BATCH AND CONTINUOUS BIOHYDROGEN PRODUCTION USING MIXED MICROBIAL CULTURE

(Spine title: Biohydrogen Production by Mixed Culture)

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by

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ABSTRACT

In this research, biological hydrogen production via dark fermentation using mixed cultures was studied in batch, repeated batch and continuous systems. The preparation of active and stable microflora, increase of hydrogen production yields and rates, and evaluation of sustainability and revivability of continuous hydrogen production were the main objectives of this research.

Batch experiments showed that, depending on the type of inocula, the heat pre-treatment temperature had a direct effect on hydrogen production yield, hydrogen production rate and microbial community. The maximum yields of 2.3 and 1.6 mol H_2/mol glucose were achieved for 65°C pretreated anaerobically digested sludge and activated sludges, respectively. Pretreatment of anaerobically digested sludge at 95°C lowered the yield up to 15% while the same pretreatment for the activated sludge led to the complete suppression of hydrogen production. Biological hydrogen production with two types of microflora, activated sludge and anaerobically digested sludge, was compared at mesophilic (37°C) and thermophilic (55°C) conditions. Hydrogen production using activated sludge proved to be higher at thermophilic conditions, with cumulative hydrogen approximately six times more than that of mesophilic temperature. However, in anaerobically digested sludge, hydrogen yield at thermophilic temperature was 15% lower than that of mesophilic temperature. Repeated batch experiments at 37°C showed that hydrogen production with activated sludge was not stable due to the presence of lactic acid bacteria in the microflora according to PCR-DGGE analysis.

The effect of addition of four metabolites, including ethanol, lactic acid, butyric acid and acetic acid, by a fractional factorial design revealed the positive impact of lactic acid on hydrogen production. Although no significant hydrogen production was observed when lactic acid was used as the sole carbon source for hydrogen production, the addition of 10 mM lactic acid to a starch-containing substrate could enhance both hydrogen production rates and yields by approximately 1.9 and 1.6 times, respectively.

Continuous hydrogen production was compared in continuously stirred tank reactors (CSTR) with and without a gravity settler at mesophilic temperature. The observed inverse relationship between hydrogen yield and biomass yield implied that biomass yield is minimized with maximization of hydrogen yield. The revivability of a continuous hydrogen production system after a period of feed interruption was studied in a CSTR bioreactor. After the feed interruption, butyric acid formation completely stopped and the hydrogen yield decreased from 1.36 to 0.29 mol H_2/ mol glucose with ethanol, acetic acid, and lactic acid as the predominant soluble metabolites. Hydrogen production yield later increased to 0.7 mol H_2/mol glucose by adjusting the organic loading rate and pH. The microbial community analysis showed complete elimination of *Clostridium* affiliated strains after the re-startup of the reactor.

Key words: biological hydrogen production, dark fermentation, mixed culture, heat pre-treatment, CSTR, PCR-DGGE

CO-AUTHORSHIP

Chapter 3: Bita Baghchehsaraee was the principal author of this chapter. It was reviewed by Dr. George Nakhla, Dr. Dimitre Karamanev and Dr. Argyrios Margaritis who provided additional recommendations for improvement. These recommendations were incorporated into the chapter by Bita Baghchehsaraee. A version of this chapter has been published in *International Journal of Hydrogen Energy*, 2008, Volume 33, 4464-4073.

Chapter 4: Bita Baghchehsaraee was the principal author of this chapter. It was reviewed by Dr. George Nakhla, Dr. Dimitre Karamanev and Dr Argyrios Margaritis who provided additional recommendations for improvement. These recommendations were incorporated into the chapter by Bita Baghchehsaraee. A version of this chapter has been submitted to *International Journal of Hydrogen Energy.*

Chapter 5: Bita Baghchehsaraee was the principal author of this chapter. It was reviewed by Dr. George Nakhla, Dr. Dimitre Karamanev and Dr Argyrios Margaritis who provided additional recommendations for improvement. These recommendations were incorporated into the chapter by Bita Baghchehsaraee. A version of this chapter has been published in *International Journal of Hydrogen Energy*, 2009, Volume 34, 2573- 2579.

Chapter 6: Hisham Hafez and Bita Baghchehsaraee were the principal authors of this chapter. It was reviewed by Dr. George Nakhla who provided additional recommendations for improvement. A version of this chapter with the title of "comparative assessment of decoupling of biomass and hydraulic retention times in hydrogen production bioreactors" has been accepted for publication in *International Journal of Hydrogen Energy*. Bita Baghchehsaraee was the second author of this paper which conducted the research on one of the CSTR rectors and analysis of microbial community. The research work on IBRCS reactors was conducted by Hisham Hafez, a Ph.D. student in the Department of Civil Engineering, The University of Western Ontario.

Chapter 7: Bita Baghchehsaraee was the principal author of this chapter. It was reviewed by Dr. George Nakhla, Dr. Dimitre Karamanev and Dr Argyrios Margaritis who provided additional recommendations for improvement. These recommendations were incorporated into the chapter by Bita Baghchehsaraee. A version of this chapter is in progress for submission to *Biotechnology and Bioengineering*.

To my husband, Mehran, for his love and encouragement

To my son, Nima, who still wonders what I do

And

To my parents and my grandmother for their support

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NOMENCLATURE

e	Euler's number (2.71828)
H	cumulative hydrogen production (mL)
P	maximum hydrogen production (mL)
P_{H2}	predicted maximum hydrogen production (mL)
R	correlation coefficient value
R_m	maximum hydrogen production rate (mL/h)
t	incubation time (h)
X_a	active biomass (g VSS/L)
X_i	inert remains of microorganisms in the reactor (g VSS/L)
X_v	total biomass (g VSS/L)
X_{HP}	biomass of hydrogen producers (g VSS/L)
X_{nHP}	biomass of non-hydrogen producers (g VSS/L)
Y_{tHP}	true yield of hydrogen producers (g VSS/ g glucose)
$Y_{x/s}$	growth yield (g VSS/g glucose)
λ	lag phase time (h)
θ_c	solid retention time (d)
μ_{max}	maximum specific growth rate (h^{-1})
η	substrate conversion efficiency (%)

LIST OF ABBREVIATIONS

ANOVA	analysis of the variance
COD	chemical oxygen demand
CSTR	continuously stirred tank reactor
CIGSB	carrier- included granular sludge bed reactor
DF	degree of freedom
DGGE	denaturing gradient gel electrophoresis
F value	Fisher's *F* value
FID	flame ionization detector
FPD	flame photometric detector
HRT	hydraulic retention time
IBRCS	integrated biohydrogen reactor clarifier system
MLVSS	mixed liquor volatiles suspended solids
MS	mean square
NAD^+	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide hydride
OLR	organic loading rate
P value	probability value
PCR	polymerase chain reaction
RID	refractive index detector
SBR	sequencing batch reactor

RMSE	root mean square error
SM	soluble metabolites
SS	sum of Squares
SRT	solid retention time
SRT_{min}	minimum solids retention time
TCD	thermal conductivity detector
TCOD	total chemical oxygen demand
TSS	total suspended solids
TVFA	total volatile fatty acids
UASB	up-flow anaerobic sludge blanket reactor
VFA	volatile fatty acids
VSS	volatile suspended solids

CHAPTER 1

General Introduction

1.1 Background

Oil reserves of the world are being depleted at an alarming rate. It is estimated that the world's oil supply would be depleted by 2057, assuming the total world oil reserves of 1.25 trillion barrels and a daily consumption of 85 million barrels [1]. The decrease in the fossil fuel reserves in recent years has been accompanied by an exponential growth in worldwide energy demand. In an article published in *Science* in 2006, Ragauskas *et al.* [2] stated that " Indeed, energy demand is projected to grow by more than 50% by 2025, with much of this increase in demand emerging from several rapidly developing nations. Clearly, increasing demand for finite petroleum resources cannot be a satisfactory policy for the long term." Another problem of oil combustion is CO_2 emission which contributes to green house effect and global warming.

Hydrogen is considered as a viable alternative fuel and "energy carrier" of the future. Hydrogen is an environmentally friendly energy carrier in comparison with fossil fuels and has a high energy yield 142 kJ/g which is 2.75 times greater than hydrocarbon fuels. It has been reported that 50 million tones of hydrogen are traded annually worldwide with a growth rate of approximately 10% per year for the time being [3]. Based on an estimation by the National Hydrogen Program of the United States, the contribution of hydrogen to the energy market will be 8-10% by 2025 [4].

Due to the increasing demand for hydrogen, development of cost-effective and sustainable technologies for hydrogen production is of major importance. Currently most of the hydrogen production in the world is by steam reforming of methane and other hydrocarbons. This method is an energy intensive process requiring temperatures more

than 850°C. A small part of produced hydrogen today is from electrolysis of water; however, electrolysis is economical only for the areas with low electricity cost since electricity accounts for 80% of the operating cost [4].

Biological hydrogen production offers the potential production of renewable hydrogen from biomass. Among biological methods, dark fermentation presents a promising method for sustainable hydrogen production due to different types of biomass including wastewater streams, food scraps, animal waste, crop residuals and energy crops which can be used as substrates for hydrogen production. The high rate of hydrogen production is another advantage of dark fermentation for biohydrogen production.

Since non-sterile biomass is the practical feed for fermentative hydrogen production, mixed cultures developed from natural resources have to be used for biohydrogen production. Using mixed cultures for hydrogen production leads to many challenges including unwanted shifts in microbial communities and metabolic pathways.

1.2 Problem Statement

In spite of intensive research on fermentative hydrogen production in the last decades, this area is still in its early stage and to date no commercial plant has been established. The contradictory experimental results which have been frequently reported in the literature reflect the complexity of hydrogen production with mixed cultures and suggest more detailed characterization of the process. Further understating of the nature of microflora and its relation to soluble metabolites, as well as hydrogen yield may provide a direction for process optimization.

One of the major challenges to large scale biological hydrogen production is the development of an economical and efficient enrichment method for the preparation of large quantities of inoculum from natural sources. Enrichment is a critical stage which affects not only start-up but also overall efficiency and the stability of continuous hydrogen production systems. The type of original microflora also plays a major role in the development of a successful continuous hydrogen production system. Although facultative anaerobes have lower yields of hydrogen production, their presence in a mixed culture reduces the sensitivity of the process to environmental conditions such as oxygen exposure and makes the system more efficient for long term operation.

Another important challenge in fermentative hydrogen production is the current low hydrogen production rates and yields. If fermentative hydrogen production systems are to become commercially competitive, they must be able to produce hydrogen at rates that are sufficient for practical applications. The organic loading rate is one of the factors which directly affect the performance of a continuous biohydrogen system and needs to be optimized. The initial organic loading rate during the start up of the system may influence the long term efficiency of the system.

Continuous fermentative hydrogen production systems are characterized by operation under low hydraulic retention times. This feature increases the wash-out risk due to the operational failure such as interruption in feeding and makes the system unstable. Characterization of microbial communities with advanced molecular biology methods plays an important role in comparison of systems working at different organic loading rates.

1.3 Objectives of the Research

In the present research, hydrogen production with mixed cultures originating from municipal sludges was investigated in batch, repeated batch and continuous mode. The specific objectives of this study are:

- Developing an efficient and economical enrichment method for biohydrogen production by investigation of the effect of heat treatment temperature.
- Investigating the impact of using activated sludge as a more diverse microflora for biohydrogen production and comparing it with anaerobic digester sludge.
- Identifying the influence of soluble metabolites on biohydrogen production yields and rates.
- Clarifying the impact of organic loading rate on the performance of continuous hydrogen production and its relationship with changing microbial structure.
- Investigating the effect of feed interruption on the performance of a continuous hydrogen production system and examining the revivablity of the system.

1.4 Thesis Overview

This thesis encompasses eight chapters and conforms to the "integrated-article" format as outlined in the Thesis Regulation Guide by the School of Graduate and Postdoctoral Studies (SGPS) of the University of Western Ontario. A review of literature including background and comparison of information on fermentative hydrogen production is presented in Chapter 2.

Chapter 3 is a paper entitled, *"The effect of heat pretreatment temperature on fermentative hydrogen production using mixed cultures"*, published in the *International*

Journal of Hydrogen Energy [5]. This chapter comprehensively compares the effect of heat pretreatment temperatures on the enrichment of municipal sludge for biohydrogen production and proposes an optimum temperature for heat treatment. In this chapter the relationship between the metabolites, microbial community and hydrogen production at different pretreatment temperature was investigated.

Chapter 4 covers the results of a comparative study on using activated sludge and anaerobically digested sludge as inocula for biohydrogen production at both mesophilic and thermophilic temperatures. The stability of the inocula in this study was investigated by conducting repeated batch experiments and the relationship between instability in hydrogen production and changes in microbial community was identified.

Chapter 5 is a paper entitled, *"Effect of extrinsic lactic acid on fermentative hydrogen production"*, published in the *International Journal of Hydrogen Energy*, [6]. In this chapter, the effect of addition of acetic acid, butyric acid, ethanol and lactic acid on fermentative hydrogen production was first investigated using a fractional factorial design. This study revealed the positive effect of lactic acid addition on hydrogen production which was further investigated to determine the main reason for enhancement in hydrogen production.

Chapter 6 is a version of the paper entitled, *" Comparative assessment of decoupling of biomass and hydraulic retention times in hydrogen production bioreactors"*, which has been published online in *International Journal of Hydrogen Energy*. This study compares sustainability of biological hydrogen production from glucose in two continuously stirred tank reactors (CSTR) and two systems comprising CSTR with gravity settlers. My contribution in this study was in the preparation of the

submitted draft of the paper, running one of the CSTR reactors, and performing the analysis of microbial community in the reactors with PCR- DGGE method.

Chapter 7 describes the results of a research on the effect of feed interruption on the performance of a continuous hydrogen production reactor. This research compared the soluble metabolites, carbon mass balances, and microbial communities before and after feed interruption and discusses the revivability of the system.

Chapter 8 summarizes the major conclusions of this research and provides recommendations for further research work based on the results of this study.

The sequential outline of the research is depicted in Figure 1.1.

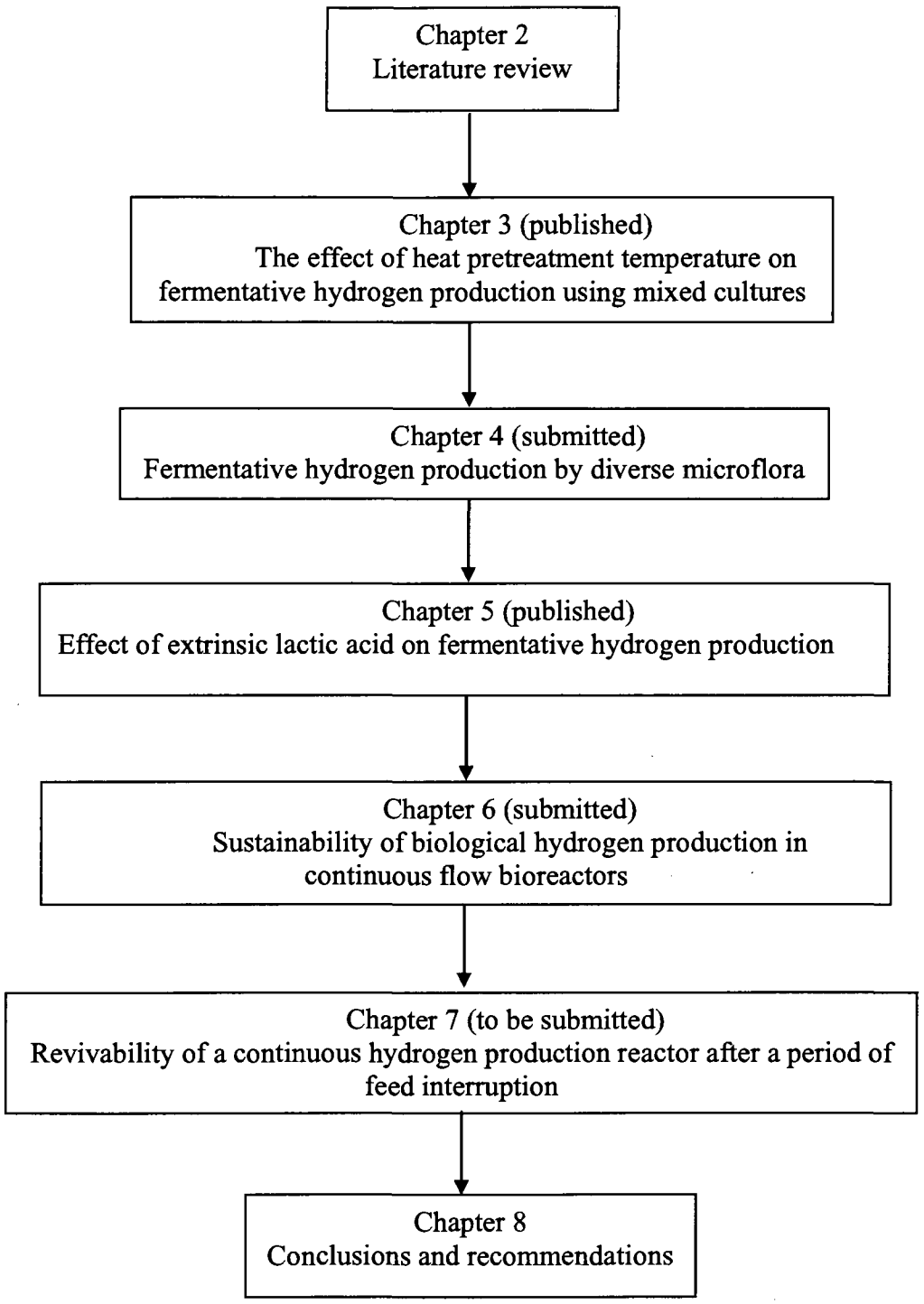

Figure 1. 1 Organization and structure of chapters

1.5 Contribution of Thesis

In the years to come with the worldwide increasing demand for clean energy and development of hydrogen based economy, biohydrogen will play an important role as a sustainable source of energy. Increasing the hydrogen production yields and rates together with development and/or modification of enrichment strategies for inocula preparations are some of the issues which have to be addressed before the commercialization of the process. Moreover, long term stability of continuous systems has to be investigated and confirmed. These major challenges were the main focus of the presented research. The contribution of this work to fermentative hydrogen production is increased knowledge related to enrichment methods and inocula preparation from mixed microflora, enhancement of hydrogen production rates and yields, and long term stability of the continuous hydrogen production reactors. The information generated from this research allows better understanding of the important parameters which affect hydrogen production by mixed cultures and provide basis for further optimization of the process in future.

1.6 References

1. Appenzeller T, National Geographic, The end of cheap oil. National Geographic. 2004(June):80-109.

2. Ragauskas AJ, Williams CK, Davison BH, Britovsek G, Cairney J, Eckert CA, The path forward for biofuels and biomaterials. Science. 2006;311(5760):484-9.

3. Winter CJ. Into the hydrogen energy economy - milestones. Int J Hydrogen Energy. 2005;30(7):681-5.

4. Armor JN. The multiple roles for catalysis in the production of H-2. Applied Catalysis A-General. 1999;176(2):159-76.

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

6. Baghchehsaraee B, Nakhla G, Karamanev D, Margaritis A. Effect of extrinsic lactic acid on fermentative hydrogen production. Int J Hydrogen Energy. 2009;34(6):2573-9.

CHAPTER 2

Literature Review

2.1 Introduction

During the last decade, much attention has been paid to the hydrogen gas as a clean and potentially renewable form of energy. The energy yield of hydrogen (142.35 KJ/g) is higher than all hydrocarbons [1] and it is the only common fuel that is not chemically bound to carbon; therefore, water is the only product of its reaction with oxygen, and its burning does not contribute to the greenhouse effect.

The importance of hydrogen as a fuel source dates back to the oil crisis in the 1970s. During that period, hydrogen was recognized as the "fuel of the future" and considerable resources were devoted to research on its viability and applications. After oil market was re-stabilized, hydrogen energy was no longer regarded as a priority. However, in the 1990s, the interest in hydrogen energy resurfaced, when it became evident that the atmospheric pollution by fossil fuels was not only health-threatening but might also cause significant global climate changes. [2]

In addition to hydrogen utilization as a source of energy, it can be employed as a reactant in hydrogenation processes, an O_2 scavenger and a coolant in electrical generators. These areas of hydrogen applications account for 3% of the energy consumption today and are subject to significant increase in years to come [3].

Hydrogen can be produced through water electrolysis and chemical cracking of hydrocarbons. However, these processes are not always environmentally friendly. As biological processes are usually carried out at ambient temperatures and pressures, they are less energy intensive than chemical or electrochemical ones. Thus, the hydrogen which is produced directly from organic materials and water by bacteria promises to be

an economical and sustainable technology for hydrogen production provided that the conversion efficiencies can be increased.

Biological production of hydrogen by fermentation can be classified into two main categories including photo fermentation and dark fermentation. In photo fermentation small-chain organic acids are used by photosynthetic bacteria as electron donors for the production of hydrogen at the expense of light energy. Photosynthetic bacteria have high theoretical conversion yields. Moreover, these bacteria do not have an oxygen-evolving activity, which causes problems of O_2 inactivation in fermentative systems [4]. Hydrogen production with photosynthetic bacteria has been the focus of some researchers so far [5, 6]. In dark fermentation hydrogen can be produced by both strict and facultative anaerobes from a variety of potentially consumable substrates, including refuse and waste materials.

Fermentative bacteria present a promising route of biological hydrogen production because they have high growth rates and can produce hydrogen in a shorter period without any light source. Moreover, different kinds of organic waste and wastewaters can be used as a substrate for hydrogen production. This process can produce hydrogen and simultaneously reduce the pollution strength of the waste.

2.2 Substrates for Fermentative Hydrogen Production

Carbohydrates are the preferred organic carbon source for hydrogen producing fermentations. Complex carbohydrates (e.g. starch and cellulose) break into hexose molecules such as glucose and then further degrade to produce the required energy for

cell. Degradation of glucose to acetic acid gives a maximum yield of 4 mol H_2/mol glucose:

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 + 184\ kJ/mol \quad (1)$$

Half of this yield is obtained with decomposition of glucose to butyrate:

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 + 257.1\ kJ/mol \quad (2)$$

It may be difficult to produce hydrogen from substrates with high lipid contents. Anaerobic hydrolysis of lipids results in the production of glycerol and long chain fatty acids (LCFA). Degradation of the LCFAs has positive free energy change and it is thermodynamically unfavorable [7], since a large portion of chemical oxygen demand (COD) of lipids is converted into long chain fatty acids during the hydrolysis reaction. Even if glycerol had a high hydrogen production potential, hydrogen production from lipids could not be high. In a study of hydrogen production from different substrates [8], it was demonstrated that hydrogen percentage of the biogas which was produced from protein degradation was much lower than the one produced from carbohydrates and even lipids. It seems the substrates which contain large amounts of proteins are not favorable for hydrogen production.

Among the preferred carbon sources, glucose and sucrose are the fermentation substrates, which have been extensively studied for hydrogen production [9, 10]. More

complicated carbohydrates such as starch or cellulose and carbohydrate rich wastes such as molasses can also be used as a substrate for hydrogen production.

Most of the studies for hydrogen production from wastes have been conducted in batch experiments, like hydrogen production from palm oil mill effluent [11] and paper mill wastes [12]. The produced gas in batch experiments from four food processing wastewaters including apple processing, potato processing and two confectioners consistently contained 60% hydrogen, and approximately 40% carbon dioxide [13]. However, the gas that was produced by a concentrated domestic wastewater in this study contained only 23±8% hydrogen. Potato processing wastewater had the highest overall gas conversion of 2.1-2.8 L/L among other wastewaters in this study.

Hydrogen production from sweet potato starch residue with the addition of 0.1% polypepton using a defined culture of *Clostridium butyricum* and *Enterobacter aerogenes* in a repeated batch culture with a yield of 2.4 mol H_2/mol hexose has been reported [14].

Continuous hydrogen production from insoluble starch in a non-sterile condition using mixed microflora gave hydrogen yield of 1.3 mol/ mol hexose [15]. Sparging the reactor with nitrogen to reduce hydrogen in the off-gas from 50% to 7% gave stable operation with a hydrogen yield of 1.9 mol/ mol hexose consumed over 18-day period. Another continuous hydrogen production by mixed microflora dominated in clostridia from soluble starch was reported by Lay [16]. Hydrogen production yield of 1.29 L/g starch-COD was obtained at pH 4.5 and 22 h HRT in a CSTR bioreactor. However, the period of these experiments lasted only a few days.

Continuous hydrogen production for over 200 days on sugar factory wastewater resulted in a yield of 14.4 mmol H_2/g carbohydrate removed [17]. Taguchi *et al.* [18]

reported continuous hydrogen production from pure strain of *Clostridium* on cellulose hydrolysate as the main substrate which was supplemented with peptone and yeast extract.

Nutrient levels should exceed the required optimum concentration during fermentation because anaerobic bacteria might be severely inhibited by even slight nutrient deficiencies. Many essential nutrients, however, can become toxic when present in high concentrations. A rough estimate of the required theoretical amount of macronutrients, i.e. nitrogen (N), phosphorous (P) and sulphur (S), can be derived from the elemental composition of bacterial cells. [19]

Lin and Lay [20] reported that hydrogen production ability by anaerobic microflora in batch experiments would depend on carbon/nitrogen (C/N) ratio. Hydrogen yield reached a maximum value of 4.8 mol/mol sucrose at a C/N ratio of 47.

Concentration of inorganic nutrients plays an important role in optimal hydrogen production. Dabrock *et al.* [21] reported that under phosphate limitation, ethanol, butanol and 1,2- propanol were the major metabolites from glucose. Iron is a component of the hydrogenase enzyme, which generates hydrogen; therefore, iron limitation decreases hydrogenase activity. Lee *et al.* [22] who conducted batch experiments with mixed culture on sucrose reported that low iron concentrations favored ethanol and butanol production. Maximum hydrogen production yields were observed when 800 mg $FeCl_3$/L was added to the growth medium.

2.3 Inoculum and Enrichment Methods

Hydrogen can be produced by anaerobic bacteria grown on carbohydrate rich substrates in dark. The bacteria known to produce hydrogen include the species of *Clostridium* [23], *Enterobacter* [24] and *Bacillus* [25].

The highest yields of hydrogen production have been reported with strains of Clostridia in pure cultures or mixed cultures where Clostridia are predominant. Pure cultures of *Clostridium butyricum* yielded 2 mol H_2/ mol glucose [26] and 2.78 mol H_2/mol sucrose [27]. Collet *et al.* [23] achieved 2.1- 3.0 mol H_2/mol lactose with *Clostridium thermolacticum*. It has been reported that *Clostridium thermocellum* could use delignified wood fibers to produce hydrogen with an average yield of 1.6 mol H_2/ mol glucose [28]. Although Clostridial species have high yields of hydrogen production, they are sensitive to the inhibition by oxygen, and require specific nutrients and environmental conditions for spore germination [29].

Facultative anaerobes including Enterobacteriace have less hydrogen production yields; however, they can be grown easier and are less sensitive to environmental conditions like the presences of oxygen. Because of these specifications, facultative anaerobes have been the focus of attention for some researchers [24, 30, 31]. *Enterobacter cloacae* which was isolated and used by Kumar and Das [24, 32] had different hydrogen yields with different substrates (2.2 mol/mol glucose, 6 mol/mol sucrose and 5.4 mol/ mol cellobiose). *Bacillus licheniformis* was also used for hydrogen production from damaged wheat grains [25].

There are also some reports of hydrogen production by co-culturing of pure species in the literature. Yokoi *et al.* [33] stated that *Enterobacter* sp. produced about 1

mol of H_2/ mol hexose, whilst *Clostridium* sp. produced around 2 mol H_2/ mol of hexose. Yokoi *et al.* [26] in another study obtained 2.6 mol H_2/mol glucose with a mixed culture of *Clostridium butyricum* and *Enterobacter aerogenes*.

Pure cultures have been shown to produce hydrogen from defined substrates such as glucose, at efficiencies about 20% to 60% of the known biochemical routes (based on 4 mol hydrogen per mol of glucose). However, application of pure cultures with waste materials is not feasible due to the different bacteria needed to break down the various organic components in the waste, and the prohibitive cost of sterilizing wastewater streams [34]. For a technically feasible process, mixed cultures obtained from natural sources which are able to operate on non sterile feedstocks are required. However, one of the obstacles for the production of hydrogen from mixed microflora is the coexistence of hydrogen consuming bacteria. Various types of hydrogen consuming anaerobes, including methanogens, acetogens, and sulfate reducing bacteria can obtain energy by utilizing molecular hydrogen [35]. In order to produce hydrogen using mixed cultures, hydrogen consumption by methanogens, homoacetogens, and sulfate reducing bacteria must be prevented and inocula must be enriched with hydrogen producing bacteria. Enrichment methods are reported to affect start-up, overall efficiency and the stability of a continuous hydrogen production system [29]. Heat treatment, acid treatment, alkaline treatment and utilization of chemical inhibitors are some of the enrichment methods that have been used for hydrogen production.

Heat treatment is the most common technique for enriching hydrogen producing bacteria. A wide range of heat-pretreated natural sources including anaerobically digested sludge [16], activated sludge [36], soil [9], cow dung [37], compost [38] and

river sediments [39] has been used for continuous hydrogen production. Heat treatment of inoculum is reported to be a rapid method for enrichment that can kill non-spore-forming hydrogen consuming bacteria, such as methanogens. It is reported that the predominant species after the heat treatment belong to the genus *Clostridia* [40]. When exposed to high temperatures, unlike methanogens, *Clostridium* species can produce protective spores and survive. However, some hydrogen consuming bacteria like *Clostridium aceticum* [41] and *Desulfotomaculum geothermicum* [42] that can produce spores will remain in the system. In spite of this fact, heat treatment is still one of the most practical methods for enrichment of inocula for hydrogen production.

Acid-base treatment is also considered as a method for enhancing hydrogen production. Chen *et al.* [43] reported that hydrogen production potential of sludge with acid or base enrichment was enhanced 200 and 333 times, compared with the control, when enrichment pH was 10 and 3, respectively. Cai *et al.* [1] also increased the hydrogen production yield from 9.1 mL H_2/ g dry solids to 16.6 mL H_2/g dry solids by alkaline pretreatment of sludge. *Eubacterium multiforme* and *Paenibacillus polymixa* were the dominant bacteria in biohydrogen production from alkaline pretreated sludge at initial pH of 11. It must be considered that acid- base treatment requires an acclimation time and sometimes the method is inadequate for avoiding subsequent biological contamination of a system with hydrogen consumers.

Applying short retention times can be useful to increase the productivity, because the growth rate of methanogens is slower than that of hydrogen producing bacteria [44]. However, in a free suspended cell bioreactor, the biomass is easily washed out and the efficiency of substrate utilization is reduced at short retention times [45, 46].

Chemical inhibitors can also suppress the methanogens and acclimation time is not generally required. 2-bromoethanesulfonate is a specific inhibitor for methanogens, but it may result in the formation of resistant mutants [47, 48]. Acetylene at 1% v/v in the head space is also reported as an effective inhibitor for methanogens [49].

The evaluation of the enrichment methods has been scantly studied. In a study comparing the heat treatment versus acid treatment, hydrogen yields observed as a result of the heat treatment were reported to be higher than those in acid treatment [50]. In a recent study [51], the effects of acid treatment, alkaline treatment and heat treatment on the hydrogen production were compared and the highest hydrogen yield was achieved with heat treatment while the lowest yield was obtained with alkaline treatment. Although some studies have been conducted with the aim of preparation of an active and stable microflora, this area has still remained as an obstacle in biological production of hydrogen from wastewater.

2.4 Metabolic Pathways for Fermentative Hydrogen Production

The microbial hydrogen production is derived by the anaerobic metabolism of pyruvate, an intermediate formed during the catabolism of various substrates by glycolysis (Embden-Meyerhof pathway):

$$C_6H_{12}O_6 + 2NAD^+ \rightarrow 2CH_3COCOOH + 2NADH + 2H^+ \quad (3)$$

The NADH which is formed during the glycolysis can be used for hydrogen evolution through the NADH oxidation to NAD^+ [3]:

$$NADH + H^+ \rightarrow H_2 + NAD^+ \quad (4)$$

The enzyme which is involved in this reaction is known as ferredoxin oxidoreductase.

Other pathways for hydrogen evolution are via the decomposition of pyruvate. The breakdown of pyruvate is catalyzed by two different enzymatic systems [52].

One of these systems is Pyruvate- formate lyase (PFL) which is used by enteric bacteria for hydrogen production. In this system, the pyruvate is converted to formic acid and acetyl-CoA. The formic acid can be further degraded with catalytic hydrogenase to hydrogen:

$$Pyruvate + CoA \rightarrow acetyl\text{-}CoA + formate \quad (5)$$

$$HCOOH \rightarrow H_2 + CO_2 \quad (6)$$

The second enzymatic system for the breakdown of pyruvate is pyruvate-ferredoxin oxidoredoctase (PFOR) which can be presented by the following reactions:

$$Pyruvate + CoA + 2Fd(ox) \rightarrow acetyl\text{-}CoA + 2Fd(red) \quad (7)$$

$$2\,Fd(red) \rightarrow 2Fd(ox) + H_2 \quad (8)$$

In this system hydrogen production is associated with the presence of an electron carrier called ferredoxin through which the electrons transfered to H^+ ions derived from water serve as electron acceptors, and are catalyzed by hydrogenase to form H_2. Strict anaerobes release hydrogen by this system [53].

2.5 Factors Affecting Fermentative Hydrogen Production

In fermentative hydrogen production, hydrogen rates and yields are influenced by environmental and physico-chemical factors including pH, temperature, hydraulic retention time, organic loading rate, concentration of soluble metabolites and hydrogen partial pressure.

2.5.1 pH

Due to the effects of pH on the hydrogenase activity [21], metabolic pathways [16] and microbial community [54] pH control is crucial to the hydrogen production. However, the reported optimal pH values for hydrogen production are conflicting, varying from initial pH of 11.0 for batch fermentation of sewage sludge [1], to controlled pH values of 7.0 and 5.5 for continuous fermentation of molasses [55] and glucose [9], respectively.

In hydrogen production with mixed cultures operation at pH values higher than 6, may increase the risk of methanogensis. Development of methanogens has been reported in a CSTR at the short HRT of 6 hours when pH increased to 6 [54]. However, some other researchers reported no methanogenic population build-up in continuous hydrogen

production systems at pH values of 6.5 and 6.6 [56, 57] which could be attributed to the duration of the process.

By comparison of the studies on continuous hydrogen production with mixed cultures at various pH values, Hawkes *et al.* [58] suggested an optimum pH range between 5.2 and 5.8 within HRT values of 6-32 hours and substrate types (sucrose, starch and beer industry waste).

Fang and Liu [59] demonstrated that the diversity in microbial community increased as pH shifted from 4.0 to 7.0 in continuous production of hydrogen from glucose. At the optimal pH of 5.5, the biogas comprised 64±2% of hydrogen with a yield of 2.1±0.1 mol H_2/mol glucose. The increase of pH resulted in the decrease of butyrate but increase of acetate. At pH 4.0-6.0, the effluent contained mostly butyrate (41.4-32.4% on carbon basis), followed by acetate (15.30-29.5%).

Although usually higher hydrogen production has been reported in a pH range of 5.0-6.8 with butyric and acetic acid as the predominant soluble metabolites there are some reports of high hydrogen production at pH range of 4.5-5.0 when ethanol and acetic acid are the predominant soluble metabolites [60, 61]. Operating at low pH values suppresses the methanogens and homo-acetogens [60] and it also reduces the base consumption for pH control [45].

2.5.2 Temperature

Most of the research on hydrogen production has been done at mesophilic temperatures around 30 to 35°C. Zoetemeyer *et al.* [62] reported that butyrate, the preferred metabolite for hydrogen production, was decreased at temperatures above 30°C while acetate and propionate were increased.

High yields of hydrogen with thermophilic regimes were also reported in literature. The hydrogen yield of 2.2 mol/ mol hexose with the hydrogen production rate of 1.0 L/L/d was achieved in a continuous conversion of food waste into hydrogen at 55°C [63]. Semi-continuous anaerobic processes with solid substrate and mesophilic regime (35°C), showed a yield of 37% of the maximum yield (based on 4 mol H_2/ mol hexose), while thermophilic regime (55°C) exhibited a yield of 80% of the maximum yield [64].

Although thermophilic hydrogen processes give high yields of hydrogen production, they are usually characterized by low volumetric production rates. It is because of the fact that many thermophilic microorganisms do not achieve high cell densities in liquid cultures. The low volumetric production rates limit the usefulness of these organisms in a practical system. [65]

2.5.3 Hydraulic Retention Time (HRT)

Hydrogen-producing bacteria usually have faster growth rate in comparison with methanogens. Therefore, at relatively high dilution rates (or low HRTs) methanogens are washed out of a mixed culture and therefore hydrogen production is increased [66]. Continuous hydrogen production in literature has usually been conducted at HRTs between 0.5 and 12 hours [58, 67]. Optimum retention time in a continuous hydrogen production system depends on other characteristics of the system such as the type of substrate and should be optimized for a specific system. Comparing two hydrogen production systems with actual wastewater, the optimum retention time for rice winery wastewater was reported to be 2 hours [68] while that of sugar factory wastewater was 12 hours [17]. Operation at too low HRTs can lead to biomass washout, while operation at

too high HRTs can lead to the inhibition of hydrogen production by accumulation of high levels of volatile fatty acids.

2.5.4 Concentration of Soluble Metabolites

Volatile fatty acids or alcohols at some concentrations may have inhibitory effects on fermentative hydrogen production. *Clostridium thermocellum* has low alcohol tolerance of less than 2% for growth [69] which is because of blockage of glycolysis which occurs after ethanol induces changes in the cell membrane [70].

