes:
1. HRT: hydraulic retention time; OLR: organic loading rate
2. The metabolite concentration plotted shows average of 4 samples (2 from each reactor R1 and R2) and the error bars represent the standard deviation.
3. HAc: acetate; HBu: butyrate; HPr: propionate; EtOH: ethanol and i-PrOH: iso-propanol
Figure E.2 Variations in operating parameters and hydrogen and methane yields under non-sparged conditions for control and LA treated cultures

Notes:
1. HRT: hydraulic retention time; OLR: organic loading rate
2. C: Control cultures without addition of linoleic acid and LA: linoleic acid fed culture.
3. The H₂ and CH₄ yields plotted shows average values for duplicate reactors R1 and R2.
4. The data is supplementary data to Chapter 8.
Figure E.3 Variations in operating parameters and hydrogen and methane yields under nitrogen-sparged conditions for control and LA treated cultures

Notes:
1. HRT: hydraulic retention time; OLR: organic loading rate
2. C: Control cultures without addition of linoleic acid and LA: linoleic acid fed culture.
3. The H$_2$ and CH$_4$ yields plotted shows average values for duplicate reactors R1 and R2
4. The data is supplementary data to Chapter 8.
APPENDIX F: COMPOSITION OF THE LIGNOCELLULOSIC BIOMASS AND PRETREATED LIQUOR

Table F.1 Chemical composition of lignocellulosic biomass

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Lignocellulosic biomass (wt% on dry basis)</th>
<th>Switchgrass (SWG)</th>
<th>Corn stover (CS)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before pre-treatment*</td>
<td>After pre-treatment*</td>
<td>Before pre-treatment*</td>
</tr>
<tr>
<td>Proximate analysis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moisture</td>
<td>3.64±0.46</td>
<td>16.48±0.78</td>
<td>0.57±0.13</td>
</tr>
<tr>
<td>Ash</td>
<td>4.63±0.43</td>
<td>4.1±0.31</td>
<td>4.35±0.24</td>
</tr>
<tr>
<td>Volatile solids</td>
<td>93±03</td>
<td>NP</td>
<td>94±02</td>
</tr>
<tr>
<td>Elemental analysis b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbon</td>
<td>47.33</td>
<td>30.53</td>
<td>46.14</td>
</tr>
<tr>
<td>Hydrogen</td>
<td>5.70</td>
<td>2.86</td>
<td>5.53</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>0.28</td>
<td>0.27</td>
<td>0.33</td>
</tr>
<tr>
<td>Sulfur</td>
<td>1.39</td>
<td>0.83</td>
<td>1.42</td>
</tr>
<tr>
<td>Oxygen c</td>
<td>45.30</td>
<td>27.52</td>
<td>46.58</td>
</tr>
<tr>
<td>Biochemical analysis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cellulose</td>
<td>45.58±5.00</td>
<td>40.30±4.96</td>
<td>38.78±5.63</td>
</tr>
<tr>
<td>Acid detergent fiber (ADF)</td>
<td>33.09±4.94</td>
<td>30.69±5.86</td>
<td>34.42±7.33</td>
</tr>
<tr>
<td>Nuteral detergent fiber (NDF)</td>
<td>68.31±5.35</td>
<td>35.65±4.68</td>
<td>78.15±4.31</td>
</tr>
<tr>
<td>Hemicellulose d</td>
<td>35.22±5.15</td>
<td>4.96±1.00</td>
<td>43.73±3.52</td>
</tr>
<tr>
<td>Lignin</td>
<td>18.35±2.62</td>
<td>13.02±1.86</td>
<td>20.61±3.84</td>
</tr>
<tr>
<td>Klason lignin</td>
<td>16.57±0.68</td>
<td>12.85±1.83</td>
<td>18.71±1.01</td>
</tr>
<tr>
<td>Acid soluble lignin</td>
<td>1.78±0.07</td>
<td>NA</td>
<td>1.90±0.15</td>
</tr>
<tr>
<td>Crude protein e</td>
<td>1.75</td>
<td>1.67</td>
<td>2.06</td>
</tr>
</tbody>
</table>