When acetic acid and butyric acid were added to the feed at concentrations of 25 mM, hydrogen production yields decreased by 13% and 22%, respectively [71]. In the same study the addition of both acids at 60 mM, which is the undissociated acid concentration, resulted in 93% reduction in hydrogen yield. The undissociated acids can cross the cell membrane, collapse the transmembrane pH gradient and, thus, cause cell death or sporulation [72].

2.5.5 Hydrogen Partial Pressure

The decrease in the partial pressure of hydrogen in the reactor can increase hydrogen production. When the hydrogen concentration in the liquid raises hydrogen production reactions are less favorable [29]. As hydrogen concentration increases, metabolic pathways shift toward the production of reduced products instead of hydrogen. In natural ecosystems, low partial pressure of hydrogen is achieved via syntrophic associations between hydrogen producing bacteria and hydrogen consuming bacteria such as methanogens [19].

In order to obtain high concentrations of hydrogen, hydrogen must be artificially removed from the system, before it leads to repression of its production or be consumed by methanogenes [30]. Continuous hydrogen production requires a partial pressure of H_2 < 50 kPa at 60°C, < 20 kPa at 70°C and < 2 kPa at 98°C [4].

Continuous versus intermittent release of gas pressure in batch tests increased hydrogen production by 43% [73]. Reducing the hydrogen partial pressure by sparging argon or nitrogen has also increased the hydrogen production yield in continuous cultures [30, 74].

A report by Tanisho *et al.* [30] demonstrated that sparging with argon results in an increase of residual NADH, which in turn increases the hydrogen production, although hydrogen was not measured. The yields were mainly estimated by calculating the amount of residual NADH. Sparging nitrogen gas was also reported as a useful method for increasing hydrogen yield [74]. They observed that the specific hydrogen production rate increased from 1.446 ml / min-g biomass to 3.131 ml / min g biomass under nitrogen sparging condition. With nitrogen sparging at a flow rate approximately 15 times the hydrogen production rate, the hydrogen yield was 1.43 mol H_2/ mol glucose. This showed a 50% increase in hydrogen yield when nitrogen was sparged into the system. In hydrogen production from a wheat starch co-product, sparging the reactor with nitrogen to reduce hydrogen in the off-gas from 50% to 7% gave stable operation with a hydrogen yield of 1.9 mol / mol hexose consumed over 18-day period. Hydrogen production yield without sparging in this reactor was 1.26 mol/ mol hexose consumed. [15]. However, too much sparging gas results in serious problems of hydrogen separation and purification. Sparging gas should be free of CO, otherwise it could inhibit hydrogenase enzyme. [4]

2.6 Bioreactor Type

Different types of bioreactors have been used in research on hydrogen production. The design parameters for continuous reactors are yet not clearly defined. Since fermentative hydrogen production is similar to the acidogenic stage of anaerobic digestion, it seems that a modified fermentor technology can be used for this purpose.

Generally, at laboratory scale continuous stirred tank reactors (CSTRs) have been used for hydrogen production, because the pH and agitation of the culture can easily be controlled in continuous performance. However, the biomass can easily be washed out at short hydraulic retention times (HRTs) resulting in failure in substrate degradation and hydrogen production efficiency.

Hussy *et al.* [15] used an anaerobic CSTR reactor for continuous fermentation of a wheat starch co-product by mixed microflora. A five day average of 1.26 mol H_2/ mol hexose consumed was the highest amount obtained without sparging, while an average of 1.87 mol H_2/ mol hexose consumed was achieved over 18 day period when nitrogen was sparged into the reactor.

It is economic to maintain higher biomass concentrations in the reactor by immobilization, granulation, membrane processes and centrifugation. Hydrogen production using immobilized *Clostridium butyricum* on porous glass beads has been reported by Yokoi *et al.* [33]. Wu *et al.* [75] used immobilized sewage sludge for hydrogen production. They achieved durable (over 24 repeated runs) and stable hydrogen production with a gel entrapment system by the addition of acrylic latex plus silicone.

Using activated carbon as support matrix, fixed bed bioreactors could enhance hydrogen production from sewage sludge and sucrose and allowed retention of hydrogen producing bacteria within the reactor [76]. Hydrogen production with immobilized sewage sludge in three-phase fluidized bed bioreactor resulted in a hydrogen production rate of 0.93 L/h/L and hydrogen yield of 2.67 mol H_2/ mol sucrose [77]. It was reported that fluidized bed could be stably used at high loading rates (HRT as low as 2 hours).

In an attempt to use membrane bioreactor for hydrogen production, it was demonstrated that hydrogen production by fermentative bacteria, grown at short detention time (3.3 hours), could be increased in the system [9]. At longer HRTs, membrane will not be necessary, as biogas production will decrease with any increase in solid retention time (SRT). Since lower hydrogen production was associated with a slight increase in the number of DNA bands in Ribosomal Intergenic Spacer Analysis (RISA) profiles, it seems that a shift in physiology of bacteria with changing the detention time is the reason for decreasing hydrogen production. Biogas composition in this study was not affected by HRT or SRT and was always in the range of 57 to 60% of hydrogen.

The up-flow anaerobic reactor was also applied for hydrogen generation from waste materials and wastewaters [68]. When winery wastewater was used in an up-flow anaerobic sludge blanket reactor (UASB), an optimum hydrogen production rate of 9.33 L/g VSS d was achieved at an HRT of 2 h, COD of 34 g/L, pH 5.5 and 55°C. The hydrogen yield in this study was 1.37-2.14 mol/mol hexose.

A remarkable hydrogen production rate of 7.3 L/h/L (7150 mmol/d/L) was obtained with a carrier- included granular sludge bed reactor (CIGSB) [78]. The hydrogen yield was 1.5 mol/ mol hexose. One of the advantages of CIGSB over UASB is

the quick sludge generation within 100 h of start-up, whereas it takes months for hydrogen producing UASB process to form granular sludge.

Practical application of some of these systems like CIGSB process with high content of suspended solid wastewater has not yet been investigated.

2.7 Metabolic Shift

Hydrogen fermentation by Clostridia occurs under a branched fermentative pathway. The ratio of product formed to substrate consumed is variable in hydrogen fermentation while it is constant in the linear fermentative pathways such as ethanol fermentations. A branched pathway produces more ATP and oxidized products than a linear pathway. The formation of products in a branched pathway is dictated by the environmental conditions especially hydrogen partial pressure [79].

Figure 2.1 shows the possible fermentation products by fermentation of carbohydrates. In practice, high H_2 yields are associated with a mixture of acetate and butyrate fermentation products, and low H_2 yields are accompanied with propionate and reduced end products such as alcohols and lactic acid [80]. However, it should be considered that under special environmental conditions such as low pH values (4.5 – 5) hydrogen can be obtained through ethanol type fermentation [81]:

$$C_6H_{12}O_6 + H_2O \rightarrow C_2H_5OH + 2CH_3COOH + 2CO_2 + 2H_2 \quad (9)$$

Therefore, ethanol formation is not always a sign of low hydrogen production yields.

It has been well investigated that metabolic pathway of hydrogen producing bacteria is significantly influenced by various environmental factors. In *C. pasteurianum* metabolism can be directed away from H_2 production toward solvent production by high glucose concentrations (12.5%), by CO (an inhibitor of hydrogenase) and by iron limitation but not by phosphate limitation [21].

Reduction of hydrogen partial pressure in the reactor can increase hydrogen production. Continuous versus intermittent release of gas pressure in batch tests increased hydrogen production by 43% [73]. Reducing the hydrogen partial pressure by sparging argon or nitrogen has also increased the hydrogen production yield in continuous cultures [30, 74].

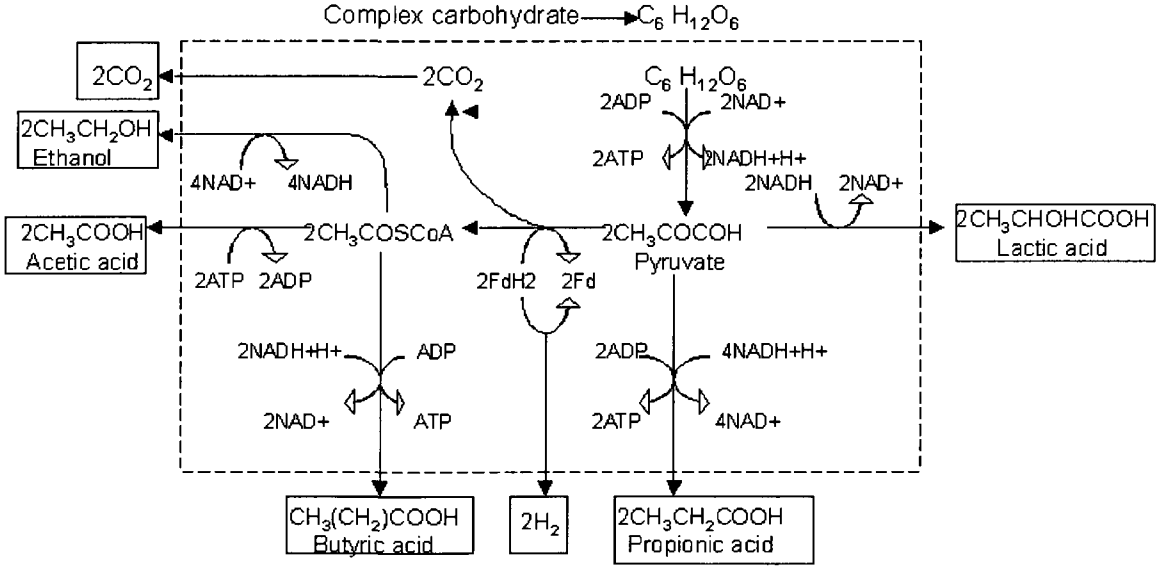

Figure 2. 1 Metabolic pathways possible for carbohydrates fermentation [61]

Tanisho *et al.* [30] observed that the removal of CO_2 from the liquid culture of *Enterobacter aerogenes* E 82005 increased the hydrogen yield from 0.52 to 1.58 mol H2/ mol glucose by redirection of metabolic pathways. In a study by Park *et al.* [34], when CO_2 in the headspace was decreased from 24.5% to 2.5% during the highest gas production phase, hydrogen yield increased by 43% (from 1.4 to 2.0 mol H_2/mol glucose).

2.8 Microbial Shift and Investigation of Microbial Community Dynamics

Maintaining an active and stable microflora is the key for sustainable hydrogen production. It is frequently observed that the change in hydrogen production yield and rate is associated with variations in microbial community [82-84].

Typically, microorganisms are identified by isolating individual cultures and examining their physiological, biological, and morphological characteristics. However, such identification is often unreliable. First, microorganisms may not be properly isolated from the artificial growth medium. Second, many microorganisms grow syntrophically with others and thus cannot be cultured individually. Third, many microorganisms share similar physiological, biochemical and morphological characteristics and, therefore, cannot be diagnosed by these methods. [85]

Molecular biological techniques offer new opportunities for the analysis of structure and species composition of microbial communities. These methods rely largely upon sequence information of genes that are universally conserved, yet sufficiently different to reflect the phylogeny of the prokaryotes [86]. Among these methods, denaturing gradient gel electrophoresis of PCR amplified community 16S rDNA have

been extensively used in the study of different microbial communities for example, in ocean mats [87], biofilms [88] and acidogenic anaerobic reactors [89].

Separation techniques based on denaturing gradient gels were first described by Fischer and Lerman [90]. In PCR-DGGE method, polymerase chain reaction (PCR) is first used to amplify a highly variable region (i.e, V3 region) of 16S rDNA gene of a mixed pool of chromosomal DNA. The result of this PCR reaction is a mixture of products with an equal size but species-specific sequence.

Denaturing gradient gels are prepared to give an increasing concentration of denaturing agent along the same direction of electrophoresis. When PCR amplified products of 16S rDNA are applied in DGGE, as a critical concentration of denaturing agent is reached the melting domains will denature and result in a separation of fragment based on the sequence. A virtual model of the separation of PCR amplified fragments via DGGE is shown in Figure 2.2.

Ueno *et al.* [91] applied PCR-DGGE for the first time to study the microbial community in hydrogen production from sludge compost. They could identify sixty-eight microorganisms in the microflora and classify them into nine distinct groups by genetic fingerprinting of the PCR-DGGE and determine the partial sequence of 16S rDNA. Most of the strains in this study were *clostridium* and *bacillus* species.

In another study conducted by Fang *et al.* [85], microbial diversity of a mesophilic hydrogen producing sludge was investigated using PCR-DGGE method. A total of ninety-six clones were selected for plasmid recovery, screened by DGGE, and sequenced for rDNA. Based on the phylogenetic analysis of rDNA sequences, 64.6% of

all the clones were affiliated with three *Clostridium* species, 18.8% with Enterobacteriaceae, and 3.1% with *Streptococcus bovis*.

Application of different environmental conditions, such as different HRTs, pH values and temperatures has a direct effect in microbial community. In each environmental condition a particular group of microorganisms become predominant and affect the overall performance of the system. Therefore, monitoring the microbial community with molecular techniques is a valuable way for fundamental investigation of fermentative hydrogen producing processes.

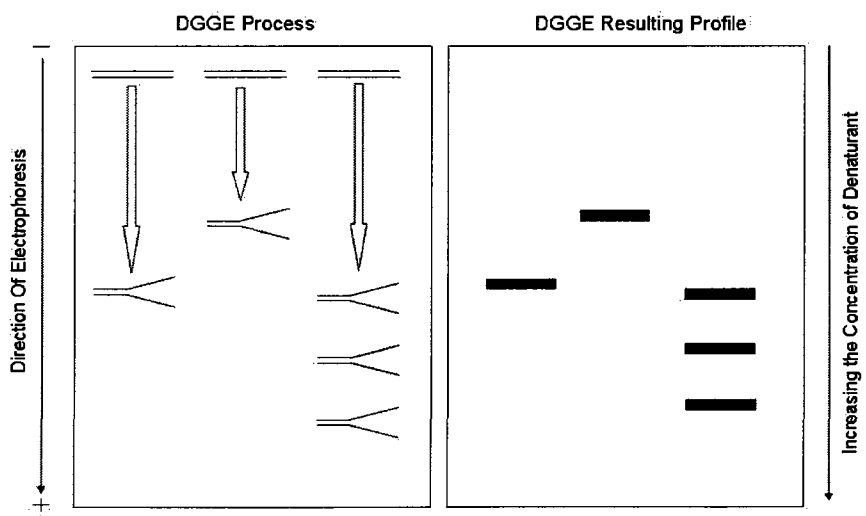

Figure 2. 2 Schematic diagram of the separation of PCR amplified fragmants via DGGE

2.9 Conclusions

Although, fermentative hydrogen production has been the focus of many research groups in recent years, reported results that are available in literature reveal that commercial hydrogen production is still not feasible at the present time. Further research and development with the aim of increasing both yields and rates of fermentative hydrogen production are required. Moreover, long term stability of the systems should be investigated. Establishment of an active and stable microflora has an important role to achieve a successful biohydrogen production process. Regarding the continuous hydrogen production enough information is not available on the revivability and sustainability of continuous reactors. Such information will play a significant roll in development of large scale biohydrogen production systems.

The above mentioned points are highlighted in the present study anticipated that the results of the research in this area will benefit current knowledge on dark fermentative hydrogen production.

2.10 References

1. Cai ML, Liu JX, Wei YS. Enhanced biohydrogen production from sewage sludge with alkaline pretreatment. Environ Sci Technol. 2004 ;38(11):3195-202.

2. Benemann J. Hydrogen biotechnology: Progress and prospects. Nat Biotechnol. 1996;14(9):1101-3.

3. Das D, Veziroglu TN. Hydrogen production by biological processes: A survey of literature. Int J Hydrogen Energy. 2001;26(1):13-28.

4. Nath K, Das D. Improvement of fermentative hydrogen production: Various approaches. Appl Microbiol Biotechnol. 2004 ;65(5):520-9.

5. Margaritis A, Vogrinetz J. The effect of glucose-concentration and pH on hydrogen-production by rhodopseudomonas-sphaeroides vm 81. Int J Hydrogen Energy. 1983;8(4):281-4.

6. Fang HHP, Liu H, Zhang T. Phototrophic hydrogen production from acetate and butyrate in wastewater. Int J Hydrogen Energy. 2005;30(7):785-93.

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

8. Okamoto M, Miyahara T, Mizuno O, Noike T. Biological hydrogen potential of materials characteristic of the organic fraction of municipal solid wastes. Water Science and Technology. 2000;41(3):25-32.

9. Oh SE, Lyer P, Bruns MA, Logan BE. Biological hydrogen production using a membrane bioreactor. Biotechnol Bioeng. 2004;87(1):119-27.

10. Chang FY, Lin CY. Biohydrogen production using an up-flow anaerobic sludge blanket reactor. Int J Hydrogen Energy. 2004;29(1):33-9.

11. Atif AAY, Fakhru'l-Razi A, Ngan MA, Morimoto M, Iyuke SE, Veziroglu NT. Fed batch production of hydrogen from palm oil mill effluent using anaerobic microflora. Int J Hydrogen Energy. 2005;30(13-14):1393-7.

12. Valdez-Vazquez I, Sparling R, Risbey D, Rinderknecht-Seijas N, Poggi-Varaldo HM. Hydrogen generation via anaerobic fermentation of paper mill wastes. Bioresour Technol. 2005;96(17):1907-13.

13. Van Ginkel SW, Oh SE, Logan BE. Biohydrogen gas production from food processing and domestic wastewaters. Int J Hydrogen Energy. 2005;30(15):1535-42.

14. Yokoi H, Saitsu A, Uchida H, Hirose J, Hayashi S, Takasaki Y. Microbial hydrogen production from sweet potato starch residue. Journal of Bioscience and Bioengineering. 2001;91(1):58-63.

15. Hussy I, Hawkes FR, Dinsdale R, Hawkes DL. Continuous fermentative hydrogen production from a wheat starch co-product by mixed microflora. Biotechnol Bioeng. 2003;84(6):619-26.

16. Lay JJ. Modeling and optimization of anaerobic digested sludge converting starch to hydrogen. Biotechnol Bioeng. 2000;68(3):269-78.

17. Ueno Y, Otsuka S, Morimoto M. Hydrogen production from industrial wastewater by anaerobic microflora in chemostat culture. J Ferment Bioeng. 1996;82(2):194-7.

18. Taguchi F, Yamada K, Hasegawa K, TakiSaito T, Hara K. Continuous hydrogen production by clostridium sp strain no 2 from cellulose hydrolysate in an aqueous two-phase system. J Ferment Bioeng. 1996;82(1):80-3.

19. Mara D, Horan N. The handbook of water and wastewater microbiology. ACADEMIC PRESS; 2003.

20. Lin CY, Lay CH. Carbon/nitrogen-ratio effect on fermentative hydrogen production by mixed microflora. Int J Hydrogen Energy. 2004;29(1):41-5.

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

22. Lee YJ, Miyahara T, Noike T. Effect of iron concentration on hydrogen fermentation. Bioresour Technol. 2001;80(3):227-31.

23. Collet C, Adler N, Schwitzguebel JP, Peringer P. Hydrogen production by clostridium thermolacticum during continuous fermentation of lactose. Int J Hydrogen Energy. 2004;29(14):1479-85.

24. Kumar N, Das D. Enhancement of hydrogen production by enterobacter cloacae IIT-BT 08. Process Biochemistry. 2000;35(6):589-93.

25. Kalia VC, Jain SR, Kumar A, Joshi AP. Fermentation of bio-waste to H_2 by *bacillus licheniformis*. World J Microbiol Biotechnol. 1994; 10,224-227.

26. Yokoi H, Tokushige T, Hirose J, Hayashi S, Takasaki Y. H-2 production from starch by a mixed culture of clostridium butyricum and enterobacter aerogenes. Biotechnol Lett. 1998;20(2):143-7.

27. Chen WM, Tseng ZJ, Lee KS, Chang JS. Fermentative hydrogen production with clostridium butyricum CGS5 isolated from anaerobic sewage sludge. Int J Hydrogen Energy. 2005;30(10):1063-70.

28. Levin DB, Islam R, Cicek N, Sparling R. Hydrogen production by clostridium thermocellum 27405 from cellulosic biomass substrates. Int J Hydrogen Energy. 2006;31(11):1496-503.

29. Hawkes FR, Dinsdale R, Hawkes DL, Hussy I. Sustainable fermentative hydrogen production: Challenges for process optimisation. Int J Hydrogen Energy. 2002;27(11-12):1339-47.

30. Tanisho S, Kuromoto M, Kadokura N. Effect of CO2 removal on hydrogen production by fermentation. Int J Hydrogen Energy. 1998;23(7):559-63.

31. Kurokawa T, Tanisho S. Effects of formate on fermentative hydrogen production by enterobacter aerogenes. Marine Biotechnology. 2005;7(2):112-8.

32. Kumar N, Das D. Continuous hydrogen production by immobilized enterobacter cloacae IIT-BT 08 using lignocellulosic materials as solid matrices. Enzyme Microb Technol. 2001;29(4-5):280-7.

33. Yokoi H, Maeda Y, Hirose J, Hayashi S, Takasaki Y. H-2 production by immobilized cells of clostridium butyricum on porous glass beads. Biotechnol Tech. 1997;11(6):431-3.

34. Park W, Hyun SH, Oh SE, Logan BE, Kim IS. Removal of headspace C0(2) increases biological hydrogen production. Environ Sci Technol. 2005;39(12):4416-20.

35. Adams MWW, Stiefel EI. Biochemistry - biological hydrogen production: Not so elementary. Science. 1998;282(5395):1842-3.

36. Cohen A, Distel B, Vandeursen A, Breure AM, Vanandel JG. Role of anaerobic spore-forming bacteria in the acidogenesis of glucose - changes induced by discontinuous or low-rate feed supply. Antonie Van Leeuwenhoek Journal of Microbiology. 1985;51(2):179-92.

37. Vijayaraghavan K, Ahmad D, Bin Ibrahim MK. Biohydrogen generation from jackfruit peel using anaerobic contact filter. Int J Hydrogen Energy. 2006;31(5):569-79.

38. Fan KS, Chen YY. H-2 production through anaerobic mixed culture: Effect of batch S-0. Chemosphere. 2004;57(9):1059-68.

39. Zuo J, Zuo Y, Zhang W, Chen J. Anaerobic bio-hydrogen production using pre-heated river sediments as seed sludge. Water Science and Technology. 2005;52(10-11):31-9.

40. Iyer P, Bruns MA, Zhang HS, Van Ginkel S, Logan BE. H-2-producing bacterial communities from a heat-treated soil inoculum. Appl Microbiol Biotechnol. 2004;66(2):166-73.

41. Menendez C, Bauer Z, Huber H, Gad'on N, Stetter KO, Fuchs G. Presence of acetyl coenzyme A (CoA) carboxylase and propionyl-CoA carboxylase in autotrophic crenarchaeota and indication for operation of a 3-hydroxypropionate cycle in autotrophic carbon fixation. J Bacteriol. 1999;181(4):1088-98.

42. Park WS, Hawang MH, Hyun SH, Kim IS. *Suppression of hydrogen consuming bacteria in order to increase H2 production potential in anaerobic fermentation.* Proceedings of 10th world congress of anaerobic digestion, Montreal, Canada.2004;1(1):642-647.

43. Chen CC, Lin CY, Lin MC. Acid-base enrichment enhances anaerobic hydrogen production process. Appl Microbiol Biotechnol. 2002;58(2):224-8.

44. Liu H, Fang HHP. Hydrogen production from wastewater by acidogenic granular sludge. Water Sci Technol. 2003;47(1):153-8.

45. Zoetemeyer RJ, Vandenheuvel JC, Cohen A. pH influence on acidogenic dissimilation of glucose in an anaerobic digester. Water Res. 1982;16(3):303-11.

46. Ueki A, Ueki K, Tanaka K, Takahashi R, Takano T. End-products and molar growth-yield of clostridium-fallax isolated from an anaerobic digester. J Ferment Bioeng. 1991;72(4):274-9.

47. Santoro N, Konisky J. Characterization of bromoethanesulfonate-resistant mutants of methanococcus-voltae - evidence of a coenzyme-M transport-system. J Bacteriol. 1987;169(2):660-5.

48. Boyd JM, Ellsworth A, Ensign SA. Characterization of 2-bromoethanesulfonate as a selective inhibitor of the coenzyme M-dependent pathway and enzymes of bacterial aliphatic epoxide metabolism. J Bacteriol. 2006;188(23):8062-9.

49. Sparling R, Risbey D, PoggiVaraldo HM. Hydrogen production from inhibited anaerobic composters. Int J Hydrogen Energy. 1997;22(6):563-6.

50. Oh SE, Van Ginkel S, Logan BE. The relative effectiveness of pH control and heat treatment for enhancing biohydrogen gas production. Environ Sci Technol. 2003 15;37(22):5186-90.

51. Mu Y, Yu HQ, Wang G. Evaluation of three methods for enriching H-2-producing cultures from anaerobic sludge. Enzyme Microb Technol. 2007;40(4):947-53.

52. Hallenbeck PC, Benemann JR. Biological hydrogen production; fundamentals and limiting processes. Int J Hydrogen Energy. 2002;27(11-12):1185-93.

53. Gaudy AF, Gaudy ET. Elements in bioenvironmental engineering. San Jose, CA: Engineering Press, Inc.; 1988.

54. Fang HHP, Liu H. Effect of pH on hydrogen production from glucose by a mixed culture. Bioresour Technol. 2002;82(1):87-93.

55. Chau CH, Lay JJ. In: A mixture design approach to hydrogen-producing anaerobs using molasses. ; Proceedings of Anaerobic Digestion, Montreal, Canada, 2004;2:856-61

56. Fan KS, Kan NR, Lay JJ. Effect of hydraulic retention time on anaerobic hydrogenesis in CSTR. Bioresour Technol. 2006;97(1):84-9.

57. Wu SY, Hung CH, Lin CN, Chen HW, Lee AS, Chang JS. Fermentative hydrogen production and bacterial community structure in high-rate anaerobic bioreactors containing silicone-immobilized and self-flocculated sludge. Biotechnol Bioeng. 2006;93(5):934-46.

58. Hawkes FR, Hussy I, Kyazze G, Dinsdale R, Hawkes DL. Continuous dark fermentative hydrogen production by mesophilic microflora: Principles and progress. Int J Hydrogen Energy. 2007;32(2):172-84.

59. Fang HHP, Liu H. Effect of pH on hydrogen production from glucose by a mixed culture. Bioresour Technol. 2002;82(1):87-93.

60. Hwang MH, Jang NJ, Hyun SH, Kim IS. Anaerobic bio-hydrogen production from ethanol fermentation: The role of pH. J Biotechnol. 2004;111(3):297-309.

61. Ren NQ, Wang BZ, Huang JC. Ethanol-type fermentation from carbohydrate in high rate acidogenic reactor. Biotechnol Bioeng. 1997;54(5):428-33.

62. Zoetemeyer RJ, Arnoldy P, Cohen A, Boelhouwer C. Influence of temperature on the anaerobic acidification of glucose in a mixed culture forming part of a 2-stage digestion process. Water Res. 1982;16(3):313-21.

63. Shin HS, Youn JH. Conversion of food waste into hydrogen by thermophilic acidogenesis. Biodegradation. 2005;16(1):33-44.

64. Valdez-Vazquez I, Rios-Leal E, Esparza-Garcia F, Cecchi F, Poggi-Varaldo HA. Semi-continuous solid substrate anaerobic reactors for H-2 production from organic waste: Mesophilic versus thermophilic regime. Int J Hydrogen Energy. 2005;30(13-14):1383-91.

65. Hallenbeck PC. Fundamentals of the fermentative production of hydrogen. Water Science and Technology. 2005;52(1-2):21-9.

66. Chen CC, Lin CY, Chang JS. Kinetics of hydrogen production with continuous anaerobic cultures utilizing sucrose as the limiting substrate. Appl Microbiol Biotechnol. 2001;57(1-2):56-64.

67. Li CL, Fang HHP. Fermentative hydrogen production from wastewater and solid wastes by mixed cultures. Crit Rev Environ Sci Technol. 2007;37(1):1-39.

68. Yu HQ, Zhu ZH, Hu WR, Zhang HS. Hydrogen production from rice winery wastewater in an upflow anaerobic reactor by using mixed anaerobic cultures. Int J Hydrogen Energy. 2002;27(11-12):1359-65.

69. Burdette DS, Jung SH, Shen GJ, Hollingsworth RI, Zeikus JG. Physiological function of alcohol dehydrogenases and long-chain (C-30) fatty acids in alcohol tolerance of thermoanaerobacter ethanolicus. Appl Environ Microbiol. 2002;68(4):1914-8.

70. Herrero AA, Gomez RF, Roberts MF. P-31 nmr-studies of clostridium-thermocellum - mechanism of end product inhibition by ethanol. J Biol Chem. 1985;260(12):7442-51.

71. Van Ginkel S, Logan BE. Inhibition of biohydrogen production by undissociated acetic and butyric acids. Environ Sci Technol. 2005;39(23):9351-6.

72. Durre P. Sporulation in clostridia (gentics). In: Handbook on Clostridia. Boca Raton London New York Singapore: Taylor & Francis Group; 2005.

73. Logan BE, Oh SE, Kim IS, Van Ginkel S. Biological hydrogen production measured in batch anaerobic respirometers. Environ Sci Technol. 2002;36(11):2530-5.

74. Mizuno O, Dinsdale R, Hawkes FR, Hawkes DL, Noike T. Enhancement of hydrogen production from glucose by nitrogen gas sparging. Bioresour Technol. 2000;73(1):59-65.

75. Wu SY, Lin CN, Chang JS, Lee KS, Lin PJ. Microbial hydrogen production with immobilized sewage sludge. Biotechnol Prog. 2002;18(5):921-6.

76. Chang JS, Lee KS, Lin PJ. Biohydrogen production with fixed-bed bioreactors. Int J Hydrogen Energy. 2002;27(11-12):1167-74.

77. Wu SY, Lin CN, Chang JS. Hydrogen production with immobilized sewage sludge in three-phase fluidized-bed bioreactors. Biotechnol Prog. 2003;19(3):828-32.

78. Lee KS, Wu JF, Lo YS, Lo YC, Lin PJ, Chang JS. Anaerobic hydrogen production with an efficient carrier-induced granular sludge bed bioreactor. Biotechnol Bioeng. 2004;87(5):648-57.

79. Kim BH, Gadd GM. Bacterial physiology and metabolism. Cambridge,New York, Melbourne, Madrid: Cambridge University Press; 2008.

80. Levin DB, Pitt L, Love M. Biohydrogen production: Prospects and limitations to practical application. Int J Hydrogen Energy. 2004;29(2):173-85.

81. Gaudy A, Gaudy E. Microbiology for environmental scientists and engineers. In: McGraw-Hill; 1980;519-66.

82. Chang JJ, Wu JH, Wen FS, Hung KY, Chen YT, Hsiao CL, Molecular, monitoring of microbes in a continuous hydrogen-producing system with different hydraulic retention time. Int J Hydrogen Energy. 2008;33(5):1579-85.

83. Koskinen PEP, Kaksonen AH, Puhakka LA. The relationship between instability of H-2 production and compositions of bacterial communities within a dark fermentation fluidized-bed bioreactor. Biotechnol Bioeng. 2007;97(4):742-58.

84. Noike T, Takabatake H, Mizuno O, Ohba M. Inhibition of hydrogen fermentation of organic wastes by lactic acid bacteria. Int J Hydrogen Energy. 2002;27(11-12):1367-71.

85. Fang HHP, Zhang T, Liu H. Microbial diversity of a mesophilic hydrogen-producing sludge. Appl Microbiol Biotechnol. 2002;58(1):112-8.

86. Ovreas L, Forney L, Daae FL, Torsvik V. Distribution of bacterioplankton in meromictic lake saelenvannet, as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. Appl Environ Microbiol. 1997;63(9):3367-73.

87. Muyzer G, Dewaal EC, Uitterlinden AG. Profiling of complex microbial-populations by denaturing gradient gel-electrophoresis analysis of polymerase chain reaction-amplified genes-coding for 16s ribosomal-rna. Appl Environ Microbiol. 1993;59(3):695-700.

88. Zhang T, Fang HHP. Digitization of DGGE (denaturing gradient gel electrophoresis) profile and cluster analysis of microbial communities. Biotechnol Lett. 2000;22(5):399-405.

89. Liu WT, Chan OC, Fang HHP. Microbial community dynamics during start-up of acidogenic anaerobic reactors. Water Res. 2002;36(13):3203-10.

90. Fischer SG, Lerman LS. Length-independent separation of DNA restriction fragments in 2-dimensional gel-electrophoresis. Cell. 1979;16(1):191-200.

91. Ueno Y, Haruta S, Ishii M, Igarashi Y. Microbial community in anaerobic hydrogen-producing microflora enriched from sludge compost. Appl Microbiol Biotechnol. 2001 ;57(4):555-62.

CHAPTER 3

The Effect of Heat Pretreatment Temperature on Fermentative Hydrogen Production Using Mixed Cultures

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3.1 Introduction

Biological hydrogen production is potentially regarded as one of the most promising alternatives for sustainable green energy production [1, 2]. Among various biological hydrogen production processes, dark fermentation is of significant importance, due to its higher rate of hydrogen evolution in the absence of any light source and its applicability to different types of organic wastes and wastewaters [3]. In spite of the intensive research on the dark fermentative hydrogen production, no commercial systems are yet available. One of the challenges for large scale biological hydrogen production is the preparation of large amounts of active and stable inocula from natural sources. To improve hydrogen production, the inocula must be enriched by the elimination of methanogens, homoacetogens, and sulfate reducing bacteria. The method by which the seed is enriched is considerably important as it may affect the start-up, overall efficiency and the stability of a continuous hydrogen production system [4].

Enrichment by heat treatment is the most common technique for screening of hydrogen producing bacteria. Heat treatment of inocula eliminates non-spore forming hydrogen consuming microorganisms such as methanogens and initiates spore germination in Clostridia by altering their germination receptors [5, 6]. Furthermore, since the germination of spores occur in a batch mode prior to the continuous operation, the risk of the inocula wash-out is minimized [5]. A wide range of heat pretreated natural sources including anaerobically digested sludge [7], activated sludge [8], soil [9], cow dung [10], compost [11] and river sediments [12] has been used for continuous hydrogen production. Taking advantage of the heat treatment, Chang and Lin [13] have reported 8 months of constant hydrogen production and substrate degradation using municipal

sewage sludge in a UASB operating at the HRT of 8 to 20 hours. Han *et al.* [14] used the heat treated sludge to inoculate four 50 L fixed-bed reactors in the acidogenic stage of a pilot-scale two-stage hydrogen and methane producing system. The volatile solids removal efficiency and hydrogen production rate were 70.9% and 3.55 $m^3/m^3/d$, respectively. Repeated heat treatment of a fraction of return sludge in a continuous hydrogen producing system was reported to increase the yield of hydrogen production, but did not significantly affect the volumetric hydrogen production rate [6].

The cost-effectiveness of the heat treatment and the predominance of the sensitive Clostridial species in heat treated inocula make this approach controversial and necessitate further research. Enrichment of inocula by heat pretreatment consumes thermal energy. The energy required for heat treatment might be economized using the excess heat in the process. Furthermore, the treatment can be less energy intensive if it is conducted at lower temperatures. Various treatment temperatures have been used in the literature for the enrichment of the hydrogen producing inocula. Pretreatment at boiling temperature is the most frequent reported enrichment method [15]. However, pretreatment at low temperatures of 75°C and 85°C [16, 17] as well as high temperature of 104 [18] have been used for hydrogen production from mixed cultures. In all of these studies a variety of natural and synthetic feedstock, various inocula and different operation conditions have been used. Natural variability of bacterial cultures coupled with the complex biological hydrogen production mechanisms do not facilitate comparison between different studies. To the best of our knowledge and based on what is stated in the literature [15], no comparative study has been conducted to evaluate the effect of heat treatment at different temperatures on hydrogen production at the same

experimental conditions. Temperatures of the heat pretreatment may significantly affect the microbial community and energy required for the process.

The purpose of this research is to comprehensively compare the effect of heat pretreatment temperatures on the enrichment of two types of natural inocula, activated sludge and anaerobically digested sludge, for hydrogen production as well as to investigate the relationship between the metabolites, microbial community and hydrogen production at each pretreatment temperature.

3.2 Materials and Methods

3.2.1 Inocula and Treatment Conditions

Two inoculum sources were investigated for hydrogen production: anaerobically digested sludge from St. Marys, Ontario, municipal wastewater treatment plant; and activated sludge from the Adelaide Pollution Control Plant in London, Ontario. Prior to their use, the sludge inocula were thickened and then sieved through 2 mm screen. In order to enrich hydrogen-producing bacteria, the inocula were heat treated at 65°C, 80°C and 95°C for 30 minutes. Untreated inocula were also used as control.

3.2.2 Batch Experiments

Hydrogen production experiments were conducted in duplicates in 320 mL-vials containing 120 mL of media. The concentration of volatile suspended solids (VSS) in each vial was 2 g/L. In all samples, the medium contained 10 g/L glucose as the carbon source and the following inorganic salts (mg/L): NH_4Cl, 2600; K_2HPO_4, 250;

$MgCl_2.6H_2O$, 125; $FeSO_4.7H_2O$, 5.0; $CoCl_2.6H_2O$, 2.5; $MnCl_2.4H_2O$, 2.5; KI, 2.5; $Na_2MoO_4.2H_2O$, 0.5; H_3BO_4, 0.5; $NiCl_2.6H_2O$, 0.5;$ZnCl_2$, 0.5. The solution was buffered with 0.07 M phosphate and the initial pH was adjusted to 6.7 using 2M NaOH and 2M HCl. Each vial was purged with nitrogen for one minute. The cultures were placed in a shaker-incubator at 37°C and 190 rpm. The volume and the composition of the produced gas and also the concentration of the soluble metabolites were measured up to several hours after hydrogen production stopped.

3.2.3 Repeated Batch Experiments

After the completion of a batch operation (see previous section for the procedures), 60 mL of the culture were collected and added to 60 mL of fresh medium (to make the total working volume of 120 mL) and the pH was adjusted to 6.7 under anaerobic conditions. The same procedure was repeated four times for other consecutive batches. During each batch, the amount and composition of the produced gas were measured. Soluble metabolites were detected at the end of each batch.

3.2.4 Analysis

The biogas composition including hydrogen, methane and nitrogen was determined by a gas chromatograph (Model 310, SRI Instruments, Torrance, CA) equipped with a thermal conductivity detector (TCD) and a molecular sieve column (Molesieve 5A, mesh 80/100, 6 ft X 1/8 in). The presence of hydrogen sulfide in the biogas was checked using another gas chromatograph (6890 series, Agilent) equipped with a flame photometric detector (FPD) and a capillary column (HP-5, 10 m × 0.32 mm

× 0.25 μm film thickness). The total gas volume was measured by releasing the gas pressure in the vials using a glass syringe (5-50 mL) to equilibrate with the room pressure as recommended by Owen *et al.* [19]. Gas volumes were corrected to standard conditions (25°C and 1 atm).

The concentrations of organic acids including acetic acid, butyric acid, iso-butyric acid, lactic acid, valeric acid, iso-valeric acid and formic acid were analyzed using a high- performance liquid chromatography system (1200 series, Agilent Technologies) equipped with Aminex HPX-87H ion exclusion column (300 mm × 7.8 mm I.D.; BIO-RAD), and a UV-detector at 210 nm. The column temperature was adjusted to 30 °C. The same instrument with a refractive index detector (RID) was used to measure the concentrations of glucose and alcohols. The temperature of the RID detector was set to 35 °C. The concentrations of total suspended solids (TSS) and volatile suspended solids (VSS) were measured according to standard methods [20].

The cumulative hydrogen production profiles were fitted with modified Gompertz equation (1). Gompertz equation (developed in 1825) is a mathematical model which shows a sigmoidal curve for a time series, where growth is slowest at the beginning and at the end of a time period. This model has been used to describe the bacterial growth. Lay *et al.* [21] derived the Equation (1) from a Gompertz equation which explained the growth of *Lactobacillus plantarum* and *Lactobacillus aciodophilus* and satisfactorily used it to describe biological methane production. The modified Gompertz equation (1) can also be used for fermentative hydrogen production:

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

where H is the cumulative hydrogen production (mL), P is the maximum hydrogen production (mL), R_m is the maximum hydrogen production rate (mL/ h), λ is the lag phase time (h) and t is the incubation time (h). The cumulative hydrogen data were fitted with Gompertz equation using CurveExpert 1.3. Pearson correlation coefficients were determined using SPSS 10.0 (SPSS Inc., USA).

3.2.5 Bacterial Community Analysis

The total genomic community DNA was extracted and purified using UltraClean Soil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA). The primer set of 349FGC (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCC TACGGGAGGCAGCAG-3') and 518R (5'–ATTACCGCGGCTGCTGG-3') at the annealing temperature of 53°C were used for the PCR amplification of the variable V3 region of 16SrDNA from the purified genomic DNA. Denaturing gradient gel electrophoresis (DGGE) of PCR products was performed with a DCode universal mutation system (Bio-Rad laboratories, Hercules, CA, USA). The PCR products were applied directly to 8% (w/v) polyacrylamide gel with 15 to 55% denaturant (urea) gradients. Electrophoresis was performed at a constant voltage of 130 V at 59°C for 4.5 hours. The DNA template of the bands of interest were re-amplified and the PCR products were purified using QIAquick PCR purification Kit (QIAGEN Sciences, Maryland, USA) in accordance with the manufacturer's protocol. The sequences of re-amplified DNA fragments were determined by dideoxy chain termination (Sequencing

Facility, John P. Robarts Research Institute, London, Ontario) and compared with available sequences in GenBank database using the BLAST program [22].

3.3 Results and Discussions

3.3.1 Effect of Pretreatment on Fermentative Hydrogen Production

Figures 3.1 (a) and (b) show the cumulative hydrogen production at different pretreatment temperatures for the activated and anaerobic sludges, respectively. The produced biogas in all of the samples only contained hydrogen and carbon dioxide. No methane and hydrogen sulfide were detected in any of the samples during the 165 hours of batch fermentation.

When activated sludge was used as an inoculum in the batch tests, hydrogen production started after a short lag time of less than 7 h (Figure 1a). Untreated activated sludge produced only 82 mL biogas which contained 39 mL of hydrogen. A significant increase in hydrogen production was observed at pretreatment temperatures of 65°C and 80°C. The maximum amounts of hydrogen produced with heat treatment at 65 and 80°C were 313 and 265 mL, respectively. However, a 21.6% and 25.7% decrease in the hydrogen production was observed after 110 and 140 h for 65°C and 80°C heat-pretreated activated sludge, respectively.

The cumulative hydrogen production curves for anaerobic sludge are shown in Figure 1b. The untreated anaerobic sludge yielded the least amount of both hydrogen (70 mL) and total gas (131 mL). Pretreated sludges at 65°C and 80°C produced 342 and 317 mL of hydrogen, respectively. The percentages of hydrogen in the evolved gas for the 65°C and 80°C pretreated sludges were 56.2% and 54.1%, respectively. The amount of

hydrogen production in the 95 °C pretreated sludge was approximately 15% less than the amount produced by the 65°C pretreated inoculum. When anaerobic sludge was used as inoculum, at all pretreatment temperatures, no decrease in cumulative hydrogen was observed after hydrogen production stopped.

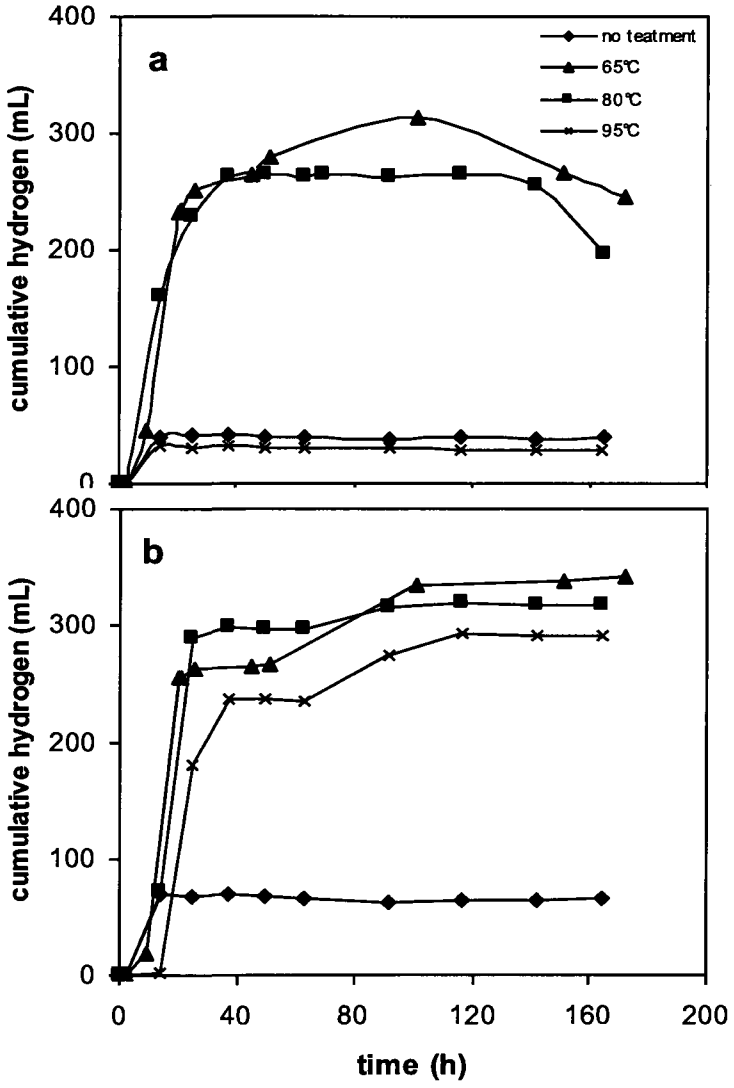

Figure 3. 1 Cumulative hydrogen production with activated sludge (a) and anaerobically digested sludge (b) pretreated at different temperatures

Hydrogen yields (mol hydrogen/mol glucose), the specific hydrogen production (mmol hydrogen/g VSS) and growth yields (g VSS/g glucose) are illustrated in Table 3.1. The highest yield (2.3 mol hydrogen/mol glucose) and specific hydrogen production (63.3 mmol hydrogen/g VSS) were achieved with the 65°C pretreated anaerobic sludge. The hydrogen yields and specific hydrogen production decreased with the increase of pretreatment temperatures in both types of inocula. Low hydrogen production was observed with both untreated activated sludge and untreated anaerobic sludge. Hydrogen production in the 95°C pretreated activated sludge was even less than the untreated inoculum, which reflects the suppressing effect of the heat pretreatment at high temperatures on this inoculum. However, the lower treatment temperatures of 65 and 80°C could enhance the hydrogen production about 7.3 and 6.7 times in comparison with untreated sample, respectively (these values were calculated based on 39 mL of evolved hydrogen in the untreated activated sludge and maximum hydrogen production in 65 and 80°C pretreated activated sludge shown in Table 3.2).

The microbial load in all the samples before the heat treatment was approximately 2 g VSS/L. No significant difference was observed in the volatile suspended solids (VSS) before and after the heat pretreatment. However, different pretreatment temperatures resulted in different VSS concentrations at the end of the fermentation, with final VSS concentrations in the untreated activated sludge and the treated activated sludge at 65, 80 and 95°C of 2.3, 2.4, 2.5 and 2.9 g VSS/L, respectively. The corresponding values for anaerobic sludge were 3.2, 3.1, 3.2 and 2.6 g VSS/L. The related growth yields are depicted in Table 3.1. Growth yields in the anaerobic sludge were higher than the ones observed in the activated sludge, except for the vials which were heat treated at 95°C.

Heat treatment at 95°C resulted in high growth yield of 0.16 g VSS/ g glucose in the activated sludge together with the production of lactic acid as the predominant metabolite (data will be presented later). The calculated yields in this study are within the range of 0.03 to 0.16 g VSS/g glucose which are close to the yields of 0.04 to 0.15 g VSS/g glucose reported by Cheong and Hansen [23].

Table 3. 1 Effect of pretreatment temperatures on hydrogen production and bacterial growth

Inoculum source	Heat treatment temperature	Hydrogen production yield (mol H_2/ mol glucose)	Specific hydrogen production (mmol H_2/gVSS)	Growth yield* $Y_{x/s}$ (g VSS/g glucose)
Activated sludge	no treatment	0.26 ± 0.02	7.2 ± 0.1	0.03
	65°C	1.64 ± 0.07	45.7 ± 2.9	0.05
	80°C	1.32 ± 0.21	36.6 ± 6.8	0.05
	95°C	0.19 ± 0.08	5.4 ± 0.1	0.16
Anaerobic sludge	no treatment	0.43 ± 0.05	12.0 ± 3.3	0.13
	65°C	2.30 ± 0.08	63.6 ± 2.6	0.12
	80°C	2.12 ± 0.05	59.0 ± 1.7	0.12
	95°C	1.95 ± 0.07	54.2 ± 2.8	0.07

3.3.2 Kinetic Analysis

The kinetic parameters estimated by modified Gompertz equation are presented in Table 3.2. The maximum hydrogen production rate (R_m) decreased with increasing heat treatment temperature for both activated sludge and anaerobically digested sludge samples. The highest R_m (26.3 mL/h) and maximum specific hydrogen production rate (109.6 mL/g VSS.h) were observed for the anaerobic sludge pretreated at 65°C. The

increase in the treatment temperature had a negative effect on the hydrogen production rate. The descending order of R_m values in all inocula tested was as follows: 65°C heat treated anaerobic sludge > 80°C treated anaerobic sludge > 65°C treated activated sludge > 95°C treated anaerobic sludge > 80°C treated activated sludge .

Table 3. 2 Kinetic parameters of hydrogen production for various pretreatment temperatures

Inoculum source	Heat treatment temperature	Maximum hydrogen production P (mL)	Maximum hydrogen production Rate R_m (mL/h)	Lag time λ (h)	Maximum specific H_2 production rate (mL/g vss.h)	R	RMSE
activated sludge	65°C	284.7	19.3	6.76	80.4	0.9944	12.62
	80°C	262.5	15.5	3.80	64.6	0.9990	4.69
anaerobic sludge	65°C	338.0	26.3	9.27	109.6	0.9999	2.25
	80°C	309.9	22.4	10.87	93.3	0.9983	7.47
	95°C	283.1	16.35	14.55	68.1	0.9963	11.87

R: correlation coefficient value, RMSE: root mean square error

3.3.3 Intermediate Metabolites Production

The concentrations of soluble metabolites were measured at various time intervals during the course of hydrogen production. The formation of metabolites together with glucose consumption for the activated sludge and anaerobic sludge are illustrated in Figures 3.2 and 3.3, respectively. The organic fatty acids detected during hydrogen production were acetate, butyrate, lactate, propionate, formate and iso-valerate, while ethanol was the sole alcohol detected in all cases.

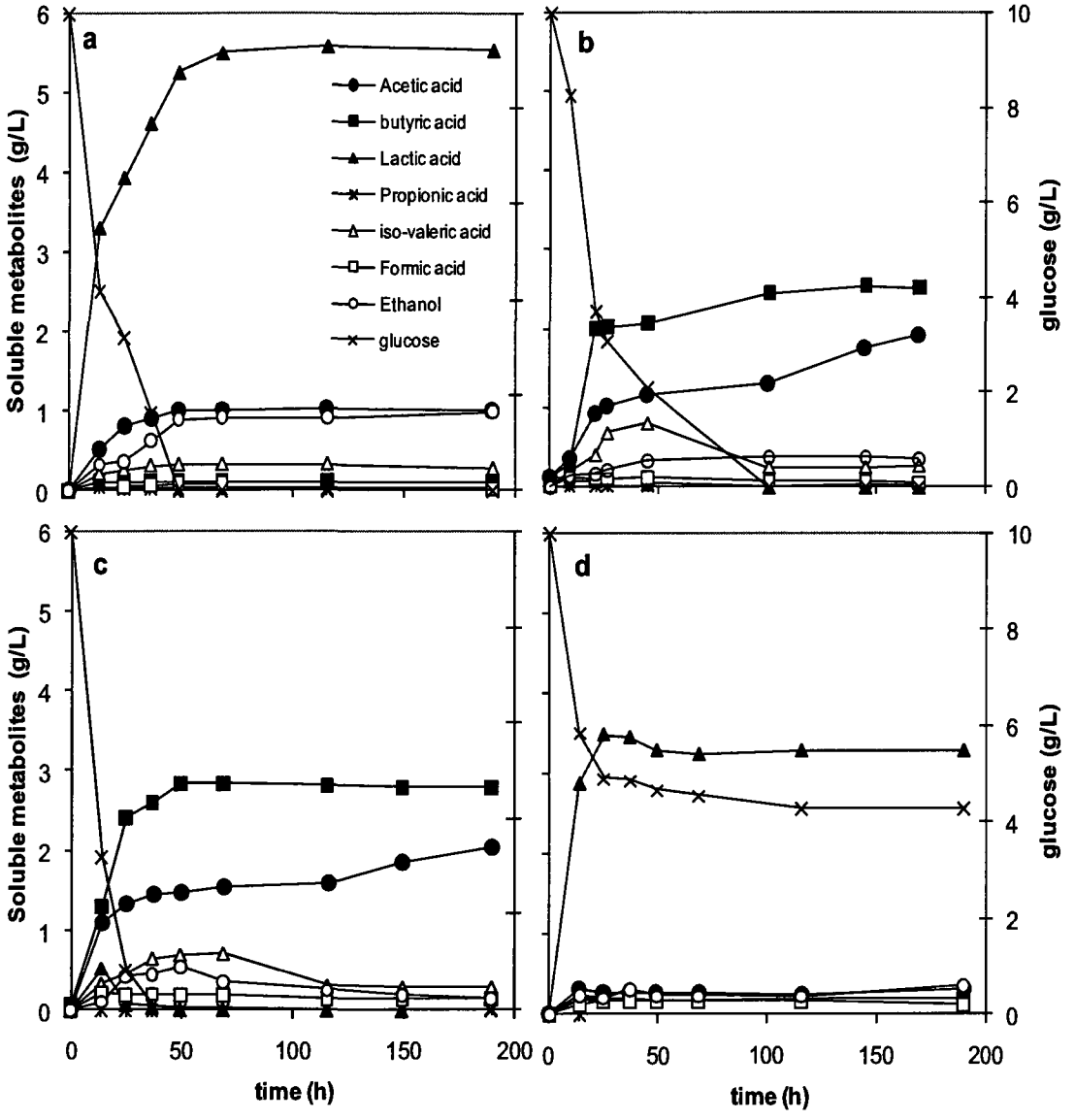

Figure 3. 2 Soluble metabolites formation and glucose consumption with activated sludge as inoculum after: (a) no treatment; (b) heat treatment at 65°C; (C) heat treatment at 80°C; (d) heat treatment at 95°C

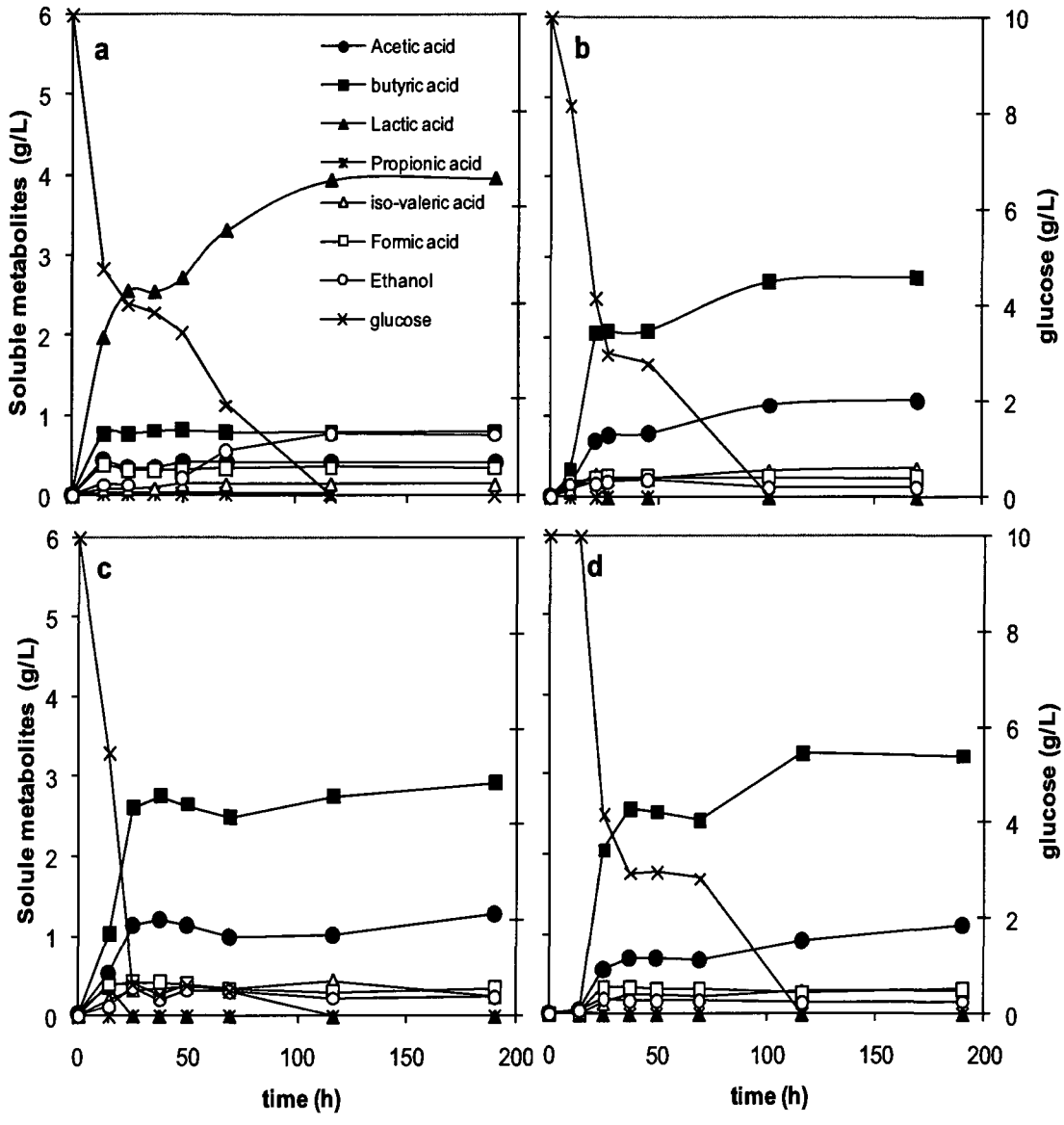

Figure 3. 3 Soluble metabolites formation and glucose consumption with anaerobically digested sludge as inoculum after: (a) no treatment; (b) heat treatment at 65°C; (c) heat treatment at 80°C; (d) heat treatment at 95°C

The intermediate metabolite formation showed different profiles depending on the pretreatment temperatures and the type of the inocula. Except for the 95°C pretreated anaerobic sludge which exhibited the largest lag time of 14.5 h (Table 3.2) other inocula started acid formation and glucose utilization almost immediately. In all the inocula with high levels of hydrogen production, including the activated sludge treated at 65°C and 80°C, and the anaerobic sludge treated at 65°C, 80°C and 95°C, hydrogen production was accompanied by the formation of high amounts of butyrate and acetate (Figures 3.2 and 3.3). High levels of ethanol were observed only in the untreated inocula (Figures 3.2a and 3.3a) with poor hydrogen production. Statistical analysis, based on Pearson correlation coefficients, showed that the cumulative hydrogen production was significantly correlated with acetate, butyrate and total VFA (Table 3.3). Pearson correlation coefficients between 0.85 and 1.0 were considered as good association. No significant correlation was observed between cumulative hydrogen production and ethanol.

Table 3. 3 Pearson correlations of cumulative hydrogen production with major soluble metabolites during the course of hydrogen production

Inoculum source	Heat treatment temperature	Acetate	Butyrate	Ethanol	Total VFAs
activated sludge	no treatment	0.868**	0.991**	0.674**	0.885**
	65 C	0.993**	0.979**	0.869	0.966**
	80 C	0.991**	0.969**	0.803	0.972**
	95 C	0.963**	0.983**	0.855**	0.989**
anaerobic sludge	no treatment	0.966**	0.995**	0.455	0.908**
	65 C	0.979**	0.998**	0.501	0.993**
	80 C	0.959**	0.990**	0.861	0.956**
	95 C	0.978**	0.998**	0.853	0.998**

**: Correlation is significant at the 0.01 level (2-tailed)

In the untreated activated sludge, the 95°C pretreated activated sludge and untreated anaerobic sludge, lactic acid was the predominant soluble metabolite (Figures 3.2a, 3.2d and 3.3a). All of these batches produced very low amount of hydrogen gas (according to Figure 3.1). These results correspond with some other studies [24, 25] which show the inhibitory effect of lactic acid fermentation on hydrogen production. Noike *et al.* [24] demonstrated the inhibitory effect of two lactic acid bacteria, *Lactobacillus paracasie* and *Entrococcus durans*, on hydrogen production caused by the lethal effect of bacteriocins excreted from these bacteria on other bacteria including hydrogen producers. Heat treatment at 50°C for 30 minutes was reported as an efficient method for the elimination of these inhibiting bacteria. Although, such bacteria could possibly be responsible for the inhibition of the hydrogen production in the untreated

inocula, they could not cause inhibition to 95°C treated activated sludge because of their inability to form heat resistant spores. However, *Clostridium butyricum* and *Clostridium acetobutyricum* which were identified in this study (will be presented in the following text) use branched metabolic pathways with the capability of lactate formation [26, 27] and are likely to be the cause of lactate production. Although lactic acid was produced in some of the other inocula during the first 24 h of fermentation, it was totally consumed later.

Comparison of soluble metabolite production curves for activated sludge pretreated at 65°C and 80°C (Figure 3.2b and 3.2c) and the corresponding hydrogen production curves (Figure 3.1a) illustrates that net decrease in hydrogen after 110 hours and 140 hours in the 65°C and 80°C pretreated activated sludge was accompanied by an increase in acetic acid concentration. This could be because of hydrogen consumption by homoacetogens through the following reaction [28]:

$$4H_2 + 2CO_2 \rightarrow CH_3COO^- + H^+ + 2H_2O \qquad (2)$$

Equation (2) indicates that 1 mol of acetate can be produced by consumption of 4 moles of hydrogen. In the activated sludge pretreated at 65°C, consumption of 67 mL (2.99 mmol) of hydrogen gas corresponded with 482 mg/L (0.889 mmol) acetate formation which is 119% of the theoretical acetate formation calculated from Equation (2). Consumption of 68 mL (3.04 mmol) hydrogen in the 80°C pretreated activated sludge was accompanied by 460 mg/L (0.804 mmol) increase in acetate which is 106% of the theoretical value. The high yield values could be because of the acetate formation by

other metabolic reactions. Although these results stoichiometrically substantiate the high probability of hydrogen loss through homoacetogensis in the activated sludge pretreated at 65 and 80°C, it is also plausible that uptake hydrogenase activities of Clostridium species may have attributed to hydrogen consumption [29].

Table 3.4 summarizes the characteristics of soluble products after 116 h of fermentation. Higher concentrations of acetate in the activated sludge samples (7.7-43.7 %) versus those in anaerobic sludge (8.0-32.1 %) could be attributed to the presence of more facultative anaerobes in the activated sludge, as the conversion of glucose to acetate is the favorable reaction of the facultative anaerobes, while the conversion to butyrate is the typical reaction observed in strict anaerobic bacteria [3]. In the vials with poor hydrogen production (untreated activated and anaerobic sludges and activated sludge pretreated at 95°C) lactic acid was the predominant metabolite with concentrations between 50.8 and 68.3% of total soluble metabolites. Propionic acid which is an undesirable metabolite in hydrogen fermentation was observed in very low amounts (0.0-0.5 %) in all the vials. Interestingly, it was observed that the pretreatment temperature affected the ratio of butyrate to acetate (Table 3.4). Increasing the pretreatment temperature from 65 to 95°C in anaerobically digested sludge caused an increase in the butyrate/ acetate ratio from 1.5 to 2.4. In activated sludge increasing the treatment temperature from 65 to 80°C raised the butyrate/acetate ratio from 0.9 to 1.2. Heat treatment at lower temperatures resulted in lower butyrate/acetate ratio accompanied by higher hydrogen production, which is consistent with the findings of Khanal *et al.* [30] and Vanandel *et al.* [31] who demonstrated that driving pathways towards a lower butyrate/acetate ratio (or a higher acetate/butyrate ratio), by changing environmental

conditions, enhances hydrogen production. This behavior can be explained by the major metabolic reactions involved in hydrogen production through butyrate fermentation:

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (3)$$

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 \quad (4)$$

Since the conversion of glucose to acetic acid gives a maximum yield of 4 mol H_2/mol glucose and half of this yield is obtained with conversion of glucose to butyrate, driving the glucose consumption reaction towards higher acetate (or lower butyrate/acetate ratio) will give rise to hydrogen production.

Table 3. 4 Characteristics of soluble metabolites after 116 h incubation using activated sludge and anaerobically digested sludge

Inoculum source	Heat treatment temperature	TVFA (mmol/l)	SM (mmol/l)	acetate (%)	butyrate (%)	lactate (%)	propionate (%)	formate (%)	valerate (%)	ethanol (%)	Butyrate/ acetate
activated sludge	no treatment	86.0	105.7	16.3	1.2	58.6	0.3	1.9	3.1	18.6	0.1
	65 C	65.3	73.2	43.7	39.2	0.0	0.0	2.2	3.6	10.8	0.9
	80 C	66.3	71.8	36.7	44.8	0.0	0.1	4.6	3.1	7.7	1.2
	95 C	48.6	53.6	7.7	4.1	68.3	0.0	6.9	3.7	9.3	0.5
anaerobic sludge	no treatment	69.2	86.2	8.0	10.4	50.8	0.5	8.8	1.7	19.7	1.3
	65 C	60.2	63.0	32.1	49.6	0.0	0.0	8.2	5.7	4.3	1.5
	80 C	59.3	64.3	26.4	48.8	0.0	0.1	10.2	6.8	7.7	1.8
	95 C	61.1	64.2	23.8	57.9	0.0	0.1	9.1	4.4	4.7	2.4

TVFA: total volatile fatty acids
SM: soluble metabolites

The glucose removal efficiency, calculated as the difference between the initial and final glucose concentrations; and its conversion efficiency to soluble metabolites, based on the initial concentration of glucose, after 24 hours of incubation are shown in the Table 3.5. As it is illustrated in Table 3.5, the carbon balance on the soluble metabolites and evolved gases was satisfactorily closed within 88% to 97% for the pretreated inocula. It was observed that the pretreatment at 80°C resulted in faster glucose removal and lower hydrogen production. This unexpected anomaly at 80°C may be attributed to the formation of higher amounts of soluble metabolites (Table 3.5) which were not related to hydrogen production. As apparent from Figure 3.2, at the end of the fermentation, glucose was completely exhausted in all of the vials except for the 95°C treated activated sludge where only 54.5% of the initial glucose was utilized.

Table 3. 5 Effect of pre treatment temperature on substrate consumption (24 hours after starting of hydrogen production)

Inoculum source	Heat treatment temperature	Carbon balance closure (%)	SM (mg C/L)	Glucose removal efficiency [1] (%)	Glucose conversion efficiency [2] (%)
activated sludge	no treatment	89	2315	67.8	57.9
	65°C	97	2029	69.1	50.7
	80°C	94	2416	91.7	60.4
	95°C	91	1860	50.9	46.5
anaerobic sludge	no treatment	77	1796	60.2	44.9
	65°C	88	1735	70.2	43.4
	80°C	91	2361	93.3	59.0
	95°C	92	1992	70.5	49.8

SM: Soluble metabolites
[1]: Calculated as the difference between initial and final glucose concentrations
[2]: Calculated as the soluble metabolites produced per initial glucose (carbon basis)

3.3.4 Effect of pH

The pH of all of the samples in batch experiments was initially buffered with 0.07 M phosphate buffer and adjusted to 6.7. The final pH values for untreated inocula were about 3.4 and those of treated inocula ranged from 3.8 to 4.6 and 4.1 to 4.5 for the activated sludge and anaerobic sludge, respectively. The major pH drop in all of the samples occurred during the first 24 hours of fermentation (Figure 3.4). Faster pH drop in

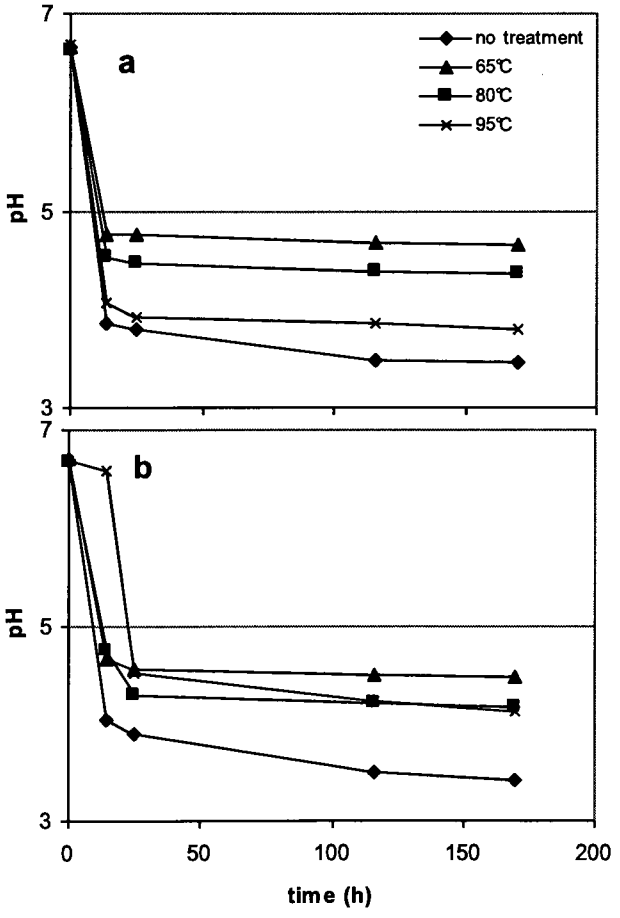

Figure 3. 4 pH profiles during hydrogen production with activated sludge (a) and anaerobically digested sludge (b) under different heat pretreatment conditions

the untreated inocula could be because of the higher production of fatty acids (Table 3.4). Since the activity of most methanogens is limited to a narrow pH range between 6.7 and 8 [32], the drop of pH could help the elimination of methanogenic activity in both treated and untreated inocula. It must be asserted that the difference in hydrogen yields could not be attributed to pH changes, since the pH in the anaerobic sludge pretreated at 95°C was almost identical to those pretreated at 65°C and 80°C despite widely disparate hydrogen production.

3.3.5 Microbial Community Analysis

The total genomic community DNA of the samples with high hydrogen production including the 65°C, 80°C pretreated activated sludge and anaerobic sludge pretreated at 65°C, 80°C and 95°C, were extracted and used for the analysis of microbial community by PCR-DGGE. The DGGE profiles of the 16S rDNA gene fragment at each treatment condition are illustrated in Figure 3.5. Table 3.6 shows the results of the sequence affiliation. In total, 11 bands and 7 species were identified. The number of the bands detected at the lower pretreatment temperatures was more than those detected at higher pretreatment temperatures, indicating that elevated pretreatment temperatures reduce species diversity.

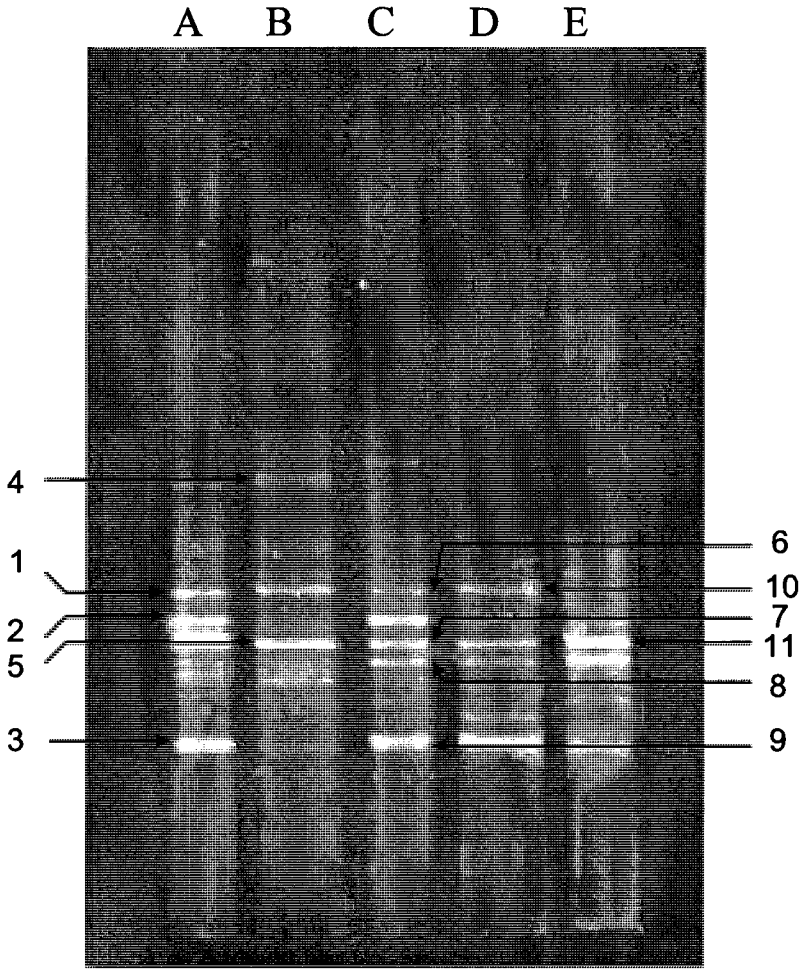

Figure 3. 5 DGGE profile of the 16S rDNA gene fragment at each treatment condition; A: activated sludge pretreated at 65°C, B: activated sludge pretreated at 80°C, C: anaerobically digested sludge pretreated at 65°C, D: anaerobically digested sludge pretreated at 80°C, E: anaerobically digested sludge pretreated at 95°C

Clostridium acetobutyricum (band1, 6 and 10) was detected in both activated sludge and anaerobic sludge samples. *Clostridium acetobutyricum* ferments carbohydrates to hydrogen and carbon dioxide with acetate and butyrate as the main soluble metabolites [33]. *Clostridium acetobutyricum* has a potential to shift from

hydrogen production towards acetone and butanol production. This shift may occur at the end of the exponential growth phase. Low growth rate, low pH and high concentrations of carbohydrates have been considered as some of the factors which stimulate solvent production by *Clostridium acetobutyricum* [34].

Table 3. 6 Affiliation of denaturing gradient gel electrophoresis (DGGE) fragments determined by their 16S rDNA sequence

Band	Affiliation (accession no.)	Similarity (%)
1	*Clostridium acetobutyricum* (AE001437.1)	94
2	*Clostridium sp.* JRI19 (EF067828.1)	93
3	*Uncultured bacterium* (DQ795258.1)	94
4	*Bacillus thuringiensis* (EF210289.1)	96
5	*Clostridium butyricum* (DQ831124.1)	100
6	*Clostridium acetobutyricum* (AE0011437.1)	99
7	*Clostridium Butyricum* (DQ831124.1)	100
8	*Clostridium sp.* HPB-21 (AY862509.1)	100
9	Uncultured *Clostridium sp.* (EF700377.1)	97
10	*Clostridium acetobutyricum (*AE001437.1)	98
11	*Clostridium butyricum* (DQ831124.1)	99

One of the bacterial species that was identified in the activated sludge inocula was *Bacillus thuringiensis* (band 4). Although, this bacterium has been reported as the predominant species in some activated sludge samples [35], to the knowledge of the authors, despite few reports of hydrogen production by some pure *Bacillus* species such

as *Bacillus licheniformis* and *Bacillus coagulans* [36, 37] or presence of other *Bacillus* species in mixed microflora of hydrogen producing reactors [38, 39], this species has not yet been reported in an anaerobic hydrogen production system.