Values are mean ± standard deviation of three replicates
* All composition calculated on the basis of the dry weight of raw material, an exception to this was the moisture content.
  a Moisture content was analyzed for processed and air dried sample
  b Results based on one time analysis
  c oxygen calculated from the composition of remaining (C,H,N and S) constituents
  d hemicellulose = NDF-ADF
  e crude protein = % total N * 6.25
NA- not applicable; NP-not performed
Table F.2 Composition of the liquid hydrolysate obtained from the steam explosion of the lignocellulosic biomass

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Concentration in hydrolysate (g L⁻¹)</th>
<th>Switch grass (SWG)</th>
<th>Corn stover (CS)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Hydrolysate</td>
<td>Hydrolysate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Resin treated</td>
<td>Resin treated</td>
</tr>
<tr>
<td></td>
<td></td>
<td>hydrolysate</td>
<td>hydrolysate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>b</td>
<td>b</td>
</tr>
<tr>
<td>Chemical analytes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total sugar</td>
<td></td>
<td>26.06±1.74</td>
<td>23.16±1.42</td>
</tr>
<tr>
<td>COD</td>
<td></td>
<td>34.5±1.15</td>
<td>27.95±3.25</td>
</tr>
<tr>
<td>Reducing sugar</td>
<td></td>
<td>25.18±1.32</td>
<td>19.18±1.03</td>
</tr>
<tr>
<td>Total phenol</td>
<td></td>
<td>1.3±0.05</td>
<td>0.45±0.07</td>
</tr>
<tr>
<td>BOD₅:COD</td>
<td></td>
<td>0.73±0.13</td>
<td>0.80±0.10</td>
</tr>
<tr>
<td>BODₚ:COD</td>
<td></td>
<td>0.84±0.15</td>
<td>0.86±0.11</td>
</tr>
<tr>
<td>Sugar analysis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arabinose</td>
<td></td>
<td>1.6±0.18</td>
<td>1.48±0.21</td>
</tr>
<tr>
<td>Glucose</td>
<td></td>
<td>1.48±0.18</td>
<td>1.43±0.15</td>
</tr>
<tr>
<td>Xylose</td>
<td></td>
<td>13.62±0.53</td>
<td>11.59±0.84</td>
</tr>
<tr>
<td>Acid and Furanas</td>
<td></td>
<td>0.6±0.08</td>
<td>0.16±0.03</td>
</tr>
<tr>
<td>5-HMF</td>
<td></td>
<td>2.2±0.15</td>
<td>0.68±0.10</td>
</tr>
<tr>
<td>Acetic acid</td>
<td></td>
<td>1.83±0.74</td>
<td>1.15±0.30</td>
</tr>
</tbody>
</table>

Values are mean ± standard deviation of three replicates
a acid catalyzed steam exploded hydrolysate, neutralized and filtered
b ion-exchange (XAD-4) resin treated hydrolysate, neutralized and filtered
c estimated by anthrone method
d estimated by DNSA method
e BOD₅: 5 day biological oxygen demand (BOD); BODₚ: Ultimate BOD
f 5-hydroxymethyl furfural
A,B,C,D,E,F and represents the statistical differences in the mean of the same row at p<0.05.
APPENDIX G: PRINCIPAL COMPONENT ANALYSIS

The bi-plot PCA (Figure 6.7) shown in Chapter 6 was tested for robustness using the Box-plot and it was indicated there were no outliers. Since the data set contains many zeros in case of lactate and methane, log transformation of the original data set was performed in STATISTICA version 8.0. The PCA was then performed using the log transformed data is shown in Figure G.1.

Figure G.1 Principal Component analysis (on the log transformed data) showing the grouping of samples from cultures A and B tested under various conditions based on their gas and liquid metabolites (a) Score plot (b) loading plot

Notes: 1. Only the first and second principal components are shown. 2. The numbers 1 to 6 and ‘C’ represent the experimental conditions in Table 6.1 and the letters ‘A’ and ‘B’ denote the culture source. 3. CH₄ = methane; EtOH = ethanol; HPr = propionate; HLa = lactic acid; i-PrOH = iso-propanol; HAc = acetic acid; HBu = butyric acid.
Table G.1 Factor coordinates of variables based on correlation for cultures fed furans plus glucose