Clostridium butyricum (band 5, 7 and 11) was found in all the hydrogen producing samples in this study. This species is one of the most frequently reported species in hydrogen producing mixed cultures [40, 41].

Although based on the stoichiometry, the hydrogen consumption in the pretreated activated sludge at 65°C and 80°C could be because of homoacetogensis, none of the identified bands in the DGGE profile of activated sludge samples belonged to the homoacetogenic species. It must also be added that there were other bands in the DGGE profile which were not identified.

3.3.6 Repeated Batch Experiments

In order to investigate the stability of hydrogen production with 65°C heat pretreated activated sludge and anaerobic sludge, theses sludges were operated in a repeated batch mode. Neither net hydrogen utilization nor methane and hydrogen sulfide formation were observed in the batches. Specific hydrogen production for the activated sludge substantially increased from 45 mmol/g VSS in the first batch to 164 mmol/g VSS in the fourth batch. The same increase in the specific hydrogen production was observed for anaerobic sludge with the corresponding values of 56 to 134 mmol/g VSS (Figure 3.6). These results show the effectiveness of heat treatment at 65°C for these two inocula. The metabolites measured at the end of each batch, indicated that other than ethanol which was in the range of 81 to 1607 mg/L in the activated sludge and 92 to

133 mg/L in the anaerobic sludge, there were no observed differences between the two sludges throughout the four consecutive batches.

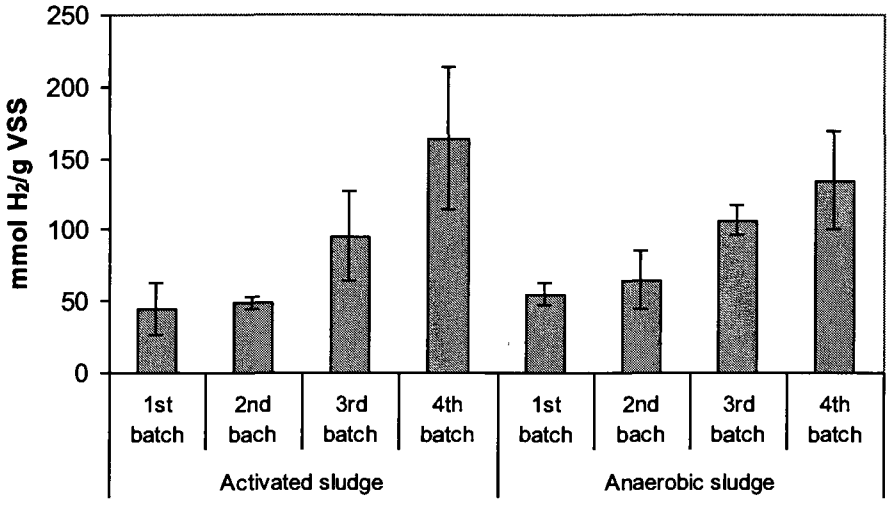

Figure 3. 6 Specific sequential hydrogen production in pretreated activated sludge and aerobically digested sludge at 65°C

3.4 Conclusions

The results of this work demonstrated that the hydrogen yield, hydrogen production rate and microbial community were influenced by various heat-pretreatment temperatures and this effect depended on the type of the inocula. Pretreatment at 65°C resulted in the highest hydrogen yield as well as the highest hydrogen production rates in both the activated sludge (1.6 mol hydrogen/mol glucose and 19.3 mL/h) and anaerobic sludge (2.3 mol hydrogen/mol glucose and 26.3 mL/h). Pretreatment at higher temperatures resulted in lower hydrogen production yields and rates and was

accompanied by higher butyrate/acetate ratio. When the pretreatment temperature was increased to 95°C in the activated sludge, hydrogen production was suppressed and metabolic pathways were observed to shift towards lactic acid formation. Our repeated batch experiments with the inocula treated at 65°C illustrated a good stability of the process during four consecutive batches. The analysis of microbial communities showed that elevated pretreatment temperatures reduced species diversity.

3.5 References

1. Zajic JE, Margaritis A, Brosseau JD. Microbial hydrogen production from replenishable resources. Int J Hydrogen Energy. 1979;4(5):385-402.

2. Margaritis A. Hydrogen energy in Canada I. Int J Hydrogen Energy. 1983;8(4).

3. Nath K, Das D. Improvement of fermentative hydrogen production: Various approaches. Appl Microbiol Biotechnol. 2004;65(5):520-9.

4. Hawkes FR, Dinsdale R, Hawkes DL, Hussy I. Sustainable fermentative hydrogen production: Challenges for process optimization. Int J Hydrogen Energy. 2002;27(11-12):1339-47.

5. Hawkes FR, Hussy I, Kyazze G, Dinsdale R, Hawkes DL. Continuous dark fermentative hydrogen production by mesophilic microflora: Principles and progress. Int J Hydrogen Energy. 2007;32(2):172-84.

6. Duangmanee T, Padmasiri SI, Simmons JJ, Raskin L, Sung S. Hydrogen production by anaerobic microbial communities exposed to repeated heat treatments. Water Environ Res. 2007;79:975-83.

7. Lay JJ. Modeling and optimization of anaerobic digested sludge converting starch to hydrogen. Biotechnol Bioeng. 2000;68(3):269-78.

8. Cohen A, Distel B, Vandeursen A, Breure AM, Vanandel JG. Role of anaerobic spore-forming bacteria in the acidogenesis of glucose - changes induced by discontinuous or low-rate feed supply. Antonie Van Leeuwenhoek Journal of Microbiology. 1985;51(2):179-92.

9. Oh SE, Lyer P, Bruns MA, Logan BE. Biological hydrogen production using a membrane bioreactor. Biotechnol Bioeng. 2004;87(1):119-27.

10. Vijayaraghavan K, Ahmad D, Bin Ibrahim MK. Biohydrogen generation from jackfruit peel using anaerobic contact filter. Int J Hydrogen Energy. 2006;31(5):569-79.

11. Fan KS, Chen YY. H-2 production through anaerobic mixed culture: Effect of batch S-0. Chemosphere. 2004;57(9):1059-68.

12. Zuo J, Zuo Y, Zhang W, Chen J. Anaerobic bio-hydrogen production using pre-heated river sediments as seed sludge. Water Science and Technology. 2005;52(10-11):31-9.

13. Chang FY, Lin CY. Biohydrogen production using an up-flow anaerobic sludge blanket reactor. Int J Hydrogen Energy. 2004;29(1):33-9.

14. Han SK, Kim SH, Kim HW, Shin HS. Pilot-scale two-stage process: A combination of acidogenic hydrogenesis and methanogenesis. Water Science and Technology. 2005;52(1-2):131-8.

15. Li CL, Fang HHP. Fermentative hydrogen production from wastewater and solid wastes by mixed cultures. Crit Rev Environ Sci Technol. 2007;37(1):1-39.

16. Chang JS, Lee KS, Lin PJ. Biohydrogen production with fixed-bed bioreactors. Int J Hydrogen Energy. 2002;27(11-12):1167-74.

17. Mu Y, Zheng XJ, Yu HQ, Zhu RF. Biological hydrogen production by anaerobic sludge at various temperatures. Int J Hydrogen Energy. 2006;31(6):780-5.

18. Khanal SK, Chen WH, Li L, Sung SW. Biological hydrogen production: Effects of pH and intermediate products. Int J Hydrogen Energy. 2004;29(11):1123-31.

19. Owen WF, Stuckey DC, Healy JB, Young LY, McCarty PL. Bioassay for monitoring biochemical methane potential and anaerobic toxicity. Water Res. 1979;13:485-492.

20. APHA, AWWA, WEF. Standard methods for examination of water and wastewater, 19th edition. ; 1995.

21. Lay JJ, Li YY, Noike T. Mathematical model for methane production from landfill bioreactor. J Environ Eng -ASCE. 1998;124(8):730-6.

22. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990;215(3):403-10.

23. Cheong DY, Hansen CL. Acidogenesis characteristics of natural, mixed anaerobes converting carbohydrate-rich synthetic wastewater to hydrogen. Process Biochemistry. 2006;41(8):1736-45.

24. Noike T, Takabatake H, Mizuno O, Ohba M. Inhibition of hydrogen fermentation of organic wastes by lactic acid bacteria. Int J Hydrogen Energy. 2002;27(11-12):1367-71.

25. Kawagoshi Y, Hino N, Fujimoto A, Nakao M, Fujita Y, Sugimura S, Effect of inoculum conditioning on hydrogen fermentation and pH effect on bacterial community relevant to hydrogen production. J Biosci Bioeng. 2005;100(5):524-30.

26. Crabbendam PM, Neijssel OM, Tempest DW. Metabolic and energetic aspects of the growth of *Clostridium butyricum* on glucose in chemostat culture. Arch Microbiol. 1985;142(4):375-82.

27. Jones DT, Woods DR. Acetone-butanol fermentation revisited. Microbiol Rev. 1986 ;50(4):484-524.

28. Diekert G, Wohlfarth G. Metabolism of homoacetogens. Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology. 1994;66(1-3):209-21.

29. Das D, Dutta T, Nath K, Kotay SM, Das AK, Veziroglu TN. Role of Fe-hydrogenase in biological hydrogen production. Curr Sci. 2006;90(12):1627-37.

30. Khanal SK, Chen WH, Li L, Sung SW. Biological hydrogen production: Effects of pH and intermediate products. Int J Hydrogen Energy. 2004;29(11):1123-31.

31. Vanandel JG, Zoutberg GR, Crabbendam PM, Breure AM. Glucose fermentation by *Clostridium butyricum* grown under a self generated gas atmosphere in chemostat culture. Appl Microbiol Biotechnol. 1985;23(1):21-6.

32. Williams RT, Crawford RL. Methanogenic bacteria, including an acid-tolerant strain, from peatlands. Appl Environ Microbiol. 1985;50(6):1542-4.

33. Woods DR, editor. The Clostridia and biotechnology. USA: Butterworth-Heinemann; 1993.

34. Bahl H, Gottschalk G. Parameters affecting solvent production by *Clostridium acetobutyricum* in continuous culture. Biotechnol Bioeng Sym. 1984;14:215-223.

35. Mizuki E, Maeda M, Tanaka R, Lee DW, Hara M, Akao T, *Bacillus thuringiensis*: A common member of microflora in activated sludges of a sewage treatment plant. Curr Microbiol. 2001;42(6):422-5.

36. Kalia VC, Jain SR, Kumar A, Joshi AP. Fermentation of bio-waste to H_2 by *Bacillus licheniformis*. World J Microbiol Biotechnol. 1994(10):224-227.

37. Kotay SM, Das D. Microbial hydrogen production with *Bacillus coagulans* IIT-BT S1 isolated from anaerobic sewage sludge. Bioresour Technol. 2007;98(6):1183-90.

38. Ueno Y, Haruta S, Ishii M, Igarashi Y. Microbial community in anaerobic hydrogen-producing microflora enriched from sludge compost. Appl Microbiol Biotechnol. 2001 ;57(4):555-62.

39. Shin HS, Youn JH, Kim SH. Hydrogen production from food waste in anaerobic mesophilic and thermophilic acidogenesis. Int J Hydrogen Energy. 2004 ;29(13):1355-63.

40. Kim DH, Han SK, Kim SH, Shin HS. Effect of gas sparging on continuous fermentative hydrogen production. Int J Hydrogen Energy. 2006;31(15):2158-69.

41. Koskinen PEP, Kaksonen AH, Puhakka LA. The relationship between instability of H_2 production and compositions of bacterial communities within a dark fermentation fluidized-bed bioreactor. Biotechnol Bioeng. 2007;97(4):742-58.

CHAPTER 4

Fermentative Hydrogen Production by Diverse Microflora

A version of this chapter is submitted to International Journal of Hydrogen Energy

4.1 Introduction

Dark fermentation presents a promising method for sustainable hydrogen production due to the potential use of different types of biomass including wastewater streams, food scraps, animal waste, crop residual and energy crops. Although high yields of hydrogen have been reported with pure species [1-3], use of pure cultures is not plausible due to the prohibitive cost of sterilization. For a technically feasible process, mixed cultures obtained from natural sources which are able to operate on non-sterile feedstocks are required. A wide range of natural sources including municipal sludge [4, 5], compost [6], soil [7] and even river sediments [8] directly or after some treatment have been used for continuous biohydrogen production. Heat treated anaerobically digested sludge can be regarded as the most frequently used inocula for hydrogen producing systems [9-11]. Clostridia, which are strict anaerobes, are usually the predominant species in mixed cultures of hydrogen producing systems [12] and make these systems more difficult to control. Clostridia are extremely vulnerable to oxygen inhibition and upon sporulation, due to unfavorable environmental condition very specific nutritional and environmental requirements are needed to trigger spore germination [13]. Facultative anaerobes, such as *Enterobacter* sp., have lower hydrogen production yields of approximately 1 mol/mol glucose [14] but they are able to consume oxygen and help maintain the anaerobic condition in reactors. Yokoi *et al.* [1] obtained 2.6 moles hydrogen per mole glucose with a mixed culture of *Clostridium butyricum* and *Enterobacter aerogenes*. Since the available oxygen in the system was utilized by the facultative anaerobe, *Enterobacter aerogenes*, the mixed culture was more tolerant to environmental conditions. Waste activated sludge is another diverse microflora which

rarely has been used for inoculation of hydrogen producing systems [15, 16] and potentially contains more facultative anaerobes.

Activated sludge and anaerobically digested sludge have been compared under different pre-treatment conditions at mesophilic temperature [17, 18]. However, the behavior of these two microflora has never been compared at different operation temperatures (mesophilic versus thermophilic). Since hydrogen production at thermophilic temperature has been repeatedly reported in literature [19-21] and sometimes regarded more favorable because of lower dissolved hydrogen concentrations at thermophilic temperatures [22] such a comparative study will not only reveal useful information about using different microflora for hydrogen production but may have practical implications. Thus, overall objective of this study is comparison of activated sludge and anaerobically digested sludge as two types of inocula for hydrogen production at mesophilic and thermophilic temperatures. In addition the stability of the cultures at mesophilic temperature has been investigated in repeated batch experiment.

4.2 Materials and Methods

4.2.1 Inoculum and Treatment Conditions

Waste activated sludge from the Adelaide Pollution Control Plant in London, Ontario and anaerobically digested sludge from St. Marys, Ontario, municipal wastewater treatment plant were used as the microbial sources for hydrogen production. Prior to inoculation, the sludge inocula were thickened. In order to enrich the hydrogen-producing bacteria and avoid the elimination of the non-spore forming facultative anaerobes, the inocula were heat-treated at 65 °C for 30 minutes.

4.2.2 Batch Experiments

Batch experiments were performed in duplicates in 320 mL-batch vials containing 140 mL of media. The nutrient medium contained 10 g/L glucose as the carbon source and the following inorganic salts (mg/L): NH_4Cl, 2600; K_2HPO_4, 250; $MgCl_2.6H_2O$, 125; $FeSO_4.7H_2O$, 5.0; $CoCl_2.6H_2O$, 2.5; $MnCl_2.4H_2O$, 2.5; KI, 2.5; $Na_2MoO_4.2H_2O$, 0.5; H_3BO_4, 0.5; $NiCl_2.6H_2O$, 0.5;$ZnCl_2$, 0.5. The amount of inoculum added to each vial was equivalent to a VSS concentration of 2 g/L. The solution was buffered with 0.05 M phosphate and the initial pH was adjusted to 7 using 2M NaOH and 2M HCl. Each vial was purged with nitrogen for one minute to provide an anaerobic condition. The vials were placed in a shaker-incubator at 37°C or 55°C and 180 rpm.

4.2.3 Repeated Batch Experiments

Repeated batch experiments were conducted at 37 °C. In repeated batch experiments, after the completion of each batch operation, 60 mL of the culture was collected and added to 60 mL of fresh medium (to make a 120 mL working volume) and the pH was adjusted to 7 using 2M NaOH and 2M HCl under anaerobic conditions. In each batch, the amount and composition of the produced gas were measured.

4.2.4 Analytical Procedures

The total gas volume was measured intermittently by releasing the gas pressure in the vials using glass syringes (Perfektum; Popper & Sons Inc., NY, USA) according to Owen *et al.* [23]. Gas volumes were corrected to standard conditions (25°C and 1 atm). Biogas composition was determined by a gas chromatograph (Model 310, SRI

Instruments, Torrance, CA) equipped with a thermal conductivity detector (TCD) and a molecular sieve column (Molesieve 5A, mesh 80/100, 6 ft X 1/8 in). The temperatures of the column and the TCD detector were 90 °C and 105 °C, respectively. Argon was used as the carrier gas at a flow rate of 30 mL/min.

In order to measure pH during the fermentation 2 mL liquid samples were taken from the vials and pH was determined using a Symphony SB70P pH meter (VWR Scientific products, Canada). The concentrations of volatile fatty acids and ethanol were measured by a gas chromatograph (Varian 8500) with a flame ionization detector (FID) equipped with a fused silica column (30m × 0.32 mm). Helium was used as the carrier gas at a flow rate of 5 mL/min. The temperatures of the column and detector were 110 and 250°C, respectively. The concentrations of lactic acid was analyzed using a high performance liquid chromatography system (1200 series, Agilent Technologies) equipped with Aminex HPX-87H ion exclusion column (300 mm × 7.8 mm I.D.; BIO-RAD), and a UV-detector at 210 nm. The column temperature was adjusted to 30 °C. Concentration of glucose was measured using a colorimetric method based on reaction with glucose oxidase enzyme. The commercial kit (220-32; Diagnostic Chemical Limited, Charlottetown, Canada) was used for this measurement. The concentrations of volatile suspended solids (VSS) were measured according to standard methods [24].

The cumulative hydrogen production profiles were fitted with the modified Gompertz equation [25]:

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

where H is the cumulative hydrogen production (mL), P is the maximum hydrogen production (mL), R_m is the maximum hydrogen production rate (mL/h), λ is the lag phase time (h) and t is the incubation time (h). The cumulative hydrogen data were fitted with Gompertz equation using the CurveExpert software (Curve Expert 1.34, Hyams D.G., Starkville, MS, USA).

4.2.5 Microbial Community Analysis

In repeated batch experiments after the first, third and fifth batches the total genomic DNA were extracted and after PCR amplification were analyzed by denaturing gradient gel electrophoresis. The detailed procedure has been described in our previous work [18].

4.3 Results and Discussions

The hydrogen production data were satisfactorily correlated (R^2 = 0.9930-0.9994) based on the modified Gompertz equation (Equation 1). The estimated kinetic parameters at different operating temperatures are presented in Table 4.1. No methane was detected in any of the samples during the course of gas production. When activated sludge used as inocula the highest yield (1.32 mol H_2/mol glucose consumed) and highest specific hydrogen production rate (2.39 mL/h.gVSS) were observed at 55 °C. However, with anaerobically digested sludge at 37°C the yield was 2.18 mol H_2/mol glucose consumed compared with 1.25 mol H_2/mol glucose consumed at 55 °C; while the specific hydrogen production rate at 37°C and 55°C were comparable at 2.94 and 3.08 mL/h. g VSS).

Although the specific hydrogen production rate is higher at thermophilic temperature as illustrated in Table 4.1, the lower biomass yield which is one of the characteristics of thermophilic bacteria [22] reduced the observed hydrogen production rate and hydrogen yield at thermophilic temperature. It is noteworthy to mention that the highest hydrogen content of biogas (70%) was observed with activated sludge at 55 °C.

Table 4. 1 Kinetic parameters of hydrogen production at different temperatures

Inocula	Temperature (°C)	Kinetic parameters				Yield (mol H_2/mol Glucose consumed)	Specific H_2 production rate (mL/h.g VSS)	H_2 Content (%)
		P (mL)	R_m (mL/h)	λ (h)	R^2			
Activated Sludge	37	44.1	4.2	8.7	0.9994	0.56	2.13	57
	55	177.5	4.2	39.6	0.9930	1.32	2.39	70
Anaerobic sludge	37	275.1	7.9	9.9	0.9935	2.18	2.94	58
	55	214.0	5.8	45.1	0.9967	1.25	3.08	59

The experimental and fitted hydrogen production profiles, using activated sludge at 37°C and 55°C are shown in Figure 4.1. This figure also shows pH profiles and hydrogen production rate profiles (which were developed based on hydrogen production data from fitted Gompertz equation) in related batch experiments. At mesophilic temperature, hydrogen production started after a lag phase of approximately 9 hours and proceeded for 20 hours before stopping when the pH reached 4 after 30 hours. However, at the thermophilic temperature hydrogen production started after a longer lag period and all hydrogen production occurred when the pH of the culture was at 4. At the

thermophilic temperature, 177 mL hydrogen was produced by activated sludge which was four times higher than the amount produced at the mesophilic temperature. The production of hydrogen at pH values below 6 is preferred because the methanogens are effectively inhibited [13]. The maximum hydrogen production rates with activated sludge at 37 °C and 55 °C were identical and equal to 4.2 mL/h.

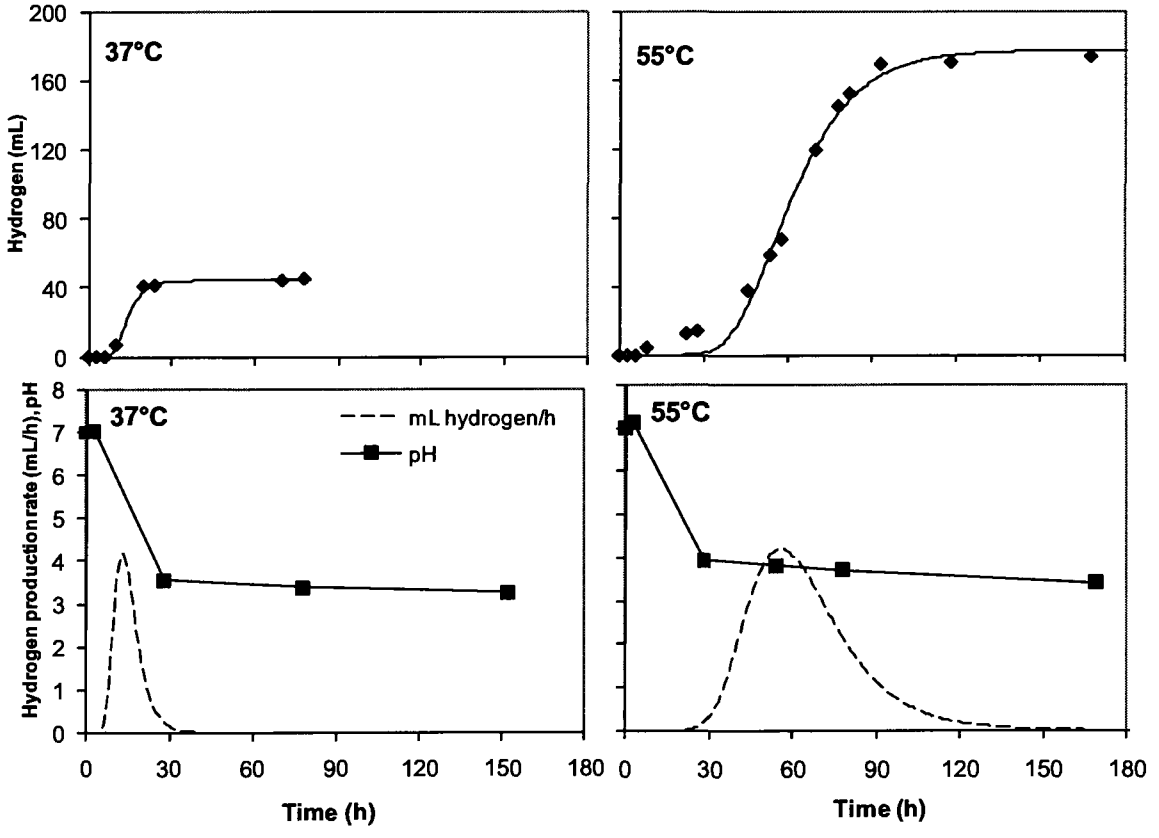

Figure 4. 1 Cumulative hydrogen production and hydrogen production rate profile using activated sludge as inoculum at mesophilic temperature (37 °C) and thermophilic temperature (55 °C)

When anaerobically digested sludge was used as the inoculum for hydrogen production (Figure 4.2), at 37 °C, hydrogen production commenced after 10 hours and reached 275 mL after 65 hours. At 55°C hydrogen production started after 45 hours and reached 213 mL. The maximum hydrogen production rates were 7.9 and 5.8 mL/h at mesophilic and thermophilic temperatures, respectively. Lower hydrogen production rates at thermophilic temperature can be attributed to the fact that many thermophilic microorganisms do not produce high cell densities in liquid cultures [22]. At 37 °C hydrogen production was insensitive to pH drop as it continued even after pH dropped to 4. The hydrogen production at 55 °C followed the observed trend with activated sludge at the thermophilic temperature, i.e. all hydrogen was produced at pH 4. The results illustrated that at thermophilic temperatures the hydrogen production with both inocula was less sensitive to pH, as opposed to mesophilic temperatures.

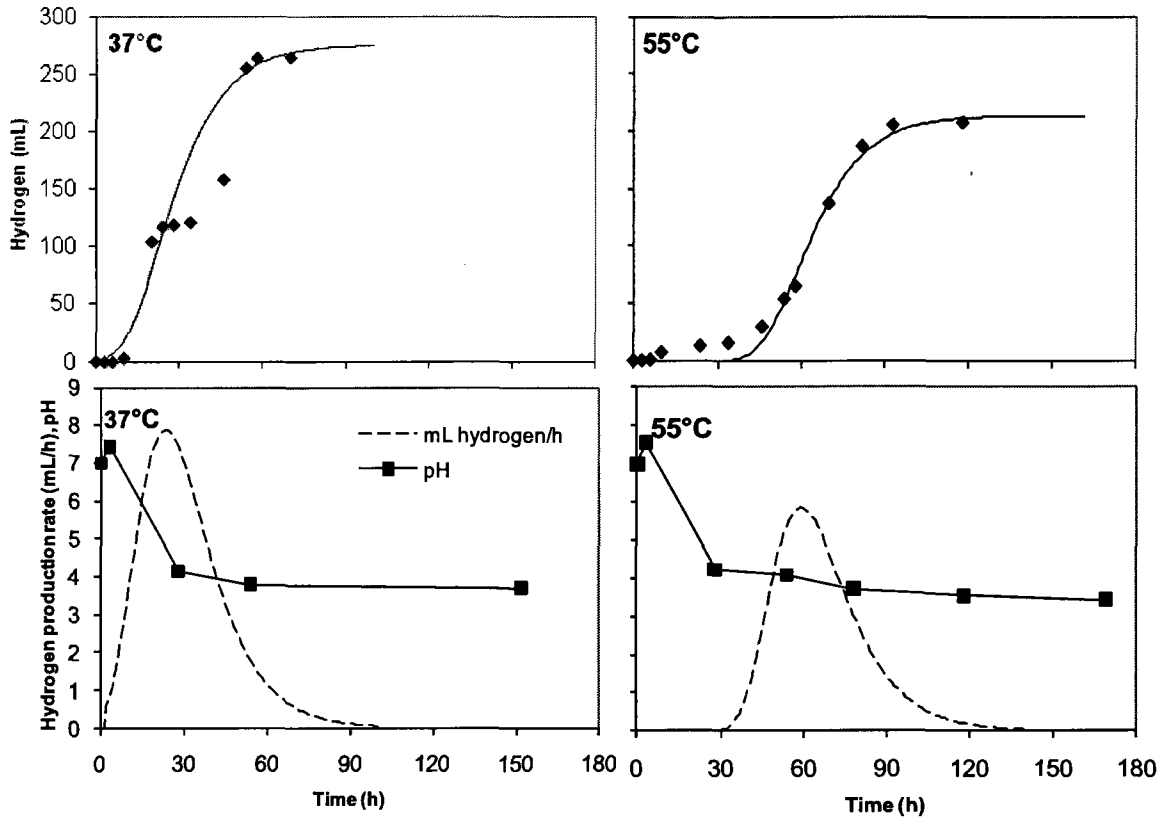

Figure 4. 2 Cumulative hydrogen production and hydrogen production rate profile using anaerobically digested sludge as inoculum at mesophilic temperature (37 °C) and thermophilic temperature (55 °C)

Table 4.2 shows the concentrations of the soluble metabolites at the end of the fermentation. Butyric acid and acetic acid were the dominant soluble metabolites with activated sludge at 55°C and anaerobic sludge at 37°C and 55°C. However, at mesophilic temperature the main product with the activated sludge was lactate at concentration of 936 mg/L which could be the main reason for the low hydrogen production yield [26]. The formation of ethanol with activated sludge (81-114 mg/L) was less than that of anaerobically digested sludge (165-326 mg/L).

Table 4. 2 Soluble metabolites formation with activated sludge and anaerobic digested sludge at 37 °C and 55 °C at the end of fermentation

Inocula	Temperature °C	Acetate (mg/L)	Butyrate (mg/L)	Propionate (mg/L)	Valerate (mg/L)	Lactate (mg/L)	Ethanol (mg/L)
Activated	37	242	327	0	0	936	81
Sludge	55	703	1014	9	8	N.T.	114
Anaerobic	37	1045	2733	50	5	25	326
sludge	55	692	1465	8	9	N.T.	165

N.T., not tested

The stability of hydrogen production with two inocula at 37°C was investigated in repeated batch experiments for 650 hours. As illustrated in Figure 4.3a, cumulative hydrogen production with activated sludge clearly increased after the first batch but it never remained constant during the five consecutive batches and significant discrepancies were observed as it was reflected by 149 mL hydrogen production in batch 2 and 74 mL in batch 3. However, when anaerobic sludge was used as inocula, although hydrogen production decreased after the first batch, the ultimate hydrogen production for the rest of batches varied within a narrow range of 110-130 mL hydrogen (Figure 4.3b).

The stability of hydrogen production with two inocula was investigated in repeated batch experiments for 650 hours. Due to extremely long lag time in repeated batch experiment at 55° C, repeated batch experiment conducted only at 37° C. As illustrated in Figure 4.3a, cumulative hydrogen production with activated sludge clearly increased after the first batch but it never remained constant during the five consecutive batches and significant discrepancies were observed as it was reflected by 149 mL hydrogen production in batch 2 and 74 mL in batch 3. However, when anaerobic sludge

was used as inocula, although hydrogen production decreased after the first batch, the ultimate hydrogen production for the rest of batches varied within a narrow range of 110-130 mL hydrogen (Figure 4.3b). The average yields of hydrogen production in activated sludge and anaerobically digested sludge were 1.21 ± 0.62 and 1.40 ± 0.16 mol H_2/ mol glucose consumed, respectively. The relatively high coefficient of variation (standard deviation/ mean) of 0.51 in activated sludge versus 0.12 in anaerobic sludge shows that hydrogen production was more stable with anaerobic sludge in comparison with activated sludge.

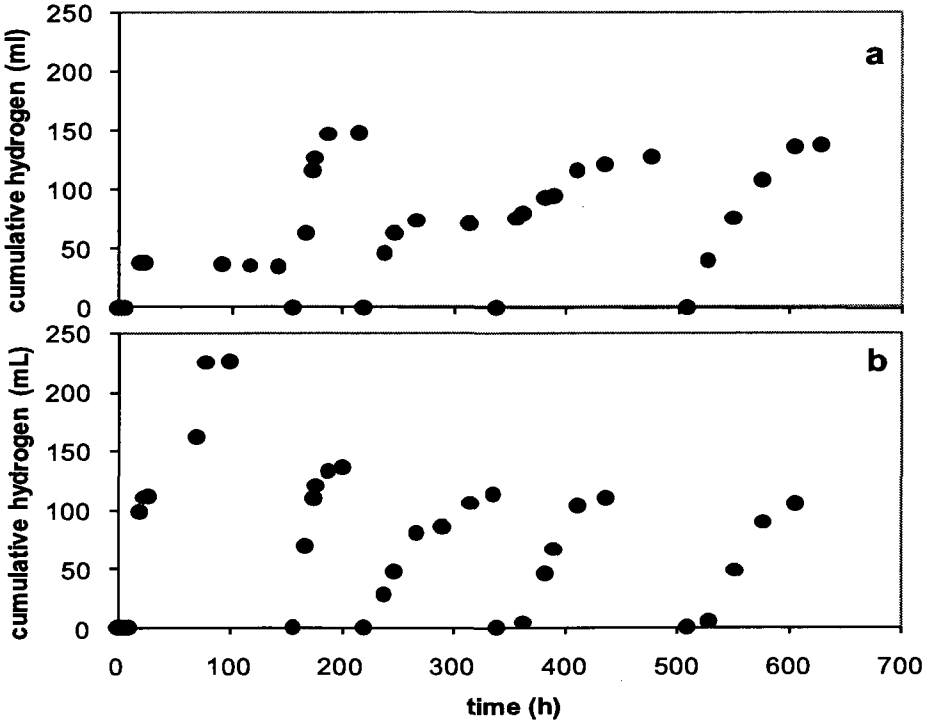

Figure 4. 3 Hydrogen production in five consecutive batches with activated sludge (a) and anaerobic digested sludge (b)

The investigation of the soluble metabolites at the end of each batch (Figure 4.4) exhibited that butyric acid and acetic acid were the predominant metabolites with anaerobic digested sludge. However, with activated sludge, high levels of ethanol and lactic acid were detected in some of the batches which indicated a shift from butyrate-type fermentation to ethanol and lactic acid fermentation. When activated sludge was used, formation of lactic acid followed a sequential pattern along the consecutive batches, it decreased from 936 mg/L in batch 1 to 634 mg/L in batch 2 and then increased to 2155 mg/L in batch 3, after that it dropped to 22 mg/L in batch 4 and again increased to 318 mg/L in batch 5. It has to be noted that in the batches with high concentrations of lactic acid, (batches 1 and 3) lowest cumulative hydrogen production was observed.

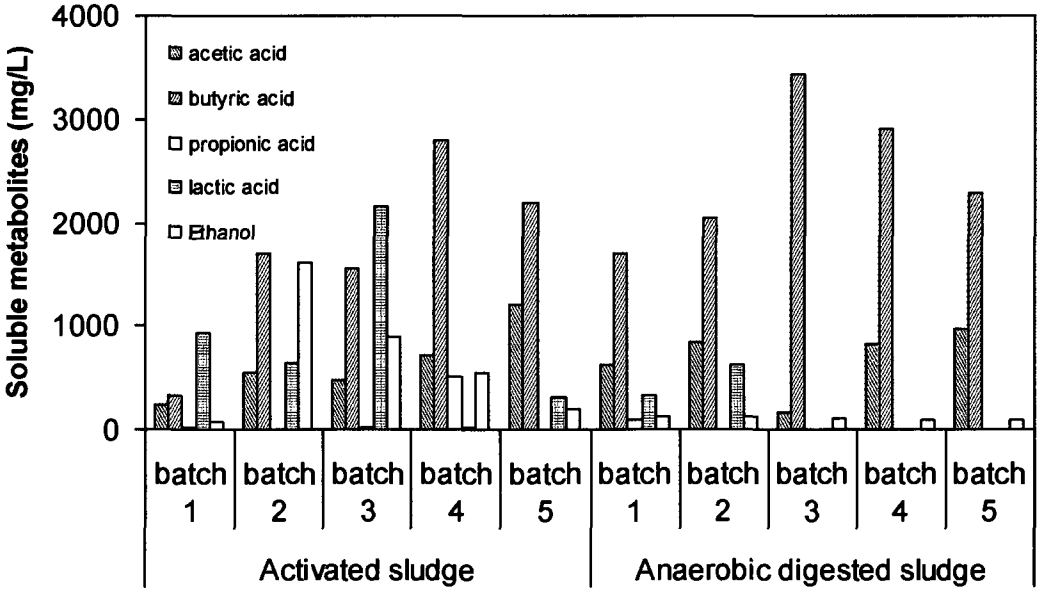

Figure 4. 4 Comparison of soluble metabolites formation during five consecutive batches with activated sludge and anaerobically digested sludge

The relationship between the hydrogen yield and lactic acid concentration at the end of consecutive batches has been illustrated in Figure 4.5. Increase in the formation of lactic acid until concentration of 634 mg/L resulted in 27% and 32% enhancement of hydrogen yield for activated sludge and anaerobic sludge, respectively. However, higher concentrations of lactic acid caused a sharp decrease in hydrogen yield. From these results it can be concluded that fluctuation in hydrogen production with activated sludge is because of formation of lactic acid as the predominant soluble metabolites in some of the batches. As for the anaerobic sludge, the highest observed concentration of lactic acid was 630 mg/L and hence the hydrogen yield did not drop drastically. This data suggests that threshold level of lactic acid in fermentative hydrogen production is approximately 650 mg/L.