<table>
<thead>
<tr>
<th>Variables</th>
<th>Factor 1</th>
<th>Factor 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>log H₂</td>
<td>-0.76</td>
<td>-0.05</td>
</tr>
<tr>
<td>log CH₄</td>
<td>0.85</td>
<td>-0.27</td>
</tr>
<tr>
<td>log HLa</td>
<td>0.12</td>
<td>0.05</td>
</tr>
<tr>
<td>log HAc</td>
<td>-0.44</td>
<td>0.64</td>
</tr>
<tr>
<td>log HPr</td>
<td>0.07</td>
<td>0.89</td>
</tr>
<tr>
<td>log HBu</td>
<td>0.48</td>
<td>-0.70</td>
</tr>
<tr>
<td>log i-PrOH</td>
<td>-0.83</td>
<td>-0.45</td>
</tr>
<tr>
<td>log EtOH</td>
<td>-0.83</td>
<td>-0.45</td>
</tr>
<tr>
<td>Explained Variance</td>
<td>38.9</td>
<td>27.1</td>
</tr>
<tr>
<td>Propotional total</td>
<td>3.11</td>
<td>2.17</td>
</tr>
<tr>
<td>Cumulative proportion</td>
<td>38.9</td>
<td>66.0</td>
</tr>
</tbody>
</table>

Table G.2 ANOVA results based on factor scores (cultures A and B)

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>SS A</th>
<th>SS B</th>
<th>MS A</th>
<th>MS B</th>
<th>F-value A</th>
<th>F-value B</th>
<th>P-value A</th>
<th>P-value B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regression</td>
<td>3</td>
<td>25.209</td>
<td>11.813</td>
<td>8.403</td>
<td>3.938</td>
<td>71.614</td>
<td>18.313</td>
<td>0.003</td>
<td>0.020</td>
</tr>
<tr>
<td>Residual</td>
<td>3</td>
<td>0.352</td>
<td>0.645</td>
<td>0.117</td>
<td>0.215</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>6</td>
<td>25.261</td>
<td>12.458</td>
<td>4.260</td>
<td>2.076</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Notes:
1. The F-value is the mean square due to regression divided by the mean square due to the residual.
2. DF = degrees of freedom; SS = sum of squares; MS = mean square
3. Factor showing maximum variance is used for conducting ANOVA

The bi-plot PCA (Figure 7.5) shown in Chapter 7 was tested for robustness using the Box-plot and it was indicated there were no outliers. Since the data set contains zeros in case of lactate, Hydrogen and methane, log transformation of the original data set was performed in STATISTICA version 8.0. The PCA was then performed using the log transformed data is shown in Figure G.2.
Figure G.2 Principal Component analysis (on the log transformed data) showing the grouping of samples under different conditions (a) Score plot (b) loading plot

Notes: 1. Only the first and second principal components are shown. 2. The first, second and third numbers of the sample labeling corresponds to the HRT (h), pH and LA concentration (g L\(^{-1}\)), respectively. 3. \(\text{CH}_4\) = methane; \(\text{EtOH}\) = ethanol; \(\text{HPr}\) = propionate; \(\text{HLa}\) = lactic acid; \(\text{HAc}\) = acetic acid and \(\text{HBu}\) = butyric acid.
Table G.3 Factor coordinates of variables based on correlation for steam exploded switch grass fed cultures

<table>
<thead>
<tr>
<th>Variables</th>
<th>Factor 1</th>
<th>Factor 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>log H2</td>
<td>0.827104</td>
<td>-0.501811</td>
</tr>
<tr>
<td>log CH4</td>
<td>-0.929291</td>
<td>0.294125</td>
</tr>
<tr>
<td>log Hla</td>
<td>0.144747</td>
<td>0.077791</td>
</tr>
<tr>
<td>log Hac</td>
<td>-0.401607</td>
<td>-0.623510</td>
</tr>
<tr>
<td>log HPr</td>
<td>-0.005846</td>
<td>-0.272223</td>
</tr>
<tr>
<td>log Hbu</td>
<td>0.902687</td>
<td>0.106950</td>
</tr>
<tr>
<td>log etoH</td>
<td>0.377670</td>
<td>0.870015</td>
</tr>
</tbody>
</table>

Explained Variance 38.4 22.5
Propotional total 2.7 1.6
Cumulative propotion 38.4 60.9
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