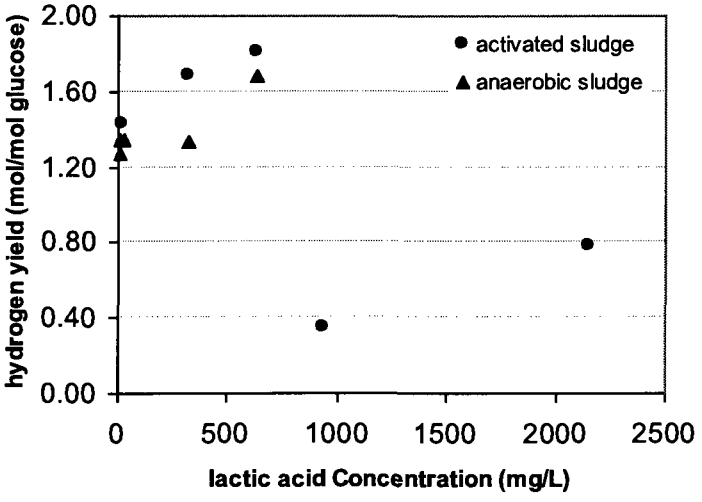

Figure 4. 5 Relationship between hydrogen yield and lactic acid concentration in repeated batch experiment with activated sludge and anaerobically digested sludge

In order to further investigate these two systems and find out the predominant species, at the end of the first, third and fifth batch, the DNA of the biomass was extracted and used for PCR-DGGE analysis. The DGGE profile of the 16S rDNA gene fragment (Figure 4.6) showed considerable variations during the re-cultivation of both types of inocula. Although activated sludge is potentially a more diverse microflora in comparison with anaerobically digested sludge, this diversity was not observed in the DGGE profile, which might be attributed to both heat treatment at 65°C and selectivity of the subsequent anaerobic cultivation process. The change in DGGE profile indicated that a different microbial community was formed in each batch and the cultures did not stabilize within 650 hours of operation in repeated batch mode.

Table 4.3 shows the results of the sequence affiliation. *C. butyricum* and *C. acetobutyricum* affiliated species were detected with both types of inocula. These two species are well known hydrogen producers which have frequently been reported in hydrogen-producing mixed microflora [9, 27]. Some of the other bands, which were affiliated to Clostridia including bands G, K and N, disappeared during the re-cultivations. The band K is affiliated to *C. aurantibutyricum* which is a potential hydrogen producer previously detected in biohydrogen production reactors [28]. *L. delbrueekii* which was detected in all consecutive batches with activated sludge is a homofermentative strain which can produce only lactic acid from glucose metabolism [29]. *L. fermentum* and *B. boum* were other detected bands which are heterofermentative species and can produce other metabolites such as acetate and ethanol in addition to lactate. Presence of lactic acid bacteria in the DGGE profiles showed that the formation of lactic acid in repeated batch experiments (Figure 4.4) was attributed to the identified

lactic acid bacteria not re-direction of metabolic pathways in hydrogen producers. In the first and fifth batch with anaerobic sludge where lactic acid was not significantly detected (Figure 4.4), no lactic acid bacteria were identified or the observed bands were weak (Figure 4.6).

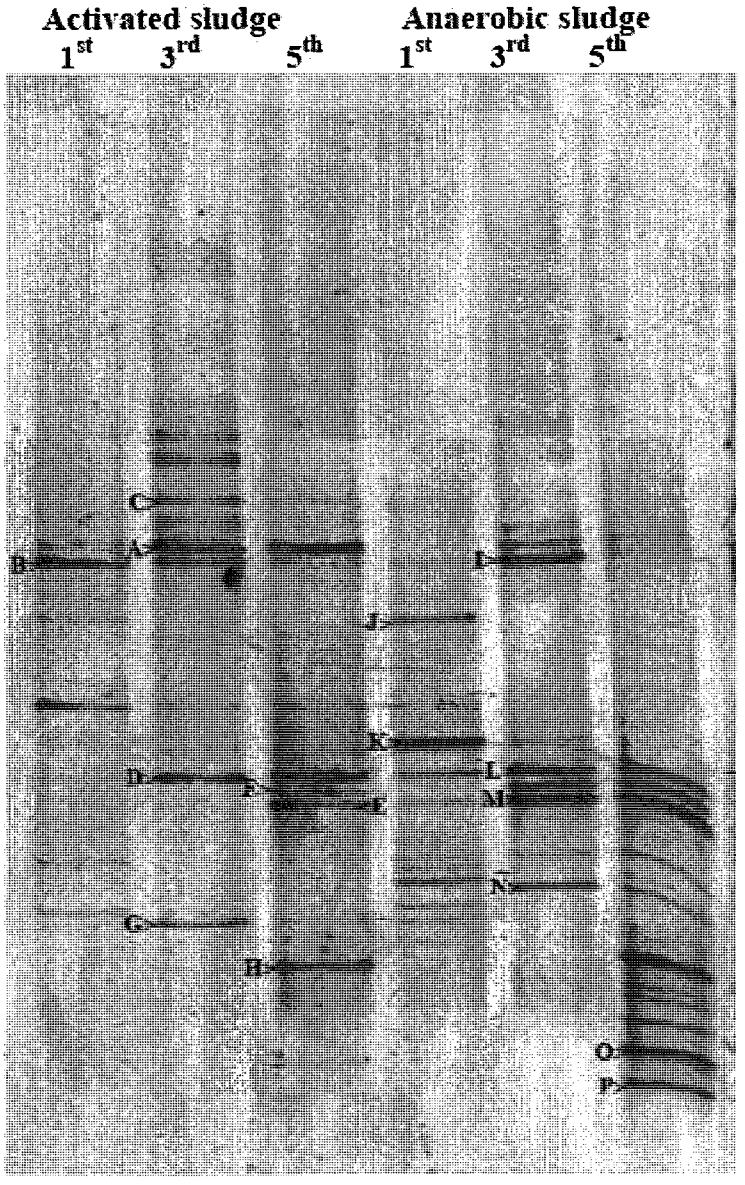

Figure 4. 6 DGGE profile of the 16S rDNA gene fragment using activated sludge and anaerobic sludge after the first, third and fifth consecutive batch

Table 4. 3 Affiliation of denaturing gradient gel electrophoresis (DGGE) fragments determined by their 16S rDNA sequence

Band	Affiliation (accession no.)	Similarity (%)
A	*L. delbrueekii* (FJ915705.1)	99
B	*L. delbrueekii* (FJ915706.1)	96
C	*L. fermentum* (GQ131282.1)	91
D	*C. butyricum* (DQ831124.1)	100
E	*C. acetobutyricum* (FM994940.1)	99
F	Uncultured bacterium (AB441617.1)	99
G	*Clostridium* sp. (FJ876436.1)	99
H	*B. boum* (AY166529.1)	98
I	*L. delbrueekii* (FJ915706.1)	98
J	Uncultured bacterium (DQ235219.1)	93
K	*C. aurantibutyricum* (FJ358641.1)	99
L	*C. butyricum* (DQ831124.1)	100
M	*C. acetobutyricum* (FM994940.1)	100
N	Uncultured *Clostridium* (DQ168846.1)	99
O	Uncultured Bacillus *sp.* (DQ168845.1)	95
P	Uncultured Bacillus *sp.* (DQ168845.1)	96

4.4 Conclusions

Preparation of an active and stable seed from natural microflora is one of the important stages of hydrogen production via dark fermentation which is influenced by the operational temperature. In this study hydrogen production with activated sludge and anaerobically digested sludge was compared at two temperatures of 37 °C and 55 °C. The highest yield of 2.18 mol H2/mol glucose consumed in batch experiments was observed

with anaerobically digested sludge at 37°C. The results of batch studies with activated sludge showed thermophilic temperatures are preferable for hydrogen production with this inoculum. For activated sludge, hydrogen yield of 1.32 mol H_2/mol glucose consumed was observed at 55°C with butyric acid (1596 mg/L) as the dominant soluble metabolite followed by acetic acid (703 mg/L). This yield was approximately 2.4 times more than hydrogen yield of 0.56 mol H_2/ mol glucose consumed at 37°C. Although with activated sludge, hydrogen production in repeated batch mode at mesophilic temperature increased after the first batch the cumulative hydrogen production did not converged to a stable trend. In anaerobic digested sludge, however, the cumulative hydrogen production consistency was observed after the first batch. The fluctuation of hydrogen production in the activated sludge was attributed to the presence of lactic acid bacteria in the microflora and their competition with hydrogen-producing bacteria. As apparent from results of this study, the potential diversity of activated sludge does not provide any advantages to hydrogen production relative to anaerobically digested sludge and is not appropriate for hydrogen production.

4.5 References

1. Yokoi H, Tokushige T, Hirose J, Hayashi S, Takasaki Y. H-2 production from starch by a mixed culture of clostridium butyricum and enterobacter aerogenes. Biotechnol Lett. 1998;20(2):143-7.

2. Chen WM, Tseng ZJ, Lee KS, Chang JS. Fermentative hydrogen production with clostridium butyricum CGS5 isolated from anaerobic sewage sludge. Int J Hydrogen Energy. 2005;30(10):1063-70.

3. Collet C, Adler N, Schwitzguebel JP, Peringer P. Hydrogen production by clostridium thermolacticum during continuous fermentation of lactose. Int J Hydrogen Energy. 2004;29(14):1479-85.

4. Lay JJ. Modeling and optimization of anaerobic digested sludge converting starch to hydrogen. Biotechnol Bioeng. 2000;68(3):269-78.

5. Cohen A, Distel B, Vandeursen A, Breure AM, Vanandel JG. Role of anaerobic spore-forming bacteria in the acidogenesis of glucose - changes induced by discontinuous or low-rate feed supply. Antonie Van Leeuwenhoek Journal of Microbiology. 1985;51(2):179-92.

6. Fan KS, Chen YY. H-2 production through anaerobic mixed culture: Effect of batch S-0. Chemosphere. 2004;57(9):1059-68.

7. Oh SE, Lyer P, Bruns MA, Logan BE. Biological hydrogen production using a membrane bioreactor. Biotechnol Bioeng. 2004;87(1):119-27.

8. Zuo J, Zuo Y, Zhang W, Chen J. Anaerobic bio-hydrogen production using pre-heated river sediments as seed sludge. Water Science and Technology. 2005;52(10-11):31-9.

9. Kim SH, Han SK, Shin HS. Effect of substrate concentration on hydrogen production and 16S rDNA-based analysis of the microbial community in a continuous fermenter. Process Biochemistry. 2006;41(1):199-207.

10. Kim DH, Kim SH, Ko IB, Lee CY, Shin HS. Start-up strategy for continuous fermentative hydrogen production: Early switchover from batch to continuous operation. Int J Hydrogen Energy. 2008;33(5):1532-41.

11. Kyazze G, Martinez-Perez N, Dinsdale R, Premier GC, Hawkes FR, Guwy AJ, Influence of substrate concentration on the stability and yield of continuous biohydrogen production. Biotechnol Bioeng. 2006;93(5):971-9.

12. Iyer P, Bruns MA, Zhang HS, Van Ginkel S, Logan BE. H2-producing bacterial communities from a heat-treated soil inoculum. Appl Microbiol Biotechnol. 2004;66(2):166-73.

13. Hawkes FR, Dinsdale R, Hawkes DL, Hussy I. Sustainable fermentative hydrogen production: Challenges for process optimisation. Int J Hydrogen Energy. 2002;27(11-12):1339-47.

14. Yokoi H, Ohkawara T, Hirose J, Hayashi S, Takasaki Y. Characteristics of hydrogen production by aciduric enterobacter aerogenes strain HO-39. J Ferment Bioeng. 1995;80(6):571-4.

15. Cohen A, Distel B, Vandeursen A, Breure AM, Vanandel JG. Role of anaerobic spore-forming bacteria in the acidogenesis of glucose - changes induced by discontinuous or low-rate feed supply. Antonie Van Leeuwenhoek Journal of Microbiology. 1985;51(2):179-92.

16. Eroglu E, Eroglu I, Gunduz U, Turker L, Yucel M. Biological hydrogen production from olive mill wastewater with two-stage processes. Int J Hydrogen Energy. 2006;31(11):1527-35.

17. Kawagoshi Y, Hino N, Fujimoto A, Nakao M, Fujita Y, Sugimura S, Effect of inoculum conditioning on hydrogen fermentation and pH effect on bacterial community relevant to hydrogen production. J Biosci Bioeng. 2005;100(5):524-30.

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

19. Oh YK, Kim SH, Kim MS, Park S. Thermophilic biohydrogen production from glucose with trickling biofilter. Biotechnol Bioeng. 2004;88(6):690-8.

20. Levin DB, Islam R, Cicek N, Sparling R. Hydrogen production by clostridium thermocellum 27405 from cellulosic biomass substrates. Int J Hydrogen Energy. 2006;31:1496-503.

21. O-Thong S, Prasertsan P, Intrasungkha N, Dhamwichukorn S, Birkeland N. Optimization of simultaneous thermophilic fermentative hydrogen production and COD reduction from palm oil mill effluent by thermoanaerobacterium-rich sludge. Int J Hydrogen Energy. 2008;33(4):1221-31.

22. Hallenbeck PC. Fundamentals of the fermentative production of hydrogen. Water Science and Technology. 2005;52(1-2):21-9.

23. Owen WF, Stuckey DC, Healy JB, Young LY, McCarty PL. Bioassay for monitoring biochemical methane potential and anaerobic toxicity. water Res. 1979;13:485-492.

24. APHA, AWWA, WEF. Standard methods for examination of water and wastewater, 19th edition. ; 1995.

25. Lay JJ, Li YY, Noike T. Mathematical model for methane production from landfill bioreactor. J Environ Eng -ASCE. 1998;124(8):730-6.

26. Noike T, Takabatake H, Mizuno O, Ohba M. Inhibition of hydrogen fermentation of organic wastes by lactic acid bacteria. Int J Hydrogen Energy. 2002;27(11-12):1367-71.

27. Koskinen PEP, Kaksonen AH, Puhakka LA. The relationship between instability of H2 production and compositions of bacterial communities within a dark fermentation fluidized-bed bioreactor. Biotechnol Bioeng. 2007;97(4):742-58.

28. Hung CH, Cheng CH, Cheng LH, Liang CM, Lin CY. Application of clostridium-specific PCR primers on the analysis of dark fermentation hydrogen-producing bacterial community. Int J Hydrogen Energy. 2008;33(5):1586-92.

29. Kim BH, Gadd GM. Bacterial physiology and metabolism. Cambridge, New York, Melbourne, Madrid: Cambridge University Press; 2008.

CHAPTER 5

Effect of Extrinsic Lactic Acid on Fermentative Hydrogen Production

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5.1 Introduction

Agricultural and food wastes, as well as carbohydrate-rich industrial wastewaters are promising substrates for biological hydrogen production [1, 2]. Acidogenic bacteria can ferment the carbohydrates in these feedstocks to hydrogen, carbon dioxide and volatile organic acids - mainly acetic and butyric acid. However, these bacteria are not able to further break down the organic acids to hydrogen due to the positive Gibbs free energy of the reaction.

Liu *et al.* [3] reported the production of hydrogen from acetate at the cathode of a microbial fuel cell. The biochemical barrier was overcome by increasing the electrochemical potential achieved by bacteria in the microbial fuel cell applying an additional voltage of at least 250 mV. Another report on hydrogen production from organic acids is from a mixture of acetic and lactic acids [4]. The *Clostridium diolis* used in that study was not able to produce hydrogen when acetic, citric, propionic and succinic acid were the only available organic substrates. However, when a mixture of acetic and lactic acid was used hydrogen and butyric acid were produced.

In dark fermentative hydrogen production, the presence of lactic acid as a metabolite during hydrogen production is frequently regarded as a sign of lower hydrogen production [5, 6, 7]. Lactic acid has been rarely studied as substrate for hydrogen production and despite reports of methane production from lactate [8], no significant hydrogen production was reported when lactate was used as the sole substrate [4, 9]. Hydrogen yield with a mixed culture using lactate as substrate was reported to be only 2.2 mL H_2/g lactate (COD basis) with a substrate conversion efficiency of 0.5% [9].

The effect of the addition of lactic acid to a carbohydrate-containing medium for hydrogen production has not been investigated yet. Our study on the effect of addition of some metabolites, including acetic acid, butyric acid, lactic acid and ethanol, to a hydrogen producing system showed that extrinsic lactic acid could enhance the hydrogen production. This paper is the first report of the enhancement in hydrogen production by addition of lactic acid to a carbohydrate-containing medium.

5.2 Materials and Methods

5.2.1 Inoculum and Treatment Condition

Municipal waste activated sludge from the Adelaide Pollution Control Plant in London, Ontario was used as the inoculum for hydrogen production. Prior to inoculation, the sludge was dewatered and then sieved through 2 mm screen. In order to enrich hydrogen-producing bacteria, the inoculum was heat treated at 70°C for 30 minutes.

5.2.2 Batch Experiments

All hydrogen production experiments were conducted in 320 mL batch vials. The nutrient medium was prepared using 5 g/L starch as a carbon source, plus the following inorganic salts (in mg/L): NH_4Cl, 2600; K_2HPO_4, 250; $MgCl_2.6H_2O$, 125; $FeSO_4.7H_2O$, 5.0; $CoCl_2.6H_2O$, 2.5; $MnCl_2.4H_2O$, 2.5; KI, 2.5; $Na_2MoO_4.2H_2O$, 0.5; H_3BO_4, 0.5; $NiCl_2.6H_2O$, 0.5;$ZnCl_2$, 0.5. The solution was buffered with 0.07 M sodium phosphate and the initial pH was adjusted to 7 using 2M NaOH or 2M HCl. After the addition of the inoculum each vial was purged with nitrogen for one minute. The cultures were placed in

a shaker-incubator at 35°C and 180 rpm. The experiments for this study were performed under two categories:

Fractional factorial experiments

These experiments included 15 batches to study the effect of addition of ethanol, lactic acid, butyric acid and acetic acid on hydrogen production. The concentration of different metabolites in each batch is presented in the "fractional factorial design section". The working volume was 120 mL and the inoculum concentration was 1.3 g VSS/L.

Lactic acid experiments

The experiments were conducted to study the effect of addition of lactic acid on hydrogen production. The concentration of lactic acid was 10 mM. The experiments were conducted at working volume of 140 mL with 0.8 g VSS/L inoculum.

5.2.3 Analytical Procedures

The total gas volume was measured by releasing the gas pressure in the vials using appropriately-sized glass syringes (Perfektum; Popper & Sons Inc., NY, USA) in the 5 to 50 mL range to equilibrate with the ambient pressure as recommended by Owen *et al.* [10]. Gas volumes were corrected to standard conditions (25°C and 1 atm). Biogas composition was determined by a gas chromatograph (Model 310, SRI Instruments, Torrance, CA) equipped with a thermal conductivity detector (TCD) and a molecular sieve column (Molesieve 5A, mesh 80/100, 6 ft × 1/8 in). The temperatures of the column and the TCD detector were 90 and 105°C, respectively. Argon was used as carrier gas at a flow rate of 30 mL/min.

The concentrations of volatile fatty acids (VFAs) were analyzed using a gas chromatograph (Varian 8500) with a flame ionization detector (FID) equipped with a

fused silica column (30m × 0.32 mm). Helium was used as carrier gas at a flow rate of 5 mL/min. The temperatures of the column and detector were 110 and 250°C, respectively. Lactic acid concentrations were measured using a high-performance liquid chromatography system (1200 series, Agilent Technologies) equipped with Aminex HPX-87H ion exclusion column (300 mm × 7.8 mm I.D.; BIO-RAD), and a UV-detector at 210 nm. The column temperature was adjusted to 30 °C. The same instrument with a refractive index detector (RID) was used to measure the concentrations of alcohols. The temperature of the RID detector was set to 35 °C. The concentrations of volatile suspended solids (VSS) were measured according to standard methods [11].

The cumulative hydrogen production profiles were fitted with the modified Gompertz equation [12]:

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

where H is the cumulative hydrogen production (mL), P is the maximum hydrogen production (mL), R_m is the maximum hydrogen production rate (mL/ h), λ is the lag phase time (h) and t is the incubation time (h). The cumulative hydrogen data were fitted with Gompertz equation using the CurveExpert software (Curve Expert 1.34, Hyams D.G., Starkville, MS, USA).

5.2.4 Fractional Factorial Design

A two-level irregular fractional factorial design (12 runs), augmented by three centre-point runs, was implemented to explore the effect of addition of ethanol (x_1) at

concentration of 5 mM and lactic acid (x_2), butyric acid (x_3) and acetic acid (x_4) at concentrations of 10 mM on the maximum hydrogen production and the hydrogen production rate. The maximum concentrations were chosen based on the average concentrations of these metabolites produced in our previous experiments. Table 5.1 summarizes the test levels of the experimental variables. The detailed experimental design with the coded values of the four variables is given in Table 5.2. The design array was set to determine the main effect of each factor and two factor interactions. The analysis of the variance was performed using the Design-Expert software (Version 7.0.0, Stat-Ease, Minneapolis, USA).

Table 5. 1 Experimental variables and their levels

Variables		Levels		
		-1	0	1
x_1	Ethanol (mM)	0	2.5	5
x_2	Lactic acid (mM)	0	5	10
x_3	Butyric acid (mM)	0	5	10
x_4	Acetic acid (mM)	0	5	10

5.3 Results and Discussion

5.3.1 Fractional Factorial Experiments

According to the design matrix (Table 5.2), fifteen batch experiments including three center points, were conducted. The cumulative hydrogen profiles in these experiments are shown in Figure 5.1. No methane was detected in any of the batches

during the course of gas production. It was observed that all the runs containing 10 mM lactic acid (level +1: runs 2, 5, 6, 9, 10 and 12) produced the highest amounts of hydrogen. The center point runs (13, 14 and 15), which contained 5 mM (level 0) lactic acid, produced slightly lower amounts of hydrogen. The lowest amounts of hydrogen, however, were evolved when no lactic acid was added to the culture (runs 1, 3, 4, 7, 8 and 11).

Table 5. 2 Design matrix with three central points of four variables with observed responses

Run [a]	x_1	x_2	x_3	x_4	Cumulative Hydrogen Production (ml)	Hydrogen Production rate (ml/h)
1	-1	-1	-1	-1	89.8	5.76
2	+1	+1	-1	-1	108.2	6.40
3	-1	-1	+1	-1	59.3	1.59
4	+1	-1	+1	-1	87.8	3.98
5	-1	+1	+1	-1	121.1	7.08
6	+1	+1	+1	-1	117.3	6.14
7	-1	-1	-1	+1	53.0	1.95
8	+1	-1	-1	+1	75.1	3.68
9	-1	+1	-1	+1	102.2	5.20
10	+1	+1	-1	+1	118.0	7.04
11	+1	-1	+1	+1	73.1	2.83
12	-1	+1	+1	+1	113.7	6.32
13	0	0	0	0	94.8	5.01
14	0	0	0	0	102.8	5.19
15	0	0	0	0	91.2	4.31

[a] The random order performed was 4, 10, 7, 15, 1, 3, 12, 5, 8, 2, 9, 6, 11,14, 13.

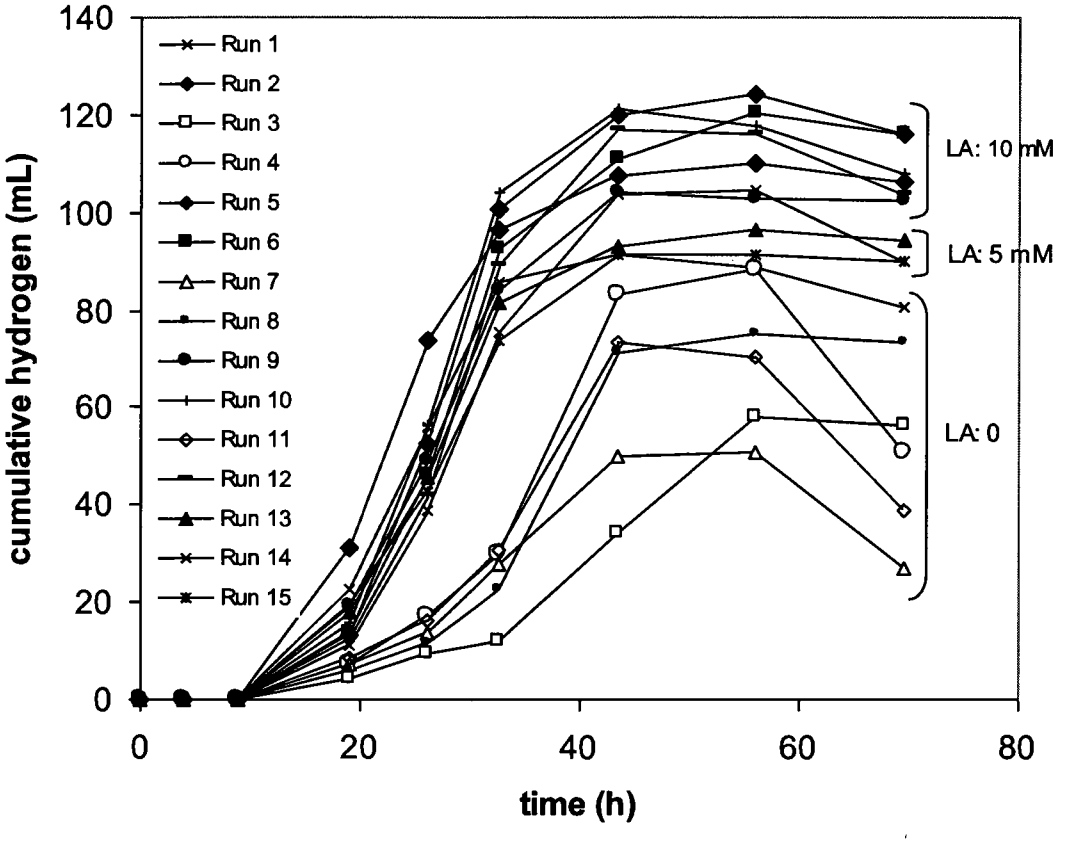

Figure 5. 1 Cumulative hydrogen production in different experimental conditions based on fractional factorial matrix. LA: Lactic acid concentration

The statistical analysis confirmed the large positive effect of lactic acid on hydrogen production. The maximum hydrogen production (P) and maximum hydrogen production rate (R_m) were estimated by the Gompertz equation (1) and used for the statistical analysis. The estimated equation for the maximum hydrogen production was obtained by multivariable regression in terms of the coded values x_1 to x_4 as follows,

$$P_{H2}=93.23 + 7.81\, x_1 + 20.19\, x_2 + 0.93\, x_3 - 3.72\, x_4 + 6.63\, x_2 x_3 + 6.74\, x_2 x_4 \qquad (2)$$

where P_{H2} is the predicted maximum hydrogen production (mL) and x_1 to x_4 are the coded values of ethanol, lactic acid, butyric acid and acetic acid concentrations as demonstrated in Table 5.1, which range from -1 to 1. The R-squared value of the model (0.9054) also shows that about 90 percent of the variability of P_{H2} is explained by x_1 to x_4, x_2x_3 and x_2x_4.

The analysis of the variance (ANOVA) is shown in Table 5.3. The calculated Fisher's F value (11.17) and a probability (*P*) value of 0.0028 in ANOVA demonstrates that the regression model was significant. Generally, if the *P* value is lower than 0.05, it indicates that the term is statistically significant [13]. It was observed from the ANOVA table that lactic acid (*P*= 0.0001) was the most significant term of the model. Ethanol (*P*= 0.0477) also showed a significant effect on hydrogen production. However, it is probably better to accept higher p-values (<0.1) to avoid the chance of missing an important factor [14]. Based on this concept, interactions of lactic acid-butyric acid (x_2-x_3) and lactic acid-acetic acid (x_2-x_4) having *P* values of 0.0811 and 0.0755, respectively, were also considered as marginally significant terms in the model. These interactions indicated that at higher concentrations of butyric and acetic acid the positive effect of lactic acid on hydrogen production increased. The butyric acid (x_3) and acetic acid (x_4) with *P* values of 0.7562 and 0.2275 were insignificant factors; however, they were included in the model to satisfy the hierarchy. The curvature *F* value of 0.26 implies that the curvature, as measured by the difference between the average of the center points and the average of the factorial points, in the design space was not significant. Therefore, the behavior can be represented by a linear change in the response between the high and low levels of experimental variables.

Table 5. 3 Analysis of Variance

Source	DF	SS	MS	*F*	*P*
Model	6	5707.47	951.24	11.18	0.0028
Ethanol (x_1)	1	488.75	488.75	5.74	0.0477
Lactic acid (x_2)	1	4890.70	4890.70	57.47	0.0001
Butyric acid (x_3)	1	9.26	9.26	0.11	0.7512
Acetic acid (x_4)	1	147.87	147.87	1.74	0.2289
$x_2 \times x_3$	1	352.17	352.17	4.14	0.0814
$x_2 \times x_4$	1	363.00	363.00	4.27	0.0778
Curvature	1	22.07	22.07	0.26	0.6262
Residual	7	595.72	85.10		
Lack of Fit	5	524.04	104.81	2.92	0.2742
Pure Error	2	71.68	35.84		
Total	14	6325.26			

DF, degree of freedom; SS, sum of Squares; MS, mean square; *F*, Fisher F value; *P*, Probability

The same statistical analysis was also applied for the maximum hydrogen production rate as the response. The results illustrated that only the lactic acid had a significant positive effect on the maximum hydrogen production rate (P= 0.000) and the effects of the other compounds were insignificant.

5.3.2 Lactic Acid Experiments

The results of the fractional factorial design confirmed that addition of lactic acid to the media could enhance both maximum hydrogen production and hydrogen production rate. In order to further study this enhancement, the cumulative hydrogen

production from starch in the presence of lactic acid (10 mM) was investigated and compared with the cases in which only starch or lactic acid were present in the culture (Figure 5.2). It was observed that the presence of lactic acid in a starch-containing media increased the hydrogen production from 89.5 to 119.9 mL/g COD starch. However, when lactic acid was the sole carbon source, only 2.9 mL (3.0 mL/g COD lactic acid) hydrogen was produced, similar to the previous observation of 2.3 mL/ g COD lactate in the literature [9].

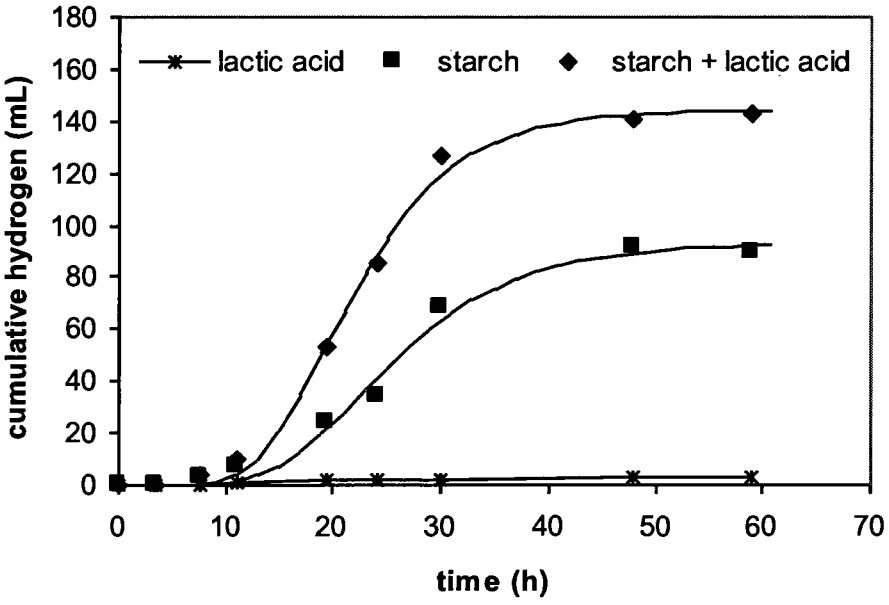

Figure 5. 2 Cumulative hydrogen production for cultures conducted with lactic acid, starch, starch and lactic acid

The kinetic parameters estimated by the modified Gompertz equation (1) are presented in Table 5.4. The lag time (λ) in the medium containing both lactic acid and

starch was slightly lower than the one with only starch. When 10 mM lactic acid was added to the starch medium the maximum hydrogen production rate (Rm) and the maximum hydrogen production (P) increased from 4.3 mL/h to 8.2 mL/h and from 93.0 mL to 144.1 mL, respectively. The hydrogen production yield also increased by 59% from 5.70 to 9.08 mmol H_2/ g starch. However, even after considering the additional carbon available for fermentation from the lactic acid, hydrogen yield increased by 35% from 14.25 to 19.25 mmol H_2/g carbon.

Table 5. 4 Performance of fermentative hydrogen production with different substrate conditions

Culture condition	Kinetic parameters				H_2 yield (mmol H_2/ g starch)	Biomass (g VSS/L)	Final pH
	P (mL)	R_m (mL/h)	λ (h)	R			
Starch	93.0	4.31	14.41	0.9944	5.70	1.31	6.1
Starch + lactic acid	144.1	8.23	12.82	0.9980	9.08	1.35	5.9
Lactic acid	2.9	NA	NA	NA	NA	0.9	6.9

For the experiments shown in Figure 5.2, the concentrations of soluble metabolites were measured at various time intervals during the course of hydrogen production (Figure 5.3). Formation of the metabolites from starch showed different patterns in the presence and absence of lactic acid. In the absence of lactic acid (Figure 5.3A), acetate, butyrate and ethanol were produced at the same level. Lactic acid was also produced but it was completely consumed later. In media containing both lactic acid and starch (Figure 5.3B), lactic acid decomposition started after 12 hours. Butyric acid exhibited the highest rate of

formation and it was the most predominant soluble metabolite. Acetic acid was the second major metabolite. Ethanol was formed in lower concentration compared to starch medium. The other difference was the formation of small amounts of propionic acid which were decomposed at the end of the fermentation. In the starch medium, propionic acid was not detected. When lactic acid was the sole carbon source (Figure 5.3C), propionate type fermentation was observed. The formation of propionic and acetic acid as the main fermentation products together with some valeric acid coupled with insignificant gas production are characteristic of propionate type fermentation [15]. All the aforementioned characteristics were observed in the culture that contained only lactic acid as carbon source (Figures 5.2, 5.3C). *Clostridium propionicum* is one of the species that has the ability to consume lactic acid through the following reaction [16]:

$$3\ CH_3CH(OH)COOH \rightarrow 2\ CH_3CH_2COOH + CH_3COOH + CO_2 \qquad (3)$$

Equation (3) indicates that from the consumption of three moles of lactic acid, two moles propionic acid and one mole acetic acid can be produced. As apparent from Table 5.5, in the medium containing only lactic acid, from 8.9 mM lactic acid, 5.13 mM propionic acid and 2.94 mM acetic acid were produced which are respectively 86.5% and 99.1% of the theoretical values calculated from Equation (3). It is noteworthy that the lactic acid present in starch containing medium showed completely different metabolic pathway and gave rise to the hydrogen production together with the formation of butyric acid as the major metabolite.

Table 5. 5 Characteristic of soluble metabolites after 48 h incubation

Culture condition	TVFA (mM)	SM (mM)	Acetate (mM)	butyrate (mM)	lactate (mM)	propionate (mM)	formate (mM)	valerate (mM)	ethanol (mM)	iso-butyrate (mM)	excess NADH (mM)
Starch	34.28	49.22	15.27	7.84	1.81	0.00	9.17	0.00	14.94	0.18	37.05
Starch + lactic acid	45.63	53.86	13.60	17.43	3.00	0.45	10.86	0.00	8.22	0.30	50.75
Lactic acid	9.64	9.64	2.94	0.17	1.10	5.13	0.00	0.05	0.00	0.25	1.08

TVFA: total volatile fatty acids
SM: soluble metabolites

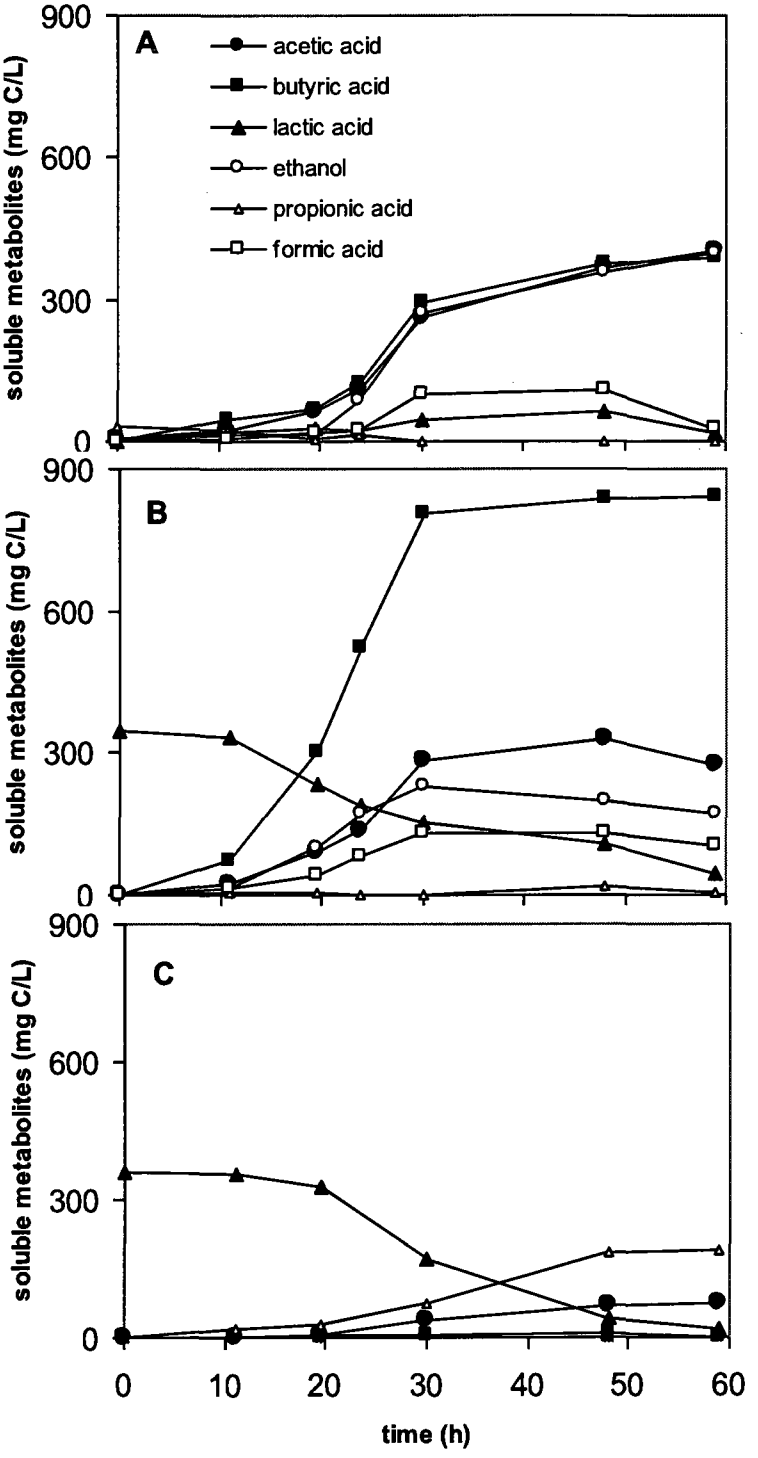

Figure 5. 3 Soluble metabolite formation and lactic acid decomposition in culture containing starch (A), starch and lactic acid (B) and only lactic acid (C)

If NADH pathway is the pathway for hydrogen evolution, hydrogen will be evolved through the re-oxidation of NADH [17]:

$$NADH + H^+ \rightarrow H_2 + NAD^+ \quad (4)$$

With increasing excess NADH, the hydrogen yield will be improved. Through the main pathway of glycolysis glucose is decomposed to pyruvate. Pyruvate is then used for formation of organic acids and alcohols. The overall reaction equations [18, 19] for the products which were detected in our experiments are presented in Table 5.6. The valerate and iso-butyrate were not considered in the calculation of excess NADH, since they are produced by Clostridial species from fermentation of amino acids not carbohydrates [16]. Excess NADH for each condition can be calculated from the difference between produced and used NADH.

Assuming that the amount of produced pyruvate is equal to that of used pyruvate [18] the excess NADH can be calculated from the molar concentrations of soluble metabolites as follows:

$$\begin{aligned}[\text{Generated NADH}] &= [\text{produced pyruvate}] + [\text{acetate}] \\ &= [\text{used pyruvate}] + [\text{acetate}] \\ &= \{[\text{acetate}] + 2[\text{butyrate}] + [\text{propionate}] + [\text{lactate}] + [\text{ethanol}]\} + \\ &\quad [\text{acetate}] \end{aligned} \quad (5)$$

$$[\text{Used NADH}] = [\text{lactate}] + 2\,[\text{propionate}] + [\text{formate}] + [\text{ethanol}] \quad (6)$$

[Excess NADH] = [Generated NADH] – [used NADH]

$$=2[\text{acetate}]+2[\text{butyrate}]-[\text{propionate}]-[\text{formate}] \quad (7)$$

Table 5. 6 Overall reaction equations for the mixed culture fermentation of carbohydrates

Pyruvate formation:	
Main pathway of glycolysis	$C_6H_{12}O_6 + 2\ NDA^+ \rightarrow 2\ CH_3COCOOH + 2\ NADH + 2\ H^+$
Pyruvate decomposition to:	
Acetate	$CH_3COCOOH + NAD^+ + H2O \rightarrow CH_3COOH + CO_2 + NADH + H^+$
Butyrate	$2\ CH_3COCOOH \rightarrow CH_3(CH_2)_2COOH + 2\ CO_2$
Lactate	$CH_3COCOOH + NADH + H^+ \rightarrow CH_3CHOHCOOH + NAD^+$
Propionate	$CH_3COCOOH + 2\ NADH + 2\ H^+ \rightarrow CH_3CH_2COOH + H_2O + 2\ NAD^+$
Formate	$CO2 + NADH + H^+ \rightarrow HCOOH$
Ethanol	$CH_3COCOOH + NADH + H^+ \rightarrow CH_3CH_2OH + CO_2 + NAD^+$

Based on the equation (7) the excess NADH concentrations were calculated from the molar concentrations of soluble metabolites after 48 hours incubation (Table 5.5). The results show that the addition of 10 mM lactic acid increased the residual NADH from 37.05 mM to 50.75 mM. It is noteworthy that the aforementioned 37% increase in residual NADH compares well with the 35% increase in hydrogen yield, on unit carbon mass basis.

The effect of the addition of different concentrations of lactic acid to a medium containing 5 g/L starch was investigated in batch tests. As illustrated in Figure 5.4A, the cumulative hydrogen production increased with increasing the concentration of lactic acid and remained constant at concentrations above 3 g/L. The addition of lactic acid even at concentration of 5 g/L did not cause inhibition to the hydrogen production. If both starch and lactic acid were considered as carbon sources, the highest hydrogen production was 16.9 mL hydrogen/ g COD removed with the addition of 1 g/L lactic acid to the starch culture. When lactic acid concentration was between 0.25 and 2 g/L, it was totally depleted by the end of fermentation. However, total consumption did not occur when lactic acid was added at the concentrations of 3 to 5 g/L (33 to 55 mM).

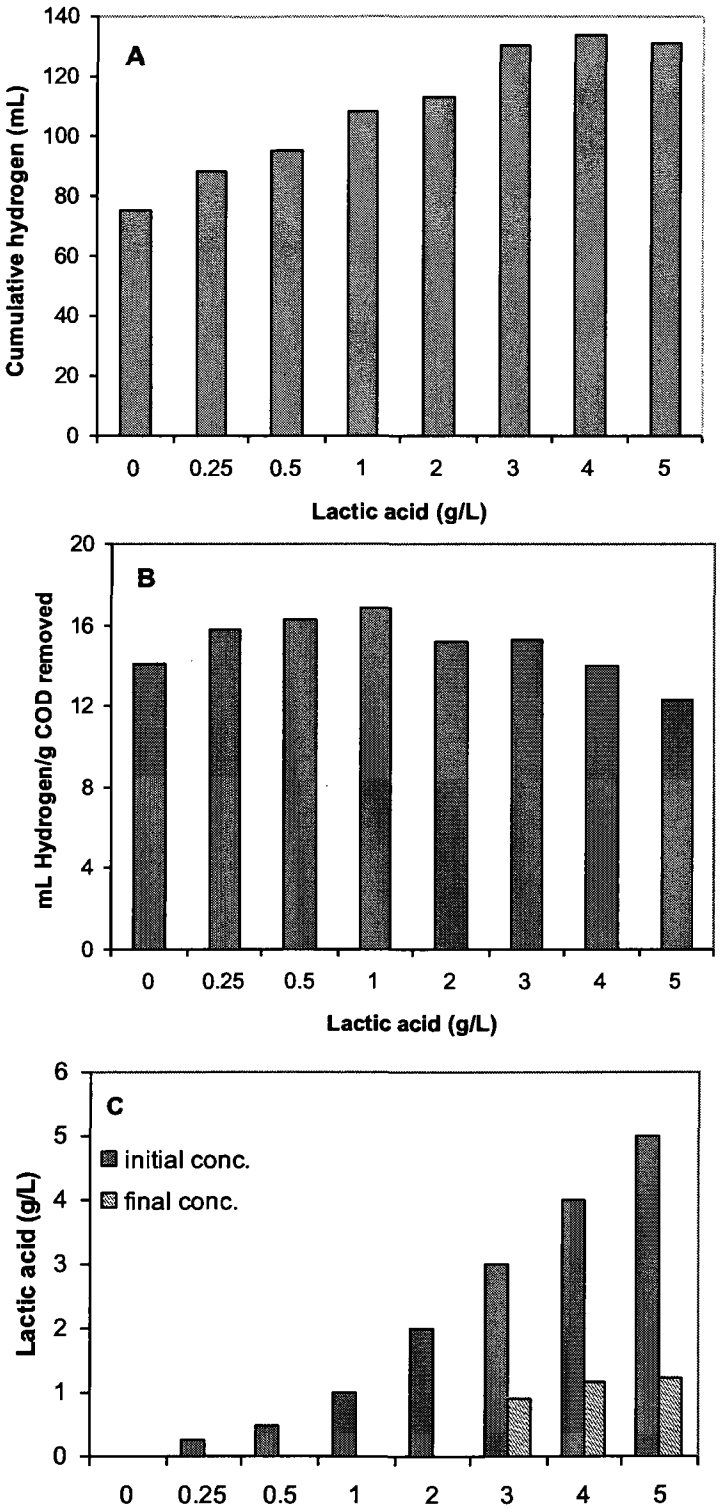

Figure 5. 4 Effect of different initial lactic acid concentration on hydrogen production

5.4 Conclusions

The results of this work demonstrated that the addition of lactic acid (up to 3 g/L) to a starch-containing medium could improve hydrogen production. The analysis of soluble metabolites showed that the metabolism of starch was affected by the addition of lactic acid. The increase in hydrogen production in the presence of lactic acid was accompanied by formation of higher amounts of butyric acid as the predominant aqueous metabolite. The increase in hydrogen production yield was assigned to the increase in the available residual NADH for hydrogen production.

5.5 References

1. Zajic JE, Margaritis A, Brosseau JD. Microbial hydrogen production from replenishable resources. Int J Hydrogen Energy 1979; 4(5): 385-402.

2. Hawkes FR, Hussy I, Kyazze G, Dinsdale R, Hawkes DL. Continuous dark fermentative hydrogen production by mesophilic microflora: Principles and progress. Int J Hydrogen Energy. 2007;32(2):172-84.

3. Liu H, Grot S, Logan BE. Electrochemically assisted microbial production of hydrogen from acetate. Environ Sci Technol 2005; 39(11):4317-20.

4. Matsumoto M, Nishimura Y. Hydrogen production by fermentation using acetic acid and lactic acid. J Biosci Bioeng 2007; 103(3):236-41.

5. Hawkes FR, Dinsdale R, Hawkes DL, Hussy I. Sustainable fermentative hydrogen production: Challenges for process optimisation. Int J Hydrogen Energy 2002; 27(11-12):1339-47.

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

7. Noike T, Takabatake H, Mizuno O, Ohba M. Inhibition of hydrogen fermentation of organic wastes by lactic acid bacteria. Int J Hydrogen Energy. 2002;27(11-12):1367-71.

8. Nishio N, Nakashimada Y. High rate production of hydrogen. Recent Progress of Biochemical and Biomedical Engineering in Japan I. 2004;90:63-87.

9. Logan BE, Oh SE, Kim IS, Van Ginkel S. Biological hydrogen production measured in batch anaerobic respirometers. Environ Sci Technol 2002;36(11):2530-5.

10. Owen WF, Stuckey DC, Healy JB, Young LY, McCarty PL. Bioassay for monitoring biochemical methane potential and anaerobic toxicity. Water Res 1979;13:485-92.

11. APHA, AWWA, WEF. Standard methods for examination of water and wastewater. 19th edition. ; 1995.

12. Lay JJ, Li YY, Noike T. Mathematical model for methane production from landfill bioreactor. J Environ Eng -ASCE 1998 ;124(8):730-6.

13. Zulkali MMD, Ahmad AL, Norulakmal NH. Oryza sativa L. husk as heavy metal adsorbent: Optimization with lead as model solution. Bioresour Technol 2006;97(1):21-5.

14. Haaland PD. Experimental design in biotechnology. MARCEL DEKKER, INC.; 1989.

15. Cohen A, Vangemert JM, Zoetemeyer RJ, Breure AM. Main characteristics and stoichiometric aspects of acidogenesis of soluble carbohydrate containing wastewaters. Process Biochemistry 1984;19(6):228-32.

16. Woods DR, editor. The Clostridia and biotechnology. USA: Butterworth-Heinemann; 1993.

17. Das D, Veziroglu TN. Hydrogen production by biological processes: A survey of literature. Int J Hydrogen Energy 2001;26(1):13-28.

18. Tanisho S, Kuromoto M, Kadokura N. Effect of CO2 removal on hydrogen production by fermentation. Int J Hydrogen Energy 1998;23(7):559-63.

19. Rodriguez J, Kleerebezem R, Lema JM, van Loosdrecht MCM. Modeling product formation in anaerobic mixed culture fermentations. Biotechnol Bioeng 2006;93(3):592-606.

CHAPTER 6

Sustainability of Biological Hydrogen Production in Continuous Flow Bioreactors

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6.1 Introduction

Hydrogen has a high energy yield (142 kJ/g) which is 2.75 times more than that of any hydrocarbon [1]. Biological hydrogen production is potentially regarded as one of the most promising alternatives for sustainable green energy production despite the feasibility of hydrogen production through water electrolysis and chemical cracking of hydrocarbons. Among different biological processes for hydrogen production dark fermentation is the most attractive one because of its potential of direct use of wastewater streams and organic wastes and its higher rate of hydrogen production in comparison with photo-fermentative processes [2, 3]. However, this process has not been commercialized yet mainly due to long term instability attributed to changes in microbial communities and metabolic shifts and also relatively low hydrogen production yields and rates [4].

One of the major obstacles for production of hydrogen from mixed microflora is the coexistence of hydrogen consuming bacteria including methanogens, acetogens, and sulfate reducing bacteria which can obtain energy by utilizing molecular hydrogen [5]. Heat treatment [6, 7], acid treatment [8], alkaline treatment [1, 8] and utilization of chemical inhibitors [9, 10] are some of the enrichment methods that have been used for suppression of hydrogen consuming bacteria such as methanogens. Although short detention times have been reported to increase the productivity, in a conventional bioreactor, the biomass is easily washed out and the efficiency of substrate utilization plummets at short detention times [11]. Proliferation of other non-spore forming bacteria such as propionate bacteria and lactic acid bacteria, concomitant with high organic

loading [12] retards hydrogen production due to competition for carbohydrates, as well as excretion of bacteriocins which inhibit hydrogen producers [13].

The maximum specific growth rate (μ_{max}) for mixed culture of 0.333 h^{-1} [14] corresponds to minimum solids retention time (SRT_{min}) of 3.0 h and thus CSTR operated for hydrogen production are characterized by HRTs of 3-8 h. However, high dilution rates result in a marked decrease in biomass content in the reactor due to severe cell washout and system failure [15]. Although, fill and draw (fed-batch) reactors have been used for hydrogen production, they invariably suffered from inconsistent hydrogen production [16] and methane production [17]. The hydrogen yield in sequencing batch reactor utilizing sucrose at an organic loading rate (OLR) of 82 g/L-d at 35 ˚C and pH 7 was 1.3 mol H_2 / mol hexose [18] compared to 1.15 mol H_2 / mol hexose observed in a CSTR at a similar OLR [19]. In order to overcome biomass washout in hydrogen reactors, decoupling of SRT from HRT in hydrogen bioreactors has been achieved primarily by using biofilms on several media including synthetic plastic media and treated anaerobic granular sludge [20], activated carbon, expanded clay and loofah sponge [6], glass beads [21] and membranes [22]. Kim *et al.* [23] using poly-vinyl alcohol as solid support medium for non-heat-treated sewage sludge seed, for the treatment of 20 g/L glucose at a pH 5.0 and HRT of 20 h found variable H_2 production in a 30 day experiment, with approximately 4 g/L of biomass retained in the reactor.

Biofilms reactors seeded with acid-shocked sewage sludge developed on activated carbon and treating 17.8 g / L sucrose at 0.5–5 h HRT, 35 ˚C, pH 6.7 yielded 7.4 L H_2 / L-h at 0.5 h HRT [24] and 1.32 L H_2 / L-h at 1h HRT [6]. At higher substrate concentrations performance declined due to competition from non-hydrogen producers but thermal

treatment restored H_2 production rates, suggesting spore-formers were part of the attached biofilm [4]. Problems with the development of methanogenic biofilms on the carrier media adversely impact process stability, which is critical for sustained hydrogen production. Oh *et al.* [25] coupled a cross-flow membrane to a CSTR operated at HRTs of 3.3 and 5 h and SRT of 3.3 to 48 h on 10 g / L glucose at pH 5.5 and 26 °C, and found an optimum volumetric hydrogen production of 0.37 L H_2 / L-h at a 12 h SRT with the yield decreasing at an SRT of 48 h. Moreover, membranes have not shown many advantages in terms of volumetric hydrogen yield and are also prone to fouling. The up-flow anaerobic sludge blanket reactor (UASB) has been used for biological hydrogen production with varying degrees of success. Fang *et al.* [26] achieved a hydrogen yield of 2.2 mol /mol hexose in a 90-day test of a USAB at an OLR of 49 g/L-d of sucrose at 6h HRT and 26 °C. The long startup time for UASB reactors and problems with particle granulation are problematic for hydrogen production in this type of bioreactors.

As depicted in table 6.1, there is no clear relationship between the hydrogen yield and the organic loading rate. In some cases higher OLRs decreased the hydrogen yield [27] whereas in some others higher OLRs increased the hydrogen yield [28]. For waste activated sludge as a seed material, it appears that increasing the OLR within the 40-160 gCOD/L-d increased hydrogen yield to an optimum of 1.67 mol H_2/mol glucose at an ORT of 120 gCOD/L-d [29], whereas hydrogen yield decreased with increasing OLR for both anaerobically digested sludge [19] and soil microorganisms [27]. Hydrogen yield with digester sludge at an OLR of 45 gCOD/L-d was 1.3 mol H_2/mol glucose [19] as compared with 0.92 mol H_2/mol glucose with waste activated sludge [29]. Moreover,

Table 6. 1 Comparison of continuous hydrogen production studies in terms of OLR and bacterial biomass retained

Reactor type	Culture Type	Substrate	OLR (g/L-d)	VSS (g/L)	Volumetric H_2 Prod. Rate (L/L-d)	H_2 yield (mol/mol hexose)	Refs.
CSTR	agricultural soil	glucose	72	2.2	0.15	0.4	[25]
CSTR	agricultural soil	glucose	80	4.6	0.8	2.06	[28]
CSTR	agricultural soil	glucose	27	0.9	3.8	0.72	[28]
CSTR	ADS *	sucrose	86	4.4	0.5	1.15	[19]
CSTR	ADS	sucrose	45	4.0	6.8	1.30	[19]
CSTR	ADS	sucrose	22	2.2	4.4	1.65	[19]
SBR	WAS **	sucrose	88	4.4	0.4	1.3	[18]
USAB	WAS	sucrose	52	20	0.5	2.2	[26]
CSTR	agricultural soil	glucose	6	2.2	1.9	2.8	[27]
CSTR	agricultural soil	glucose	12	4.2	3.5	2.5	[27]
CSTR	agricultural soil	glucose	18	6.3	5.3	2.4	[27]
CSTR	agricultural soil	glucose	24	8	6	2.2	[27]
CSTR	WAS	sucrose	160	4.9	13.4	0.68	[29]
CSTR	WAS	sucrose	120	7.8	25.2	1.67	[29]
CSTR	WAS	sucrose	80	3.5	15.4	1.58	[29]
CSTR	WAS	sucrose	40	2.3	5.0	0.92	[29]

* Anaerobic digester sludge
** Waste activated sludge

comparing the biomass concentration in the two CSTRs utilizing agricultural soil as the seed and glucose as a substrate under approximately same OLRs, Van Ginkel and Logan [27] achieved much higher hydrogen yield (2.2 mol/mol) at a biomass concentration of 8 g/L compared to Zhang *et al.* [28] who reported 0.72 mol H_2 /mol hexose with 0.9 g/L biomass. Oh *et al.* [25] achieved a hydrogen yield of 0.4 mol/mol at a biomass concentration of 2.2 g/L in a CSTR and Wu *et al.* [29] using a CSTR seeded with silicone-immobilized sludge realized a hydrogen yield of 1.58 mol/mol at 3.5 g/L of biomass compared to a hydrogen yield of 2.06 mol/mol achieved by Zhang *et al.* [28] at a similar OLR with a higher biomass concentration (4.6 g/L). It is thus clear that the higher biomass concentration in the reactors improved the hydrogen yield, which in essence shows that one of the key factors affecting the stability of hydrogen producing systems is maintaining higher biomass concentrations in the system. In addition, the low hydrogen yield and system failure was attributed to low concentrations of biomass due to washout [27].

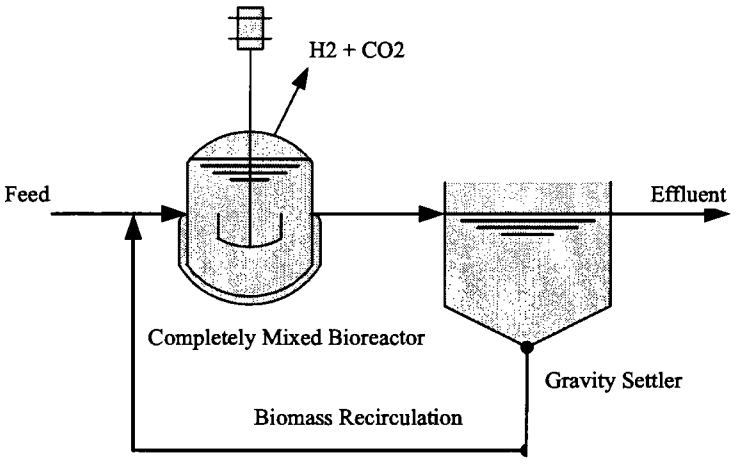
The literature review has highlighted the importance of organic loading and maintaining active biomass in the reactor for biohydrogen production. This paper has two objectives; the first objective focuses primarily on the assessment of decoupling of SRT from HRT using simple conventional clarification that is widely used in the anaerobic contact and activated sludge processes for wastewater treatment, while the other objective of this paper is to asses the impact of organic loading on four systems, utilizing anaerobic and waste activated sludges, as seed sludges. Additionally, this paper will compare the impact of seed sludges on hydrogen yield and microbial communities. The paper will include detailed COD mass balances and microbial characterization using denaturing

gradient gel electrophoresis (DGGE). While there is significant literature on individual systems optimization, there are few side by side comparisons that offer opportunity for assessment of long-term performance and sustainability, a widely reported concern with biohydrogen production. This is particularly important in light of the various metabolic pathways and microbial shifts involved in biological hydrogen production.

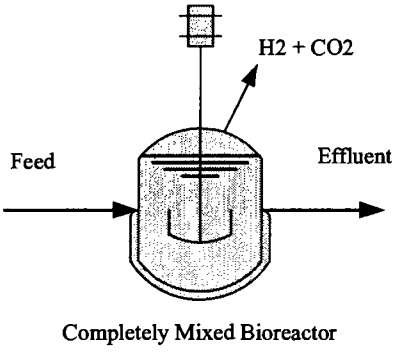
6.2 Materials and Methods

6.2.1 Systems Setup and Operation

Four lab-scale systems were operated at 37° C for 65 days (Figure 6.1). Two integrated biohydrogen reactor clarifier systems (1 and 2) [30], denoted henceforth as IBRCS-1 and IBRCS -2, comprised a continuously stirred reactor (CSTR) for biological hydrogen production (5 L working volume), followed by uncovered gravity settler (volume 8 L) i.e. open to atmosphere, while CSTR-1 and 2 consisted of a CSTR only with a working volumes of 5 L and 2 L, respectively. Details of the operational conditions for the four systems are listed in Table 6.2. In order to enrich hydrogen producing bacteria, the sludges were heat treated at 70° C for 30 minutes. The systems were monitored for total chemical oxygen demand (TCOD), soluble COD, volatile fatty acids (VFA), ethanol, lactate, glucose, volatile suspended solids (VSS), total suspended solids (TSS) and biogas composition including hydrogen, methane and nitrogen. The quantity of produced biogas was recorded daily using a wet tip gas meter (Rebel wet-tip gas meter company, Nashville, TN, USA).



IBRCS



CSTR

Figure 6. 1 Experimental Setup for the biohydrogen production systems

Table 6. 2 Operational Conditions in the hydrogen producing systems

	Glucose (g/L)	HRT (h)	SRT (h)	OLR (gCOD/L-d)	pH
IBRCS-1	2	8	48±3.2	6.5	5.5-6.5
IBRCS-2	8	8	46±4.2	25.7	5.5-6.5
CSTR-1	8	8	8	25.7	5.5-6.5
CSTR-2	20	12	12	42.8	5.5 (controlled)

6.2.2 Inocula and Media Compositions

For the IBRCSs and CSTR-1, anaerobically-digested sludge from the St. Marys wastewater treatment plant (St. Marys, Ontario, Canada) was used as the seed. These three systems (IBRCS-1, IBRCS-2 and CSTR-1) were seeded with 5 liters of sludge and started up in a continuous mode with the feed containing glucose at different concentrations as highlighted in Table 6.2. It must be emphasized that there was no sludge wastage from the clarifier for systems IBRCS-1 and IBRCS-2 throughout the operation, and the values of SRTs presented in Table 6.2 represent the average ± standard deviation (SD) during steady state operation. It is noteworthy that the reactors operation was consistent over time and accordingly the average SRT with SD of less than 10% of the mean SRT is representative of the overall SRT during the run. As expected the clarifier effluent VSS was substantially lower than the reactor VSS and remained unchanged during steady-state operation. The feed contained sufficient inorganics (mg/L): $NaHCO_3$, 2000- 4000; $CaCl_2$, 140; $MgCl_2.6H_2O$, 160; NH_4HCO_3, 600; $MgSO_4.7H_2O$, 160 ; urea, 500; Na_2CO_3, 124; $KHCO_3$, 156; K_2HPO_4, 15; trace mineral solution, 500; H_3PO_4, 250. For CSTR-2, waste activated sludge from the Adelaide Pollution Control Plant in London, Ontario was used as the inoculum for hydrogen production. The medium contained 20 g/L glucose as the carbon source and the following inorganic salts (mg/L): NH_4Cl, 2600; K_2HPO_4, 250; $MgCl_2.6H_2O$, 125; $FeSO_4.7H_2O$, 5.0; $CoCl_2.6H_2O$, 2.5; $MnCl_2.4H_2O$, 2.5; KI, 2.5; $Na_2MoO_4.2H_2O$, 0.5; H_3BO_4, 0.5; $NiCl_2.6H_2O$, 0.5;$ZnCl_2$, 0.5. The sludge was added to achieve an initial biomass concentration of 4.2 g/L in the reactor containing the growth medium. The reactor was

first operated in batch mode for 15 hours, after which the reactor was shifted to continuous mode with an HRT of 12 hours. As apparent from Table 6.2, in order to evaluate the impact of seed sludge on hydrogen yield, the two CSTRs were operated, albeit at different OLRs. The rational for using different OLR is that at the same OLR of 40-45 gCOD/L-d the anaerobically digested sludge showed approximately 50% higher hydrogen yield [19] than the waste activated sludge [29], and the general trend discussed elaborately before indicated that hydrogen yield for anaerobically digested sludge as a seed decreased with OLR, and vice versa with waste activated sludge. Hence increasing the OLR in the waste activated sludge (CSTR-2) was anticipated to cause an increase in hydrogen yield to a comparable level with the waste activated sludge; minimizing the influence of OLR in the seed assessment.

6.2.3 Analytical Methods

The biogas composition including hydrogen , methane and nitrogen was determined by a gas chromatograph (Model 310, SRI Instruments, Torrance, CA) equipped with a thermal conductivity detector (TCD) and a molecular sieve column (Molesieve 5A, mesh 80/100, 6 ft X 1/8 in). Argon was used as carrier gas at a flow rate of 30 mL/min. The temperatures of the column and the TCD detector were 90 and 105°C, respectively. Presence of oxygen was also examined by this GC in order to ensure anaerobic condition in the bioreactor.

The concentrations of volatile fatty acids (VFAs) were analyzed using a gas chromatograph (Varian 8500, Varian Inc., Toronto, Canada) with a flame ionization detector (FID) equipped with a fused silica column (30m × 0.32 mm). Helium was used as carrier gas at a flow rate of 5 mL/min. The temperatures of the column and detector

were 110 and 250°C, respectively. Lactic acid concentrations were measured using a high- performance liquid chromatography system (1200 series, Agilent Technologies) equipped with Aminex HPX-87H ion exclusion column (300 mm × 7.8 mm I.D.; BIO-RAD), and a UV-detector at 210 nm. The column temperature was adjusted to 30 °C. The same instrument with a refractive index detector (RID) was used to measure the concentrations of glucose. The temperature of the RID detector was set to 35 °C. The amount of volatile suspended solids (VSS) and chemical oxygen demand (COD) were measured according to standard methods [31].

6.2.4 Microbial Community Analysis

In all four reactors, after 50 days the total genomic community DNA were extracted using UltraClean Soil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) and after PCR amplification were analyzed by denaturing gradient gel electrophoresis (DGGE). The primer set of 357FGC (5'-CGCCCGCCGCGCGCGGCG GGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG-3') and 518R (5'–ATTACCGCGGCTGCTGG-3') at the annealing temperature of 53°C were used for the PCR amplification of the variable V3 region of 16SrDNA from the purified genomic DNA. Denaturing gradient gel electrophoresis (DGGE) of PCR products was performed with a DCode universal mutation system (Bio-Rad laboratories, Hercules, CA, USA). The PCR products were applied directly to 8% (w/v) polyacrylamide gel with 15 % to 55% denaturant (urea) gradients. Electrophoresis was performed at a constant voltage of 130 V at 58°C for 5 hours. The DNA templates of the bands of interest were re-amplified and the PCR products were purified using QIAquick PCR purification Kit (QIAGEN Sciences, Maryland, USA) in accordance with the manufacturer's protocol. The

sequences of re-amplified DNA fragments were determined by dideoxy chain termination (Sequencing Facility, John P. Robarts Research Institute, London, Ontario) and compared with available sequences in GenBank database using the BLAST program [32].

6.3 Results and Discussions

Figure 6.2 shows the hydrogen production profiles for the four reactors throughout the 65 days of operations. All the reactors showed stable hydrogen production during the two months of operation and methane was not detected in any of the reactors. However, the coefficient of variation (calculated as standard deviation divided by the average) for hydrogen production rate in CSTR-2 was 20% in comparison with approximately 10% variation in other reactors. IBRCS-2 showed a drastically higher hydrogen production rate than other systems. Comparing IBRCS-2 and CSTR-1, which were operated at same OLR, hydrogen production rate in IBRCS-2 gradually increased during the first 10 days of operation from 5.5 L/L-d to 11 L/L-d; while in CSTR-1 hydrogen production decreased during the first ten days of operation and stabilized at 1.8 L/L-d. However, in the CSTR with higher organic loading rate (CSTR-2) hydrogen production rate dropped to approximately 0.6 L/L-d after 7 days. Comparing IBRCS-1 and IBRCS-2 (see Table 6.3) under two different OLRs (6.5 and 25.7 g COD/L-d), respectively, the hydrogen yield was the same in both systems during steady state operation. The average hydrogen production rate in IBRCS-1 was 2.4 L/L-d.

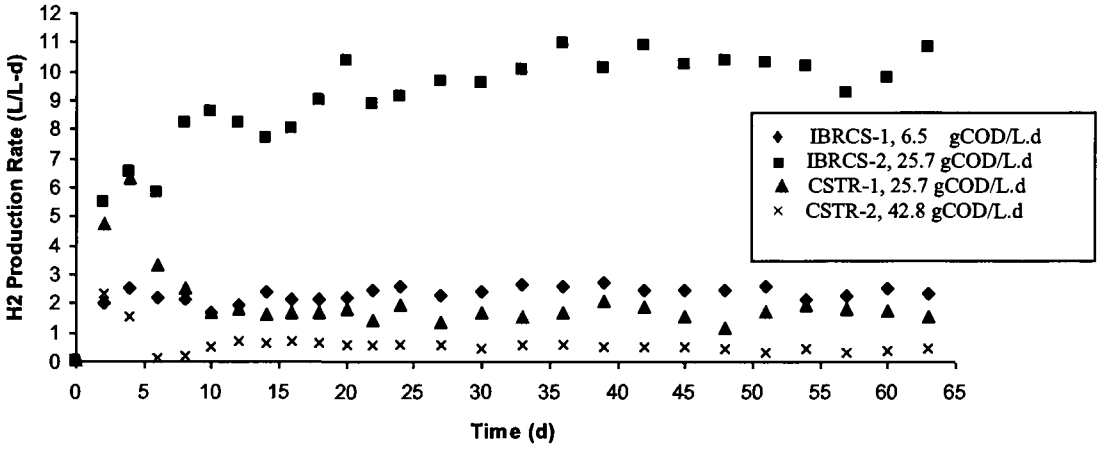


Figure 6. 2 Profiles of hydrogen production in the systems

As illustrated in Figure 6.3, in IBRCS-1, the biomass concentration in the reactor was maintained at 1.5 g/L, while in IBRCS-2 the concentration of VSS decreased from 5.2 g/L at start-up to approximately 4.0 g/L after the 5th day and remained constant thereafter with an average value of 4.2 g/L. In CSTR-1 which was working at the same OLR of IBRCS-2 (OLR = 25.7 g COD/L-d) VSS concentration decreased to approximately 0.75 g/L after 7 days and remained stable for the rest of the study. These results substantiated the effectiveness of gravity settler in maintaining the biomass in the system. Comparison of CSTR 1 and 2 which worked at different OLRs showed that the system with higher organic loading rate (CSTR-2) could sustain more biomass; this result is in agreement with other researchers' findings [27]. The drop in biomass concentrations during the first days of operations observed in both CSTRs was attributed to biomass washout.

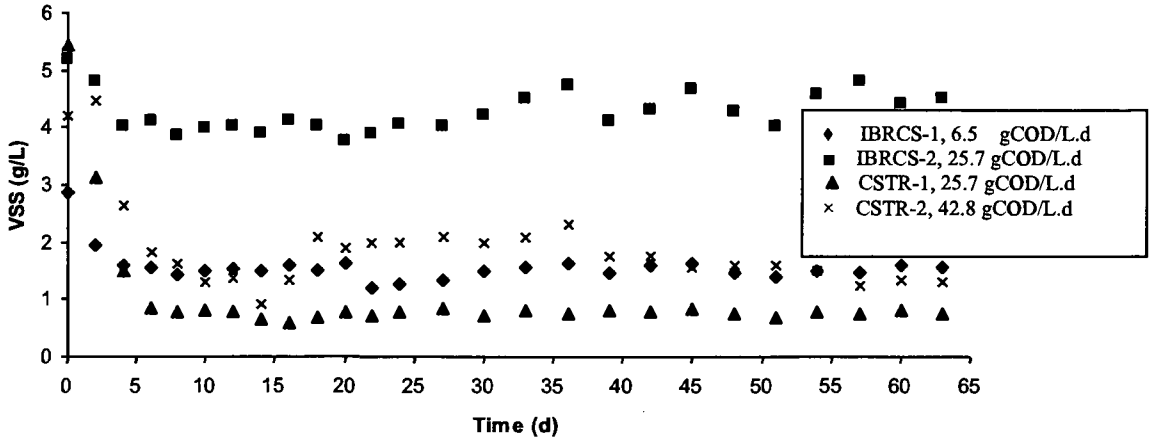


Figure 6. 3 Variations of biomass with time in the hydrogen production systems

Table 6. 3 Summary of steady state data in the hydrogen production systems

	Hydrogen Gas (%)	Hydrogen Gas (L/L/d)	Hydrogen Yield (mol/mol)	% Glucose converted	Biomass Yield (gVSS/gglucose)
Bio-1	71 ± 0.9	2.4 ± 0.2	2.8 ± 0.3	99.9 ± 1.0	0.12 ± 0.02
Bio-2	73 ± 2.7	9.6 ± 0.9	2.8 ± 0.3	99.9 ± 1.5	0.09 ± 0.01
CSTR-1	66 ± 5.3	1.8 ± 0.2	1.0 ± 0.1	50 ± 3.5	0.19 ± 0.02
CSTR-2	76 ± 3.6	0.55 ± 0.11	0.5 ± 0.1	29 ± 5.7	0.29 ± 0.02

The steady state data as summarized in table 6.3 shows that the IBRCSs achieved a hydrogen yield of 2.8 mol/mol, much higher than a hydrogen yield of 1.0 and 0.5 mol/mol for CSTR-1 and CSTR-2, respectively. While the glucose conversion was complete in the IBRCSs, it was only 50% and 29% in CSTR -1 and CSTR-2,

respectively. The COD mass balance for the four systems is shown in Table 6.4, computed considering the measured influent and effluent CODs, and the equivalent CODs for both gas and biomass. The closure of COD balances at 97%-109% validates the reliability of the data. Using the stoichiometric yield of 4 and 2 mol H_2 / mol glucose from the Eq. (1 and 2), and according to the measured concentrations of acetate and butyrate, the contribution of the two pathways was estimated.

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (1)$$

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 \quad (2)$$

In IBRCS-1, 65 % and 35 % of the hydrogen produced were through the acetate and butyrate pathways, respectively. The main liquid products in IBRCS-2 were acetate and butyrate at concentrations of 2494 mg/L and 1594 mg/L respectively, with approximately 68 % and 32% of the hydrogen yield through the acetate and butyrate pathways, respectively. In CSTR-1, the acetate and butyrate concentrations were 650 mg/L and 1366 mg/L, respectively, and both acetate and butyrate pathways equally contributed to hydrogen production at 50% each. Furthermore, in CSTR-2 lactate was detected at an average concentration of 460 mg/L, while acetate and butyrate concentrations were much lower than the other systems The hydrogen yield followed the same pattern of CSTR -1 with 50/50 % through acetate/butyrate pathways.

Table 6. 4 Summary of products and COD mass balance

	IBRCS-1	IBRCS-2	CSTR-1	CSTR-2
VSS (mg/L)	1489 ± 116	4190 ± 308	757 ± 61	1640 ± 360
VSS out (mg/L)	247 ± 46	744 ± 50	757 ± 61	1640 ± 360
VSS out (mgCOD/L)	350 ± 65	1056 ± 71	1075 ± 87	2329 ± 511
SCOD out (mg/L)	1492 ± 79	6023 ± 194	7760 ± 323	18266 ± 2072
Acetate (mg/L)	638 ± 105	2494 ± 217	650 ± 113	321 ± 56
Propionate (mg/L)	29 ±16	132 ± 61	109 ± 46	105 ± 99
Iso-butyrate (mg/L)	0	0	0	0
Butyrate (mg/L)	398 ± 55	1594 ± 126	1366 ± 96	270 ± 68
Iso-valerate (mg/L)	6 ±7	4 ± 3	40 ± 19	14 ± 3
Valerate (mg/L)	0	0	0	0
Ethanol (mg/L)	14 ± 7	73 ± 46	60 ± 16	179 ± 66
Lactate (mg/L)	0	0	0	459 ± 104
VFA (mgCOD/L)	1491 ± 87	5924 ± 257	3550 ± 264	1882 ± 587
Glucose Out (mg/L)	0	0	4001 ± 272	14270 ± 1140
Hydrogen Gas (L/d)	12 ± 1.3	48.1 ± 4.7	9 ± 1.0	1.1 ± 0.2
Hydrogen Gas (gCOD/d) **	7.6 ± 0.8	30 ± 3	5.7 ± 0.6	0.7 ± 0.13
COD balance (%)**	109 ± 5	106 ± 3	107 ± 4	97 ± 12

* Based on 1.42 gCOD/gVSS

** Based on 8 gCOD/g H_2

*** COD balance (%) = (VSS out (gCOD/d) + H_2 (gCOD/d) + SCOD out (gCOD/d)) / (TCOD in (gCOD/L))

Figure 6.4 shows the biomass yields for the four systems, calculated as the slope of the cumulative biomass produced versus the cumulative glucose converted. It should be noted that biomass production incorporated both the temporal changes in bioreactor mixed liquor volatile suspended solids (MLVSS) and the solids leaving in the clarifier liquid effluent for IBRCS-1 and IBRCS-2, and solids leaving the bioreactor in CSTR 1 and 2. The observed biomass yields in IBRCS-1 and IBRCS-2 of 0.12 and 0.09 g VSS/g glucose were substantially lower than the CSTRs of 0.19 to 0.29 g VSS/g glucose. The high biomass yield of 0.29 g VSS/g glucose could be also attributed to possible contamination of feed with aerobic bacteria and/or presence of very small amount of soluble oxygen in the feed. Although the observed yield is impacted by SRTs, the statistically significant differences between the IBRCSs and CSTRs at the 95% confidence level can not be rationalized only on the basis of SRT.

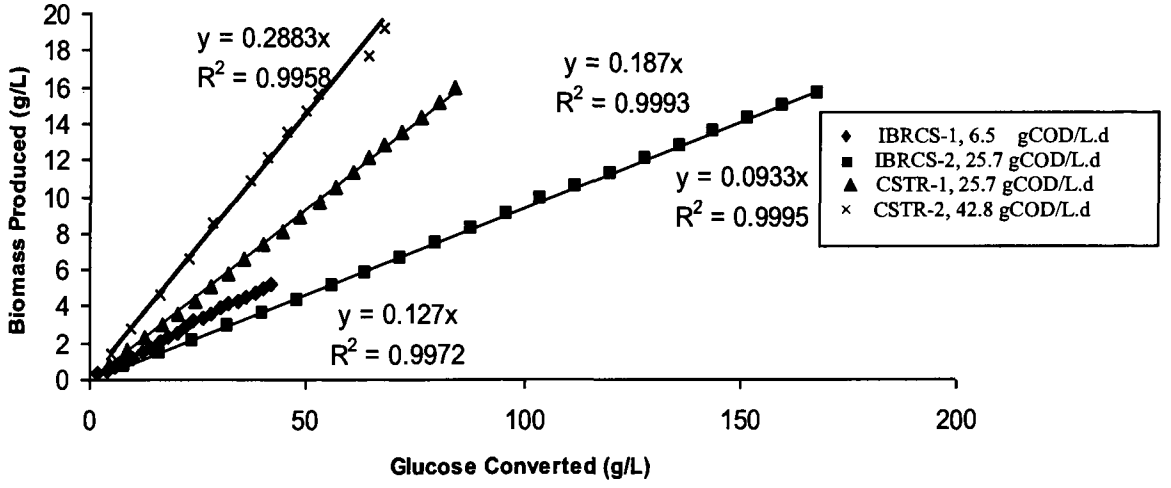


Figure 6. 4 Biomass yield estimation for the four systems

Figure 6.5 shows the inverse relationship between the biomass and hydrogen yields which emphatically demonstrates that the higher biomass yield is attributed to different microorganisms other than hydrogen producers. Using Equation (3) and the biomass yield reported in the literature for hydrogen producers of 0.1 g VSS/g glucose [11], it was estimated that the non-hydrogen producing bacteria constituted 12.5%, 0%, 40%, and 60% of the measured bioreactor VSS in IBRCS-1, IBRCS-2, CSTR-1, and CSTR-2, respectively.

$$X_v = X_a + X_i = X_{HP} + X_{nHP} + X_i = \theta_c . Y_{tHP} . OLR\eta + X_{nHP} + X_i \qquad (3)$$

where X_v is the total biomass, X_a is the active microbial population in the reactor which in this case consisted of the biomass of hydrogen producers (X_{HP}) and biomass of non-hydrogen producers (X_{nHP}), X_i is the inert remains of microorganisms in the reactor, θ_c is solid retention time, Y_{tHP} is true yield of hydrogen producers, OLR is the organic loading rate and η is the substrate conversion efficiency. It was estimated that the non-hydrogen producing bacteria constituted 12.5%, 0%, 40%, and 60% of the measured bioreactor VSS in Bio-1, Bio-2, CSTR-1, and CSTR-2, respectively. It should be noted that these values also included the inert biomass in the bioreactors.

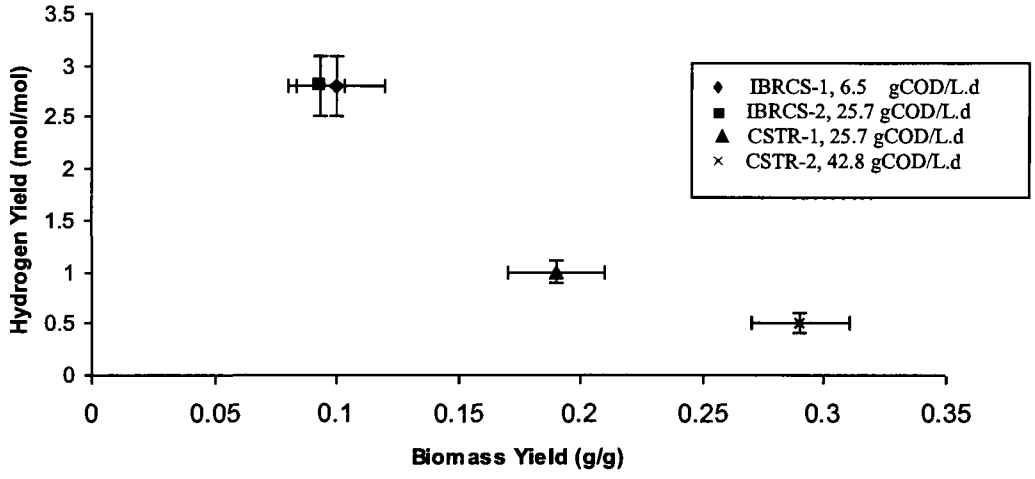


Figure 6. 5 Relation between the biomass yield and the hydrogen yield

6.3.1 Microbial Community Analysis

The DGGE profiles of the 16S rDNA gene fragments in the four continuous reactors are illustrated in Figure 6.6. Table 6.5 shows the results of the sequence affiliation. The number of the bands detected in IBRCS-2 which was operating at OLR of 25.7 gCOD/L-d was more than those detected in IBRCS-1 at an OLR of 6.5 gCOD/L-d. The increase in the number of the bands with the increase in the OLR was also observed in CSTRs as CSTR-2 with higher OLR had higher number of bands in comparison with CSTR-1. This observation may indicate increased species diversity in the continuous flow CSTR. The comparison of DGGE profile of IBRCS-2 and CSTR-1 operated at the same organic loading rates shows different microbial community structure in these reactors. Considering the higher yield of hydrogen production in IBRCS-2 and the different microbial community, it appears that the gravity settler in the IBRCSs allowed for selective enrichment of high-hydrogen producing species.

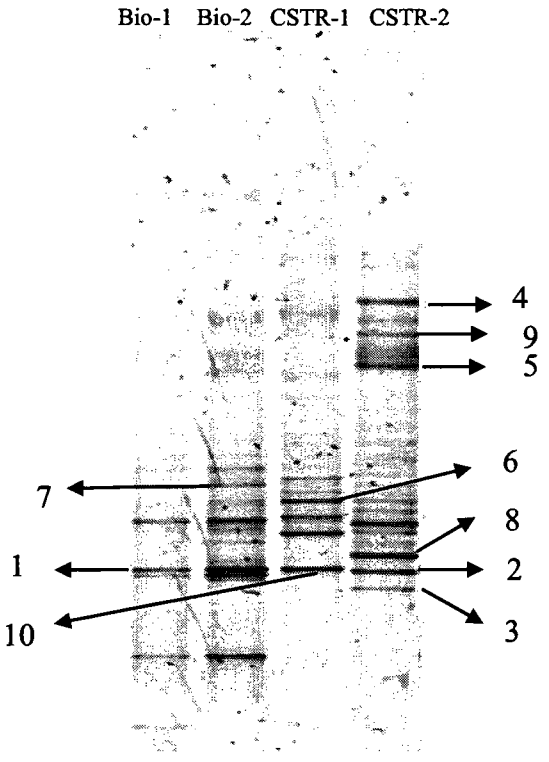


Figure 6. 6 DGGE profile of the 16S rDNA gene fragment at each treatment condition

Table 6. 5 Affiliation of denaturing gradient gel electrophoresis (DGGE) fragments determined by their 16S rDNA sequence

Band	Affiliation (accession no.)	Similarity (%)
1	*Klebsiella pneumonia*	100
2	*Klebsiella pneumonia*	100
3	*Klebsiella sp.*	99
4	*Lactobacillus fermentum*	91
5	*Pseudomonas veronii*	99
6	*Clostridium pasteurianum*	99
7	*Clostridium acetobutyricum*	99
8	*Clostridium mesophilum*	99
9	*Lactobacillus fermentum*	95
10	*Klebsiella pneumonia*	100

Klebsiella pneumonia (bands 1, 2 and 10) which has been observed in all of the reactors is a facultative anaerobic bacterium that is frequently used for hydrogen production as a pure culture [33, 34] or detected as one of the active microorganisms in mixed cultures of hydrogen production systems [35]. Band 3 in CSTR-2 is also a *Klebsiella sp.* This band has not been observed in other reactors. Presence of more facultative anaerobes in CSTR-2 may be due to using activated sludge as the microbial seed in this reactor. *Clostridium pasteurianum* (band 6) which is a hydrogen producer with a yield of 1.5 mol glucose/mol hydrogen [36] was detected in IBRCS-2 and CSTR-1. *Clostridium acetobutyricum* (band 7) was observed only in IBRCS-2. This bacterium is one of the frequently reported species in hydrogen producing reactors [37, 38].

Clostridium mesophilum (band 8) was observed only in CSTR 2. Although most of the Clostridium species have the ability of hydrogen production there is no specific report of hydrogen production by *Clostridium mesophilum*. CSTR-2 which exhibited the lowest hydrogen yield among other reactors showed the most diverse DGGE profile. *Lactobacillus fermentum* (band 4 and 9) and *Pseudomonas veronii* (band 5) are two non-hydrogen producers which were present only in CSTR-2 and could potentially use glucose for production of other metabolites rather than hydrogen and decrease the hydrogen yield in this reactor. The lactic acid production in CSTR-2 (Table 4) can be attributed to presence of lactic acid bacteria *Lactobacillus fermentum*. This finding is in agreement with Oh *et al.* [12] who reported that the concentration and/or the activities of lactate-forming bacteria increase as organic loading increases. However, these researchers did not analyze the microbial culture. Comparing CSTR-1 and CSTR-2, it is apparent that the waste activated sludge, though more microbially diverse than anaerobically digested sludge, is not advantageous for biohydrogen production due to the presence of non hydrogen producers such as *Lactobacillus* species. It has been reported that lactic acid bacteria reduces the hydrogen production through the inhibition of hydrogen producers [13]. Resistance of pre-existing lactic acid bacteria after heat treatment at 65°C and their appearance after the third consecutive batch in a repeated batch experiment using waste activated sludge has been reported in our previous work [39].

6.4 Conclusions

The decoupling of SRT from HRT in biohydrogen production systems, evaluated in this work, validated the promise of using a gravity settler after a CSTR to maintain high biomass retention in the system and decrease biomass washout, thus improving hydrogen yield and long term sustainability of hydrogen production. Using a gravity settler after a CSTR drastically increased the hydrogen production rate and hydrogen yield from 1.8 to 9.6 L/L/d and from 1 to 2.8 mol/mol glucose, respectively. The diversity of waste activated sludge does not provide any advantages to biohydrogen production relative to anaerobically digested sludge as reflected by 50% lower hydrogen yield and 50% higher biomass yield. In addition, the IBRCSs were capable of producing hydrogen at the same yield with approximately full conversion of glucose at two different OLRs in the range of 6.5-25.7 gCOD/L-d. Although further work is needed to optimize the IBRCS, and evaluate the impact of higher OLRs on the performance of the system, the inverse relationship between hydrogen and biomass yields observed in this study shows that the higher biomass yield is attributed to presence of non-hydrogen producing biomass, and the widely reported failure of biohydrogen reactors due to biomass washout corroborate the need for decoupling of SRT from HRT in order to maximize biohydrogen production.

6.5 References

1. Cai ML, Liu JX, Wei YS. Enhanced biohydrogen production from sewage sludge with alkaline pretreatment. Environ Sci Technol. 2004 JUN 1;38(11):3195-202.

2. Levin DB, Pitt L, Love M. Biohydrogen production: Prospects and limitations to practical application. Int J Hydrogen Energy. 2004;29(2):173-85.

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

4. Hawkes FR, Hussy I, Kyazze G, Dinsdale R, Hawkes DL. Continuous dark fermentative hydrogen production by mesophilic microflora: Principles and progress. Int J Hydrogen Energy. 2007;32(2):172-84.

5. Adams MWW, Stiefel EI. Biochemistry - biological hydrogen production: Not so elementary. Science. 1998 DEC 4;282(5395):1842-3.

6. Chang JS, Lee KS, Lin PJ. Biohydrogen production with fixed-bed bioreactors. Int J Hydrogen Energy. 2002 NOV-DEC;27(11-12):1167-74.

7. Baghchehsaraee B, Nakhla G, Karamanev D, Margaritis A, Reid G. The effect of heat pretreatment temperature on fermentative hydrogen production using mixed cultures. Int J Hydrogen Energy. 2008;334064-73.

8. Chen CC, Lin CY, Lin MC. Acid-base enrichment enhances anaerobic hydrogen production process. Appl Microbiol Biotechnol. 2002 FEB;58(2):224-8.

9. Park WS, Hwang MH, Hyun SH, Kim IS. Suppression of hydrogen consuming bacteria in order to increase H2 production potential in anaerobic fermentation. Proceedings of 10th world congress of anaerobic digestion-Montreal, Canada2004. 2004;1(1):642-7.

10. Sparling R, Risbey D, Poggi Varaldo HM. Hydrogen production from inhibited anaerobic composters. Int J Hydrogen Energy. 1997;22(6):563-6.

11. Chen CC, Lin CY, Chang JS. Kinetics of hydrogen production with continuous anaerobic cultures utilizing sucrose as the limiting substrate. Appl Microbiol Biotechnol. 2001;57(1-2):56-64.

12. Oh YK, Kim SH, Kim MS, Park S. Thermophilic biohydrogen production from glucose with trickling biofilter. Biotechnol Bioeng. 2004;88(6):690-8.

13. Noike T, Takabatake H, Mizuno O, Ohba M. Inhibition of hydrogen fermentation of organic wastes by lactic acid bacteria. Int J Hydrogen Energy. 2002;27(11-12):1367-71.

14. Horiuchi JI, Shimizu T, Tada K, Kanno T, Kobayashi M. Selective production of organic acids in anaerobic acid reactor by pH control. Bioresour Technol. 2002;82(3):209-13.

15. Wu SY, Hung CH, Lin CY, Lin PJ, Lee KS, Lin CN, HRT-dependent hydrogen production and bacterial community structure of mixed anaerobic microflora in suspended, granular and immobilized sludge systems using glucose as the carbon substrate. Int J Hydrogen Energy. 2008;33(5):1542-9.

16. Fan KS, Chen YY. H-2 production through anaerobic mixed culture: Effect of batch S-0. Chemosphere. 2004;57(9):1059-68.

17. Youn JH, Shin HS. Comparative performance between temperature-phased and conventional mesophilic two-phased processes in terms of anaerobically produced bioenergy from food waste. Waste Manage Res. 2005;23(1):32-8.

18. Lin CY, Jo CH. Hydrogen production from sucrose using an anaerobic sequencing batch reactor process. Journal of Chemical Technology and Biotechnology. 2003;78(6):678-84.

19. Kyazze G, Martinez-Perez N, Dinsdale R, Premier GC, Hawkes FR, Guwy AJ, Influence of substrate concentration on the stability and yield of continuous biohydrogen production. Biotechnol Bioeng. 2006;93(5):971-9.

20. Das D, Khanna N, Veziroglu TN. Recent developments in biological hydrogen production processes. Chemical Industry & Chemical Engineering Quarterly. 2008;14(2):57,+.

21. Zhang HS, Bruns MA, Logan BE. Biological hydrogen production by clostridium acetobutylicum in an unsaturated flow reactor. Water Res. 2006;40(4):728-34.

22. Vallero MVG, Lettinga G, Lens PNL. High rate sulfate reduction in a submerged anaerobic membrane bioreactor (SAMBaR) at high salinity. J Membr Sci. 2005;253(1-2):217-32.

23. Kim JO, Kim YH, Ryu JY, Song BK, Kim IH, Yeom SH. Immobilization methods for continuous hydrogen gas production biofilm formation versus granulation. Process Biochemistry. 2005;40(3-4):1331-7.

24. Lee KS, Lo YS, Lo YC, Lin PJ, Chang JS. H-2 production with anaerobic sludge using activated-carbon supported packed-bed bioreactors. Biotechnol Lett. 2003;25(2):133-8.

25. Oh SE, Lyer P, Bruns MA, Logan BE. Biological hydrogen production using a membrane bioreactor. Biotechnol Bioeng. 2004;87(1):119-27.

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

27. Van Ginkel SW, Logan B. Increased biological hydrogen production with reduced organic loading. Water Res. 2005;39(16):3819-26.

28. Zhang JJ, Li XY, Oh SE, Logan BE. Physical and hydrodynamic properties of flocs produced during biological hydrogen production. Biotechnol Bioeng. 2004;88(7):854-60.

29. Wu SY, Hung CH, Lin CN, Chen HW, Lee AS, Chang JS. Fermentative hydrogen production and bacterial community structure in high-rate anaerobic bioreactors containing silicone-immobilized and self-flocculated sludge. Biotechnol Bioeng. 2006;93(5):934-46.

30. Hafez, H.; Nakhla, G.; El Naggar, H. Integrated system for hydrogen and methane production during landfill leachate treatment. Pending patent, US Patent Application US 61/202,137, 2009.

31. APHA, AWWA, WEF. Standard methods for examination of water and wastewater, 19th edition. ; 1995.

32. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990 OCT 5;215(3):403-10.

33. Liu F, Fang B. Optimization of bio-hydrogen production from biodiesel wastes by klebsiella pneumoniae. Biotechnology Journal. 2007;2(3):374-80.

34. Chen X, Sun YQ, Xiu ZL, Li XH, Zhang DJ. Stoichiometric analysis of biological hydrogen production by fermentative bacteria. Int J Hydrogen Energy. 2006;31(4):539-49.

35. Wu SY, Lin CY, Lee KS, Hung CH, Chang JS, Lin PJ, Dark fermentative hydrogen production from xylose in different bioreactors using sewage sludge microflora. Energy Fuels. 2008;22(1):113-9.

36. Brosseau JD, Zajic JE. Hydrogen-gas production with citrobacter-intermedius and clostridium-pasteurianum. Journal of Chemical Technology and Biotechnology. 1982;32(3):496-502.

37. Kim SH, Han SK, Shin HS. Effect of substrate concentration on hydrogen production and 16S rDNA-based analysis of the microbial community in a continuous fermenter. Process Biochemistry. 2006;41(1):199-207.

38. Chin HL, Chen ZS, Chou CP. Fedbatch operation using clostridium acetobutylicum suspension culture as biocatalyst for enhancing hydrogen production. Biotechnol Prog. 2003;19(2):383-8.

39. Baghchehsaraee B, Nakhla G, Karamanev D, Margaritis A. Fermentative hydrogen production by diverse microflora, Proceedings of the International Conference on Hydrogen Production Oshawa, Canada, 2009,: 281-287

CHAPTER 7

Revivability of Biological Hydrogen Producing Bioreactors

In progress for submission to Biotechnology and Bioengineering

7.1 Introduction

Most of the world's energy demand today is supplied by fast depleting fossil fuels. The atmospheric pollution by fossil fuels is not only unhealthy but might also cause significant climate changes globally [1]. Therefore, fossil fuels should be substituted with sustainable energy sources that do not contribute to pollutiont. Hydrogen has the highest energy yield (142 kJ/g) which is 2.75 times more than that of any hydrocarbon [2]. Among different biological methods for hydrogen production dark fermentation is regarded as one of the most promising alternatives for sustainable energy generation because of the high rate of hydrogen production and its potential of direct use of wastewater streams and organic wastes [3-5].

One of the characteristics of bio-hydrogen production systems is their sensitivity to environmental conditions such as feed interruption which is mainly because of the fact that most of the hydrogen producers are strict anaerobes. Therefore, any failure in the reactor operation may cause sporulation and washout of the hydrogen producing community [5]. Working under low hydraulic retention times which is an inevitable feature of hydrogen production with mixed cultures makes this concept more critical. The hydraulic retention time of the dark fermentative hydrogen production systems is usually maintained between 3 to 12 hours [6] to prevent the growth of slow growing methanogens in the reactor. Washout of reactors at low hydraulic retention times even without any operational failure has been reported in the literature [7, 8].

Considering the large scale continuous hydrogen production, if an operational failure causes wash out in the system, re-inoculation and start-up of the system will be accompanied by huge cost and long term shutdown of the system. Reactor start-up is an

important and time-consuming phase for successful operation of continuous flow systems. Although, some techniques such as heat or acid-base treatment can considerably decrease the duration of start-up, the long term stability of the systems started with these methods has not yet been confirmed [9]. A successful startup of continuous hydrogen production systems by inoculation with non treated inocula and operating the reactor at low HRT and low pH values may take two to three months [10].

Due to the inevitable cost and time for re-inoculation of the hydrogen production systems, information on revivability of hydrogen producing systems after a period of shut-down is valuable in order to find out if these systems are revivable or whether re-inoculation is necessary. Based on the authors' knowledge, no such information is available in the literature.

The aim of this study is to investigate the performance of a continuous stirred tank bioreactor before and after feed interruption with emphasis on observed shifts in formation of soluble metabolites and microbial communities and their relationship with hydrogen production in order to examine the revivability of the system.

7.2 Materials and Methods

7.2.1 Inoculum and Composition

Anaerobically-digested sludge from the St. Marys' wastewater treatment plant (St. Marys, Ontario, Canada) was used for inoculation of the reactor. Prior to inoculation, the sludge was heat treated at 65°C for 30 minutes to enrich the hydrogen producing bacteria [11]. The nutrient medium contained glucose as the carbon source at

concentrations between 10 to 20 g/L and the following inorganic salts (mg/L): NH_4Cl, 2600; K_2HPO_4, 250; $MgCl_2.6H_2O$, 125; $FeSO_4.7H_2O$, 5.0; $CoCl_2.6H_2O$, 2.5; $MnCl_2.4H_2O$, 2.5; KI, 2.5; $Na_2MoO_4.2H_2O$, 0.5; H_3BO_4, 0.5; $NiCl_2.6H_2O$, 0.5; $ZnCl_2$, 0.5. Yeast extract at a concentration of 0.25 g/L also added to the medium.

7.2.2 Reactor Start-up and Operation

A stirred tank bioreactor (New Brunswick BioFlo 110, New Brunswick Scientific Co.) with a working volume of 2.2 liters was used in this study. Temperature was controlled at 35 ± 0.1°C and the reactor mixed at 150 rpm. The pH was maintained at 5.5 ± 0.1 with 3N potassium hydroxide. The reactor was inoculated with 300 mL of thickened anaerobically digested sludge to achieve an initial biomass concentration of 3 g/L in the reactor containing the growth medium. Subsequently the reactor was filled with the feed to the working volume and the reactor headspace was flushed with nitrogen for 30 minutes to ensure anaerobic conditions. The quantity of produced biogas was recorded daily using a wet tip gas meter (Rebel wet-tip gas meter company, Nashville, TN, USA). A biogas sampling port was installed between the reactor and the gas meter to allow direct biogas sampling with a gas tight syringe. The feed tank was kept at 4°C using F12-ED refrigerated circulator bath (Julabo, UK, Ltd.). The reactor was first operated in a batch mode for 15 hours, after which the reactor was changed to continuous flow mode.

7.2.3 Analytical Methods

The biogas composition including hydrogen, methane and nitrogen was determined by a gas chromatograph (Model 310, SRI Instruments, Torrance, CA) equipped with a thermal conductivity detector (TCD) and a molecular sieve column (Molesieve 5A, mesh 80/100, 6 ft X 1/8 in). Argon was used as carrier gas at a flow rate of 30 mL/min. The temperatures of the column and the TCD detector were 90 and 105°C, respectively. Presence of oxygen was also examined by this GC in order to ensure anaerobic condition in the bioreactor.

The concentrations of organic acids including acetic acid, butyric acid, iso-butyric acid, lactic acid, valeric acid, iso-valeric acid and formic acid were analyzed using a high-performance liquid chromatography system (1200 series, Agilent Technologies) equipped with Aminex HPX-87H ion exclusion column (300 mm × 7.8 mm I.D.; BIO-RAD), and a UV-detector at 210 nm. The column temperature was adjusted to 30 °C. The same instrument with a refractive index detector (RID) was used to measure the concentrations of glucose and alcohols. The temperature of the RID detector was set to 35 °C. Sulfuric acid at the concentration of 9 mM was used as the carrier phase. The amount of volatile suspended solids (VSS) was measured according to standard methods [12].

7.2.4 Microbial Community Analysis

At different times during the operation of the continuous reactor, intervals total genomic community DNA of biomass samples was extracted and purified using

UltraClean Soil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA). The primer set of 349FGC (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGG GGGGCCTACGGGAGGCAGCAG-3') and 518R (5'–ATTACCGCGGCTGCTGG-3') at the annealing temperature of 53°C were used for the PCR amplification of the variable V3 region of 16SrDNA from the purified genomic DNA. Denaturing gradient gel electrophoresis (DGGE) of PCR products was performed with a DCode universal mutation system (Bio-Rad laboratories, Hercules, CA, USA). The PCR products were applied directly to 8% (w/v) polyacrylamide gel with 15 to 55% denaturant (urea) gradients. Electrophoresis was performed at a constant voltage of 130 V at 59°C for 4.5 hours. The DNA template of the bands of interest were re-amplified and the PCR products were purified using QIAquick PCR purification Kit (QIAGEN Sciences, Maryland, USA) in accordance with the manufacturer's protocol. The sequences of re-amplified DNA fragments were determined by dideoxy chain termination (Sequencing Facility, John P. Robarts Research Institute, London, Ontario) and compared with available sequences in GenBank database using the BLAST program [13].

7.3 Results and Discussion

The continuously stirred tank reactor (CSTR) was started-up with an organic loading rate (OLR) of 40 g/L.d at hydraulic retention time (HRT) of 12 hours; and during the 135 days of operation went through different experimental conditions as shown in Table 7.1. Figure 7.1 illustrates the variations of fermentation parameters including hydrogen production rate, hydrogen concentration, conversion efficiency and

concentrations of biomass and soluble metabolites in the effluent during the operation of the continuous reactor.

Table 7. 1 Experimental progress of CSTR

		Experimental conditions				
Stage	Purpose	Glucose (g/L)	HRT (h)	OLR (g/L-d)	pH	Operational period (d)
I	Start-up and continuous operation	20	12	40	5.5	0-22
II	Feed interruption	---	---	---	4.5	23-33
III	Re-start up	20	12	40	4.5	34-58
IV	Decreasing the OLR	12	12	20	4.5	59-82
V	Using same OLR with lower HRT	10	10	20	4.5	83-102
VI	Increasing pH	10	10	20	4.8	103-135

7.3.1 Effect of Feed Interruption

Hydrogen production became stable after 6 days of operations and continued until day 19 when an accidental feed interruption for 12 hours happened and resulted in a sharp decrease and finally complete cessation of biogas production on day 21 (Figure 7.1-a). On day 22, continuous feeding was stopped and the reactor was operated in a batch mode (without addition of any substrate) for 10 days. During this period pH was adjusted to 4.5 to avoid development of methanogens during the operation at batch mode.

Methanogens have lower growth rate in comparison with hydrogen producers, and because of this characteristic operation at low HRT values during continuous operation can inhibit them. However, during the batch operation for several days, methanogens may find enough time to become active in the reactor. It has been reported that operation at pH values below 4.5 can inhibit not only methanogens [5] but also hydrogen consuming acetogens [14]. Therefore, pH after feed interruption was adjusted to 4.5.

After 6 days of operation in batch mode, gas production gradually recovered and biomass concentration in the effluent increased from 0.34 to 1.15 g/L. The concentration of hydrogen in the biogas decreased from 65% before the feed interruption to 25% during the batch operation.

When feeding restarted (stage III) hydrogen production rate reached to 0.6 ± 0.1 L/L.d in comparison with 3.2 ± 0.2 L/L.d before feed interruption and hydrogen concentration in the biogas reached to only 27%. Characteristics of the soluble metabolites (Figure 7.1-d) before and after feed interruption show that butyrate formation completely stopped after feed interruption and ethanol, lactic acid and acetic acid became the main soluble metabolites. Therefore, after the re-start up a shift from butyrate type fermentation to ethanol type fermentation was observed. Hydrogen production through butyrate fermentation gives higher yields of hydrogen production [15]:

$$4C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 3CH_3(CH_2)_2COOH + 8CO_2 + 10H_2 \quad (1)$$

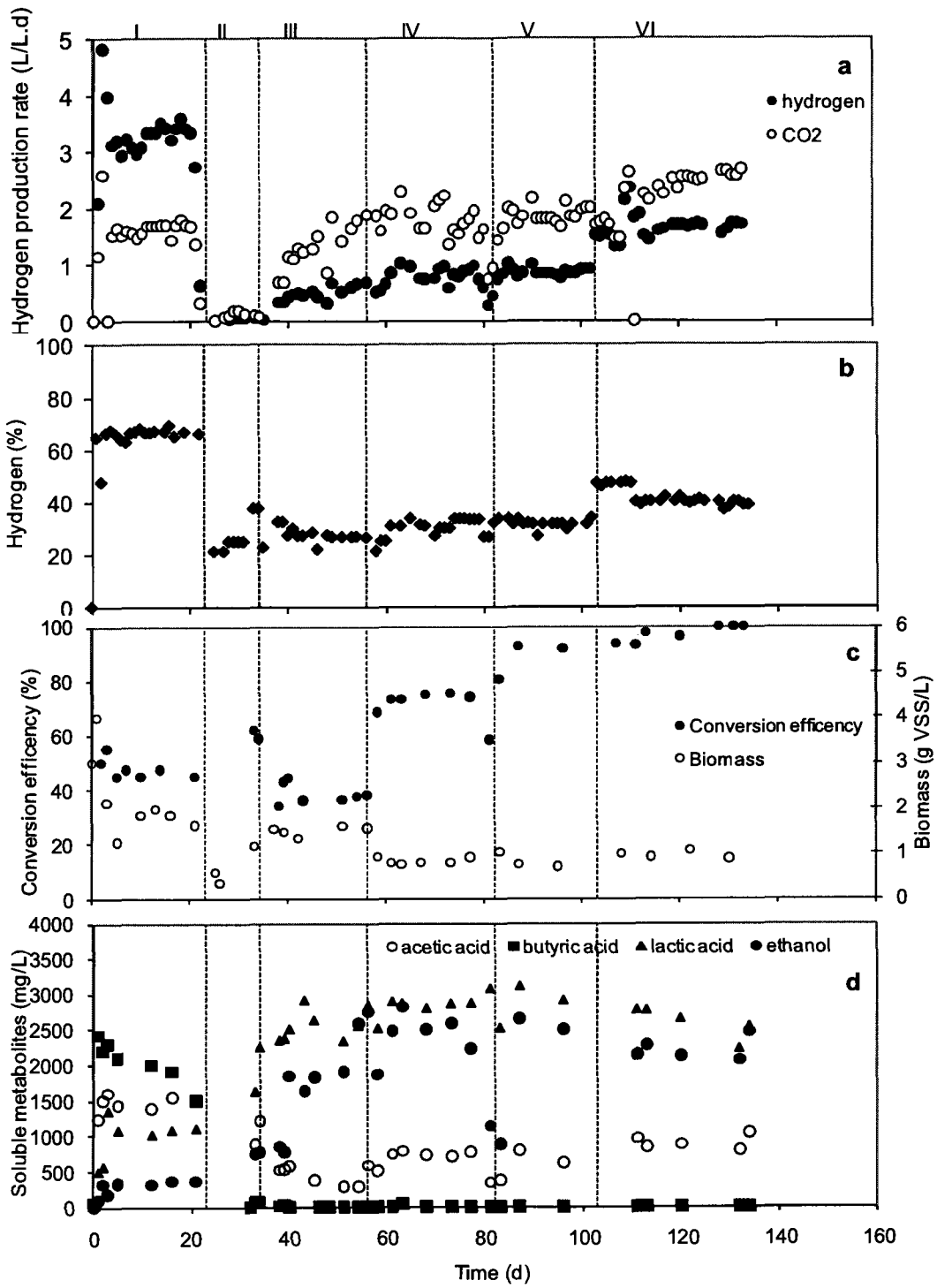


Figure 7. 1 Daily variation in: (a) hydrogen production rate; (b) hydrogen concentration; (c) biomass in the effluent; (d) concentration of soluble metabolites

However, lower hydrogen yields of 2 mol H_2/mol glucose can be obtained through ethanol type fermentation [16]:

$$C_6H_{12}O_6 + H_2O \rightarrow C_2H_5OH + CH_3COOH + 2CO_2 + 2H_2 \quad (2)$$

Although equations 1 and 2 can help for understanding of the main hydrogen production mechanism, they cannot be individually used for stoichiometric purposes because of the consumption of glucose for formation of other metabolites such as lactic acid which have been detected in the reactor. Hydrogen production through ethanol type fermentation has been rarely reported in the literature [17, 18]. This type of fermentation has been mostly investigated for its potential for ethanol production [19] rather than hydrogen. The main advantage of this type of fermentation is its low optimum pH of 4.5-5.0 [17] which is preferable for elimination of methanogens and homo-acetogens in the mixed culture and reduction of base requirements for pH control [20]. Considering the theoretical yield of 2 mol H_2/ mol glucose (Equation 2) the low yield of 0.29 mol H_2/ mol glucose could be because of formation of high amounts of lactic acid (2600 mg/L) as a reduced end product after re-start up of the reactor.

As illustrated in Table 7.2, feed interruption resulted in a significant decrease in the yield of hydrogen production and hydrogen concentration from 1.36 to 0.29 mol H_2/mol glucose and from 66.6 to 27.6%, respectively. The biomass yield after re-start up increased from 0.19 to 0.22 gVSS/g glucose and glucose conversion efficiency dropped from 46% to 37%. This could be related to a shift in microbial community in the bioreactor.

Table 7. 2 Effect of feed interruption on hydrogen production and bacterial growth (comparison of steady-state values based on stages I and III)

	Hydrogen production yield (mol H_2/ mol glucose consumed)	H_2 concentration (%)	Specific hydrogen production (mL H_2/gVSS.d)	Growth yield $Y_{x/s}$ (g VSS/g glucose)	Conversion efficiency(%)
Before feed interruption (stage I)	1.36 ± 0.05	66.6 ± 0.9	968 ± 213	0.19 ± 0.03	46 ± 1
After feed interruption (stage III)	0.29 ± 0.04	27.6 ± 2.6	178 ± 34	0.22 ± 0.00	37 ± 1

7.3.2 Effect of Organic Loading Rate

After the re-start up of the reactor, lactic acid became one of the major metabolic products. It has been observed that the concentration and/or activity of lactate-forming bacteria increased with increase in the organic loading [10]. Noike *et al.* [21] reported that hydrogen yield is reduced when lactic acid bacteria became active. In order to shift the fermentation pathway from lactic acid formation, in stage IV organic loading rate (OLR) was reduced to 20 g glucose/L.d. This reduction in the OLR was accompanied by an increase in hydrogen production rate from 0.65 to 1 L/L.d as well as hydrogen concentration from 27 to 34% (Figure 7.1 a and b). Biomass concentration in the effluent decreased from 1.5 to 1 g VSS/L (Figure 7.1c). However, as illustrated in Figure 7.1 d, concentration of lactic acid did not decrease in the stage IV.

We examined the same OLR of 20 g glucose/L.d with lower HRT in stage V. According to Figure 7.1, no significant differences were observed in hydrogen production rate, hydrogen concentration, biomass concentration and soluble metabolite including lactic acid, acetic acid and ethanol. Comparison of steady-state values in Table 7.3 shows an increase in glucose conversion efficiency from 37% to 74% by reduction of OLR from 40 to 20 g glucose/L.d. Reduction of HRT and glucose concentration in the feed by maintaining the same OLR (stage V) did not have any significant effect on hydrogen production yield and biomass yield although it raised conversion efficiency from 74% to 88%. Biomass yield decreased with reduction in OLR and remained constant in stages IV and V.

Table 7. 3 Effect of organic loading rate on hydrogen production and bacterial growth after feed interruption (comparison of steady-state values)

OLR	Hydrogen production yield (mol H_2/ mol glucose)	Hydrogen concentration (%)	Specific H_2 production (mL H_2/gVSS.d)	Growth yield $Y_{x/s}$ (g VSS/g glucose)	Conversion efficiency(%)
40 (stage III)	0.29 ± 0.04	27.6 ± 2.6	178 ± 34	0.22 ± 0.00	37 ± 1
20 (a)- (stage IV)	0.37 ± 0.09	31.1 ± 2.5	485 ± 39	0.09 ± 0.00	74 ± 1
20 (b)- (stage V)	0.35 ± 0.04	31.8 ± 2.5	434 ± 97	0.09 ± 0.01	88 ± 6

7.3.3 Effect of pH

After 103 days of operation (stage VI) pH was increased from 4.5 to 4.8. With increasing the pH, a sharp increase in hydrogen gas was observed while the content of CO_2 in the biogas decreased (Figure 7.1a). In stage VI acetic acid formation increased from 619 to 958 mg/L and a decline in formation of both lactic acid and ethanol was observed (Figure 7.1d). No butyric acid formation was detected upon increasing the pH. As illustrated in Table 7.4, changing the pH could enhance hydrogen yield from 0.35 to 0.7 mol H_2/ mol glucose and hydrogen concentration of biogas from 32 to 40%. In addition, glucose conversion efficiency improved from 88% to 99%. The significant increase in specific hydrogen production from 434 to 662 mL H2/g VSS.d together with only a small improvement in bacterial yield from 0.09 to 0.1 g VSS/ g glucose could be because of a shift in microflora or a shift in the metabolic pathways within the existing microflora rather than improvement of growth condition for available hydrogen producers. Although a pH range of 4.5 to 5.0 has been regarded as the optimum pH for ethanol type fermentation in the literature [17, 18], our results show a considerable difference in hydrogen production at pH values of 4.5 and 4.8.

Table 7. 4 Effect of pH on hydrogen production and bacterial growth after feed interruption (comparison of steady-state values)

pH	Hydrogen production yield (mol H_2/ mol glucose)	Hydrogen concentration (%)	Specific hydrogen production (mL H_2/gVSS.d)	Growth yield $Y_{x/s}$ (g VSS/g glucose)	Conversion efficiency (%)
4.5 (stage V)	0.35 ± 0.04	31.8 ± 2.5	434 ± 97	0.09 ± 0.01	88 ± 6
4.8- (stage VI)	0.70 ± 0.08	40.0 ± 2.6	662 ± 91	0.10 ± 0.01	99 ± 1

7.3.4 Carbon Mass Balance

Table 7.5 summarizes the carbon mass balance for the CSTR before and after the feed interruption (stages I and III in Table 7.1) and after improvement of hydrogen production (stage VI). It was assumed that the carbon from the yeast extract (0.25 g/L) was negligible and the cellular composition of the biomass was assumed to be $C_5H_7O_2N$ [22]. As show in Table 7.5 carbon recovery was high, in a range of 90-99%. Before feed interruption most carbons from glucose metabolism were directed toward butyric acid (28.1%), biomass (27.0%), acetic acid (15.4%), lactic acid (10.7%), ethanol (4.2%) and carbon dioxide (9.8%). Direction of carbon to propionic acid was insignificant under all test conditions (< 0.11%).

After feed interruption, the direction of carbon toward biomass remained almost constant while formation of butyric acid completely stopped and most of the carbon was converted to lactic acid (27.0%) and ethanol (24.1%), which rationalizes the low observed yield of 0.29 mol H_2/mol glucose. However, after reduction of organic loading rate from 40 to 20 g glucose/ L.d and increasing the pH from 4.5 to 4.8 the carbon conversion of glucose to biomass was reduced to only 14.1% and formation of acetic acid and ethanol increased. These changes in carbon conversion resulted in an increase in hydrogen production yield from 0.29 to 0.7 mol H_2/mol glucose.

Table 7. 5 Carbon mass balance for glucose fermentation before and after feed interruption

stage	Loaded glucose (mg C/d)	Consumed Glucose (mg C/d)	Biomass (mg VSS/L)	CO2	Butyrate	Acetate	Lactate	Propionate	Ethanol	Carbon recovery (%)
			Caron mass produced (mg C/d)							
I	35200	17072	4617 ± 771	1676 ± 115	4802 ± 676	2634 ± 133	1826 ± 453	18 ± 5	719 ± 161	95.4
III	35200	16643	4822 ± 388	2013 ± 596	28 ± 0.2	760 ± 229	4492 ± 364	3 ± 2	4009 ± 1397	98.7
VI	21120	21701	3053 ± 306	2239 ± 447	0 ± 0	1895 ± 207	5479 ± 477	0 ± 0	6092 ± 440	89.7
			Carbon distribution from glucose carbon consumed (%)							
I			27.0 ± 4.7	9.8 ± 0.0	28.1 ± 4.0	15.4 ± 0.8	10.7 ± 2.6	0.11± 0	4.2 ± 0.9	
III			29.0 ± 2.3	12.1 ± 3.6	0.2 ± 0.0	4.6 ± 1.4	27.0 ± 2.1	0.02 ± 0	24.1 ± 8.4	
VI			14.1 ± 1.4	10.3 ± 2.1	0 ± 0	8.7 ± 0.9	25.2 ± 2.2	0 ± 0	28.1 ± 2.0	

7.3.5 Analysis of Microflora Population

During all six different phases of the CSTR operation (Table 7.1), DNA samples from the whole community were used for PCR-DGGE analysis in order to investigate the changes in the microbial community. The DGGE profiles of the 16S rDNA gene fragment are shown in Figure 7.2. Table 7.6 shows the results of the sequence affiliation. Appearance of new bands during the operation of the CSTR is because of enrichment of the organisms which were already present in the reactor at undetectable concentrations. The DGGE profiles of the 16S rDNA gene (Figure 7.2) show a rapid change in the microbial community after feed interruption. This change is obvious by comparison of microbial communities before day 16 and after day 34. Most of the detected species before feed interruption, were affiliated with Clostridia. *C. butyricum* affiliated strain A (100% similarity) appeared at the third day of operation and was present in the reactor at the day 16. Band B was another *C. butyricum* affiliated strain which differed from *C. butyricum* DQ831124.1 by three mismatches. *C. acetobutyricum* affiliated bands (bands C and D) with 98% similarity to *C. acetobutyricum* FM994940.1 were also present in the reactor until the day 16. *C. acetobutyricum* and *C. butyricum* are well known hydrogen producers which ferment carbohydrates to hydrogen and carbon dioxide with acetate and butyrate as the main soluble metabolites [23]. High yields of 2 mol hydrogen/ mol glucose have been reported with pure cultures of *C. acetobutyricum* and *C. butyricum* [24]. The higher yield of hydrogen production before the feed interruption together with presence of butyrate and acetate as the main soluble metabolite could be because of presence of these species. Band E on days 3 and 16 was also identified as uncultured

Clostridia. All of the aforementioned species disappeared after the feed interruption at the day 34.

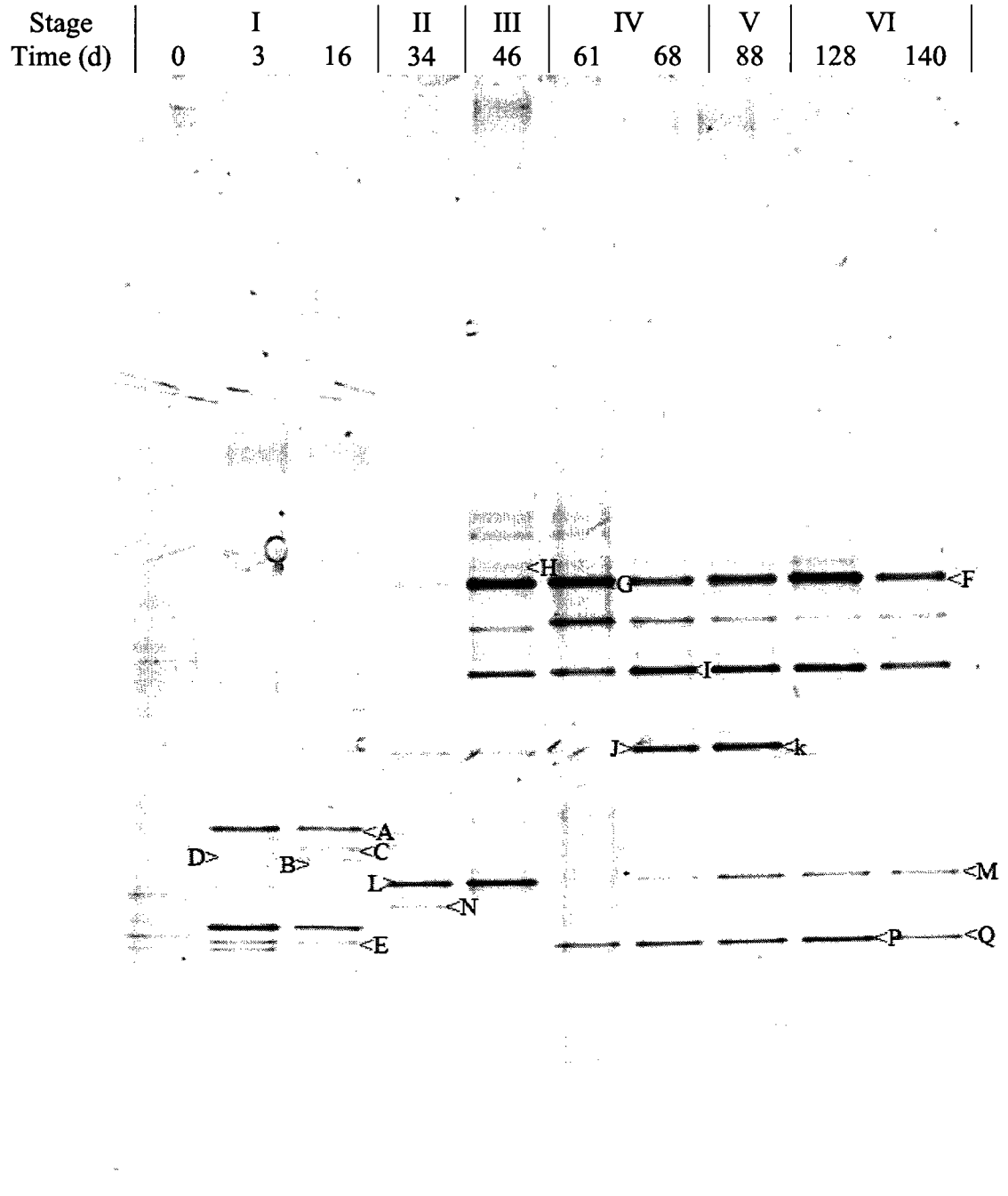


Figure 7. 2 DGGE profile of the 16S rDNA gene fragment

Table 7. 6 Affiliation of denaturing gradient gel electrophoresis (DGGE) fragments determined by their 16S rDNA sequence

Band	Affiliation (accession no.)	Similarity (%)
A	*Clostridium butyricum* (DQ831124.1)	100
B	*Clostridium butyricum* (DQ831124.1)	97
C	*Clostridium acetobutyricum* (FM994940.1)	98
D	*Clostridium acetobutyricum* (FM994940.1)	98
E	Uncultured *Clostridium* (DQ168164.1)	88
F	*Lactobacillus fermentum* (EU931244.1)	100
G	*Lactobacillus fermentum* (EU931244.1)	99
H	*Lactobacillus fermentum* (EU931244.1)	100
I	*Pseudomonas sp.* (FJ979851.1)	96
J	*Lactobacillus parabuchneri* (AB429372.1)	99
K	*Lactobacillus parabuchneri* (AB429372.1)	99
L	*Lactobacillus Casei* (FJ915819.1)	98
M	*Lactobacillus Casei* (FJ915819.1)	97
N	*Lactobacillus Casei* (FJ915819.1)	99
P	Uncultured bacterium (DQ464539.1)	100
Q	Uncultured bacterium (DQ464539.1)	99

On day 34 a *L. parabuchneri* affiliated strain and a *L. casei* affiliated strain were detected, these Lactobacillus related bands were available before the feed interruption but they were hardly visible. Band N which appeared on day 34 was also a *L. casei* affiliated strain. Operation of the reactor at an OLR of 40 g glucose/L.d after feed

interruption (stage III) resulted in formation of new bands including *L. fermentum* and *Pseudomonas* sp. affiliated strains. With changing of OLR from 40 to 20 g glucose/L.d a new strain was detected in the reactor which was, 99-100% affiliated with uncultured bacterium DQ464539.1 (bands P and Q). The uncultured bacterium DQ464539.1 was reported in an acidophilic ethanol-H_2-coproducing system. This organism could be the main hydrogen producer in the reactor after the feed interruption since its appearance increased hydrogen production rate. By comparison of DGGE profile on days 61, 68 and 88 it can be concluded that changing the HRT with a constant organic loading rate did not affect the microorganisms present in the reactor. This is in good agreement with the observed hydrogen and biomass yield in stages IV and V (days 59 to 102 in Figure 7.1a and 1c). Changing the pH from 4.5 to 4.8 which resulted in an increase in the hydrogen production yield from 0.35 to 0.7 mol H_2/mol glucose was accompanied by elimination of *L. parabuchneri* (band K) from the culture. Lactic acid bacteria compete with hydrogen–producing bacteria for carbohydrates, and thus their presence reduces hydrogen yields.

7.4 Conclusions

The results of this work demonstrated that a period of feed interruption in a continuous hydrogen producing system caused an entire stop in butyric acid formation along with a decrease in hydrogen production yield from 1.36 to 0.29 mol H_2/mol glucose. Reduction of organic loading rate together with increasing the pH after the feed interruption increased the hydrogen yield to 0.7 mol H_2/mol glucose. The main soluble

metabolites after the feed interruption were acetate, lactate and ethanol. The analysis of microbial community showed the predominance of hydrogen producing *Clostridia* in the reactor before feed interruption. However, after the feed interruption most of the specious were affiliated to *Lactobacillus*. It seems that hydrogen producing reactors are not readily revivable unless activity of lactic acid bacteria can be eliminated.

7.5 References

1. Benemann J. Hydrogen biotechnology: Progress and prospects. Nat Biotechnol. 1996;14(9):1101-3.

2. Cai ML, Liu JX, Wei YS. Enhanced biohydrogen production from sewage sludge with alkaline pretreatment. Environ Sci Technol. 2004;38(11):3195-202.

3. Kapdan IK, Kargi F. Bio-hydrogen production from waste materials. Enzyme Microb Technol. 2006;38(5):569-82.

4. Levin DB, Pitt L, Love M. Biohydrogen production: Prospects and limitations to practical application. Int J Hydrogen Energy. 2004;29(2):173-85.

5. Hawkes FR, Dinsdale R, Hawkes DL, Hussy I. Sustainable fermentative hydrogen production: Challenges for process optimisation. Int J Hydrogen Energy. 2002;27(11-12):1339-47.

6. Li CL, Fang HHP. Fermentative hydrogen production from wastewater and solid wastes by mixed cultures. Crit Rev Environ Sci Technol. 2007;37(1):1-39.

7. Chen CC, Lin CY, Chang JS. Kinetics of hydrogen production with continuous anaerobic cultures utilizing sucrose as the limiting substrate. Appl Microbiol Biotechnol. 2001;57(1-2):56-64.

8. Chen CC, Chen HP, Wu JH, Lin CY. Fermentative hydrogen production at high sulfate concentration. Int J Hydrogen Energy. 2008;33(5):1573-8.

9. Oh SE, Van Ginkel S, Logan BE. The relative effectiveness of pH control and heat treatment for enhancing biohydrogen gas production. Environ Sci Technol. 2003 NOV 15;37(22):5186-90.

10. Oh YK, Kim SH, Kim MS, Park S. Thermophilic biohydrogen production from glucose with trickling biofilter. Biotechnol Bioeng. 2004;88(6):690-8.

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

12. APHA, AWWA, WEF. Standard methods for examination of water and wastewater, 19th edition. ; 1995.

13. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990;215(3):403-10.

14. Hussy I, Hawkes FR, Dinsdale R, Hawkes DL. Continuous fermentative hydrogen production from a wheat starch co-product by mixed microflora. Biotechnol Bioeng. 2003;84(6):619-26.

15. Lungdahl, L.G., Hugenholtz, J., Wiegel, J. Acetogenic and acid-producing clostridia. In: Clarke DJ, editor. Clostridia. 1 st ed. New York: Plenum Press; 1989. p. 145-91.

16. Gaudy A, Gaudy E. Microbiology for environmental scientists and engineers. In: McGraw-Hill; 1980. p. 519-66.

17. Hwang MH, Jang NJ, Hyun SH, Kim IS. Anaerobic bio-hydrogen production from ethanol fermentation: The role of pH. J Biotechnol. 2004;111(3):297-309.

18. Ren NQ, Wang BZ, Huang JC. Ethanol-type fermentation from carbohydrate in high rate acidogenic reactor. Biotechnol Bioeng. 1997;54(5):428-33.

19. Yokoi H, Aratake T, Hirose J, Hayashi S, Takasaki Y. Simultaneous production of hydrogen and bioflocculant by enterobacter sp BY-29. World J Microbiol Biotechnol. 2001;17(6):609-13.

20. Zoetemeyer RJ, Vandenheuvel JC, Cohen A. pH influence on acidogenic dissimilation of glucose in an anaerobic digester. Water Res. 1982;16(3):303-11.

21. Noike T, Takabatake H, Mizuno O, Ohba M. Inhibition of hydrogen fermentation of organic wastes by lactic acid bacteria. Int J Hydrogen Energy. 2002;27(11-12):1367-71.

22. Orhon D, Artan N. Modelling of activated sludge systems. In: Lan-caster, Pennsylvania: Technomic Publishing Company; 1994. p. 78-87.

23. Cohen A, Vangemert JM, Zoetemeyer RJ, Breure AM. Main characteristics and stoichiometric aspects of acidogenesis of soluble carbohydrate containing wastewaters. Process Biochemistry. 1984;19(6):228-32.

24. Yokoi H, Tokushige T, Hirose J, Hayashi S, Takasaki Y. H-2 production from starch by a mixed culture of *Clostridium butyricum* and *Enterobacter aerogenes*. Biotechnol Lett. 1998;20(2):143-7.

CHAPTER 8

Conclusions and Recommendations

8.1 Conclusions

In the present research, biological hydrogen production via dark fermentation was studied in batch, repeated batch and continuous systems. The fermentation inocula consisted of mixed cultures which originated from municipal sludges. The key objectives of this thesis incorporate (i) the modification and improvement of enrichment methods to obtain active and stable inocula, (ii) the increase of hydrogen production rates and yields, and (iii) the investigation of sustainability and revivability of continuous biohydrogen production. These issues are critical to biohydrogen commercialization.

In this research, the effect of heat pre-treatment on the inocula at various temperatures was investigated and the best temperature which led to the highest hydrogen production was employed for the heat pre-treatments. Then, hydrogen production with two types of inocula, waste activated sludge and anaerobically digested sludge, were compared at mesophilic and thermophilic temperatures. In another phase of the research, the positive impact of extrinsic lactic acid on biohydrogen production was examined with starch-containing media. Continuous hydrogen production experiments were also conducted to compare sustainability of hydrogen production in continuously stirred tank reactors under different organic loading rates. The last part of this research was the investigation of revivability in continuous hydrogen production after a period of feed interruption by comparison of soluble metabolites, carbon mass balance and microbial community before and after the feed interruption. Based upon the findings of this research, the following specific conclusions were drawn:

• The effect of heat treatment at different temperatures on two types of inocula, activated sludge and anaerobically digested sludge, was investigated in batch cultures. Heat treatments were conducted at 65°C, 80°C and 95°C for 30 minutes. The untreated inocula produced less amount of hydrogen than the pretreated inocula, with lactic acid as the main metabolite. Conducting pre-treatment at 65°C resulted in the highest hydrogen yield as well as the highest maximum specific hydrogen production rate in both activated sludge (1.6 mol H_2/ mol glucose and 80.4 mL hydrogen/g VSS h) and anaerobically digested sludge (2.3 mol H_2/mol glucose and 109.6 mL hydrogen/g VSS h) in batch experiments. Approximately a 15% decrease in yield was observed with increasing pretreatment temperature from 65°C to 95°C concomitant with an increase in butyrate/acetate ratio from 1.5 to 2.4 for anaerobically digested sludge. The increase of pretreatment temperature of activated sludge to 95°C suppressed the hydrogen production while induced the production of lactic acid. The DNA analysis of the microbial community exhibited that the elevated pretreatment temperatures reduced the species diversity.

• Hydrogen production with activated sludge and anaerobically digested sludge at both thermophilic (55°C) and mesophilic (37°C) conditions was compared in batch experiments. The results revealed that the production of hydrogen with activated sludge was more efficient at the thermophilic temperature and had a yield of 0.93 mol H_2/ mol glucose. However, when anaerobically digested sludge was used for hydrogen production at thermophilic temperature, the hydrogen yield decreased to 1.28 mol H_2/ mol glucose, which was 15% less than that of mesophilic temperature. To investigate the stability of hydrogen production with the two types of inocula a series of repeated batch experiments

were conducted at 37°C. The results showed no major difference in the average hydrogen yield between the two types of inocula (1.18 ± 0.62 mol H_2/ mol glucose with activated sludge versus 1.35 ± 0.14 mol H_2/ mol glucose with anaerobic sludge), however, the hydrogen production with the activated sludge was not stable and widely fluctuated during the consecutive batches. The observed instability was attributed to formation of lactic acid.

- The effects of the addition of ethanol (5mM), acetic acid (10 mM), butyric acid (10 mM), and lactic acid (10 mM) were studied using a fractional factorial design. The addition of ethanol, acetic acid and butyric acid did not significantly affect the hydrogen production. However, lactic acid had a significant positive effect on the cumulative hydrogen production and maximum hydrogen production rate.

- The effect of extrinsic lactic acid on fermentative hydrogen production from starch-containing media was further studied in batch experiments. The addition of 10 mM lactic acid to a batch containing starch increased the hydrogen production rate and hydrogen production yield from 4.31 to 8.23 mL/h and 5.70 to 9.08 mmol H_2/ g starch, respectively. This improvement in hydrogen production rate and yield was associated with a shift from acetic acid and ethanol formation to the production of butyric acid as the predominant metabolite. The increase in the hydrogen production yield was attributed to the increase in the available residual NADH for hydrogen production. When lactic acid was used as the sole carbon source, no significant hydrogen production was observed.

• The revivability of a continuous hydrogen production system after a period of feed interruption was studied. After the feed interruption, butyric acid formation completely stopped and ethanol, acetic acid and lactic acid became the predominant soluble metabolites. The microbial community analysis showed the complete elimination of *Clostridium* affiliated strains and predominance of *Lactobacillus* affiliated strains after the re-startup of the reactor. The hydrogen production yield after the feed interruption decreased from 1.36 mol H_2/ mol glucose to 0.29 mol H_2/ mol glucose. The operation of the reactor at lower organic loading rate of 20 g glucose/L.d and pH of 4.8 could increase the yield to 0.7 mol H_2/ mol glucose.

8.2 Recommendations

The following recommendations can be made based on the results of this study:

- The addition of lactic acid to carbohydrate containing media in batch experiments resulted in a significant enhancement of hydrogen production. Further research on the use of lactic acid in continuous biohydrogen reactors using pure lactic acid and lactic acid containing wastes such as whey and yogurt waste is recommended.

- Based on the results of this study start-up at high organic loading rate of 40 g glucose/L could be the main cause of low hydrogen production in continuous systems. Additional research on the start-up strategy including gradual increase in the organic loading rate is required.

- Further information on ethanol type fermentation for biohydrogen production should be developed and the process should be optimized.

- Effective strategies for inhibition of lactic acid bacteria are underreported in the literature of hydrogen production from mixed cultures. More research is required to develop an appropriate method to minimize the presence of lactic acid bacteria, and such a method needs further assessment not only in batch but in long term continuous operation.

- Hydrogen production from mixed cultures of known bacteria may provide a deeper knowledge on dark fermentative hydrogen production and make the system more predictable and easier to control.

- Dark fermentative hydrogen production has always been studied with the aim of increasing the yields and rates of hydrogen production. However, this method can not be used individually and in the future biohydrogen production systems, dark fermentation has to be integrated with a second stage such as photo fermentative hydrogen production; therefore the soluble metabolites of the first stage should not have an inhibitory effect on the second stage. It is recommended to consider the limiting ranges of some parameters such as ammonia concentration on the second stage while conducting the research on dark fermentative hydrogen production.

APPENDIX

Supplementary Materials

APPENDIX A

Supplementary Figures for fractional factorial experiment (Chapter 5)

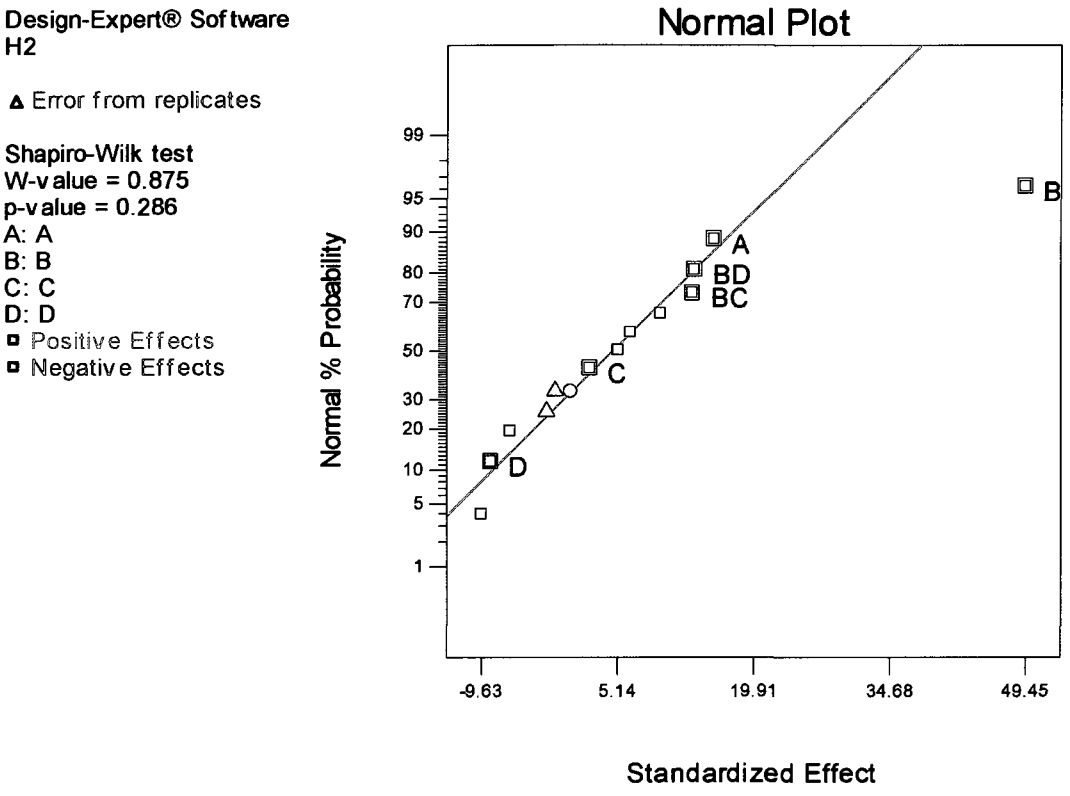


Figure A. 1 Normal plot of removed data after power transformation; A: ethanol, B: lactic acid; C: butyric acid; D: acetic acid; orange square symbols: Positive Effects; blue square symbols: Negative Effects

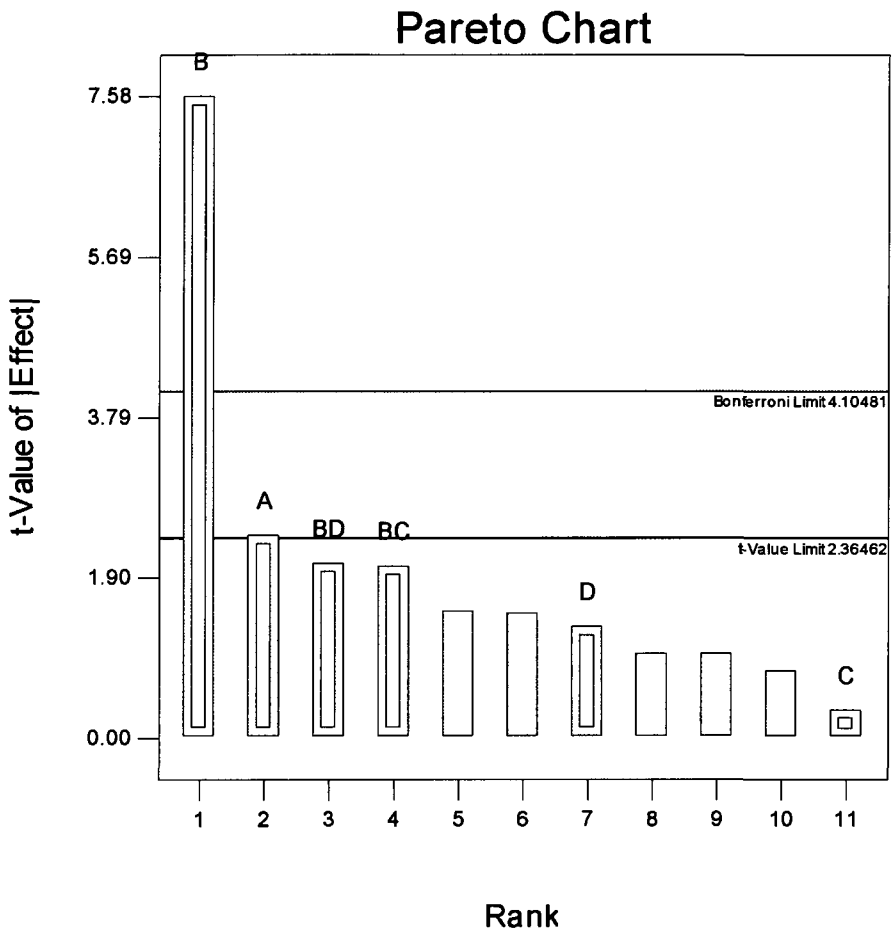


Figure A. 2 Pareto chart of examined effects of four parameters, A: ethanol, B: lactic acid; C: butyric acid; D: acetic acid; BC: interaction of lactic acid and butyric acid; BD: interaction of lactic acid and acetic acid;

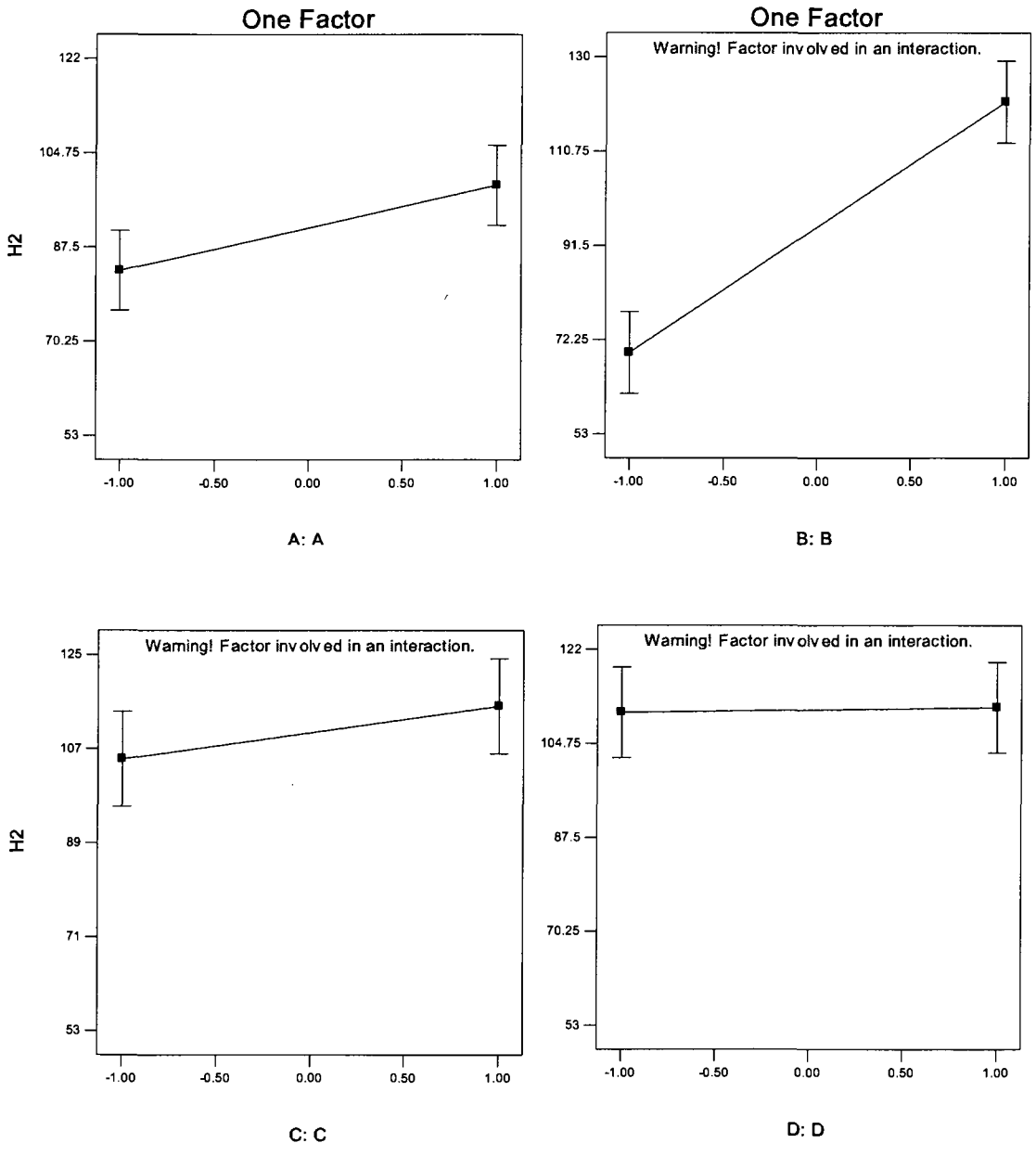
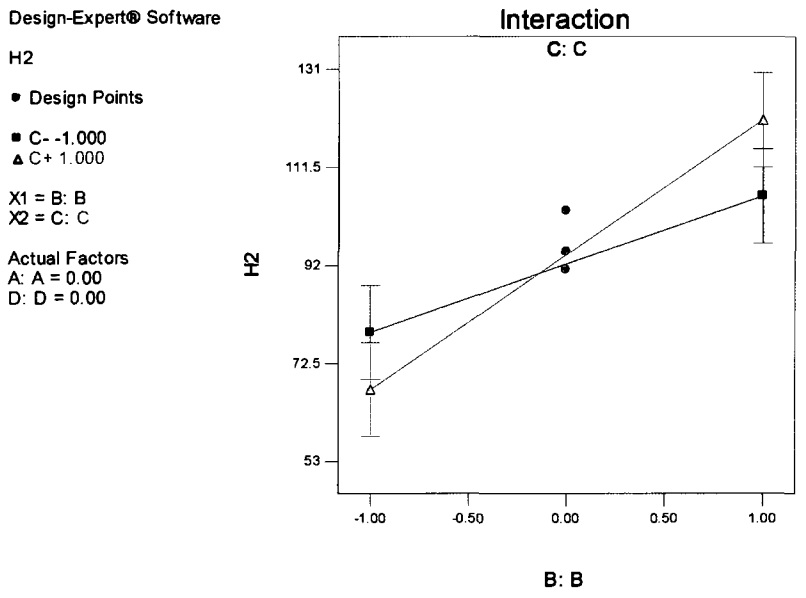


Figure A. 3 The linear effect of changing the level of a single factor, A: ethanol, B: lactic acid; C: butyric acid; D: acetic acid



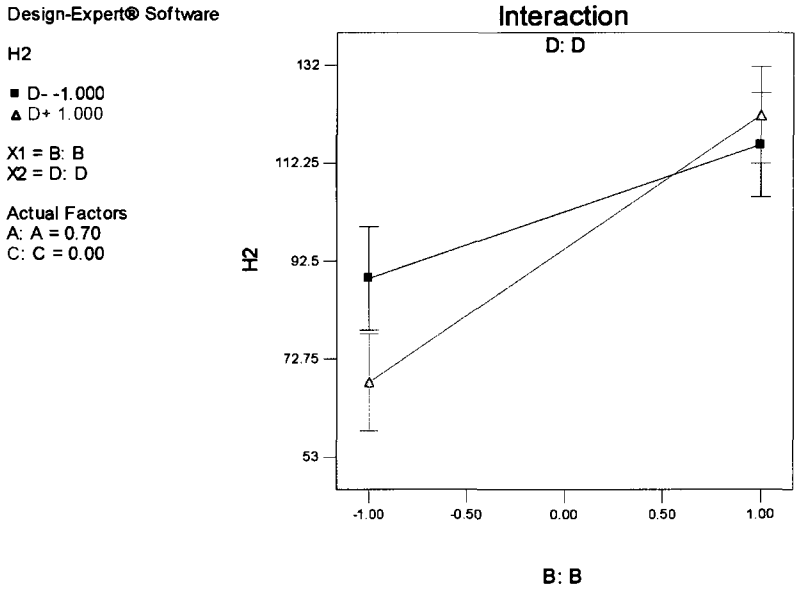


Figure A. 4 Interaction graphs for easier interpretation of two factor interaction

APPENDIX B

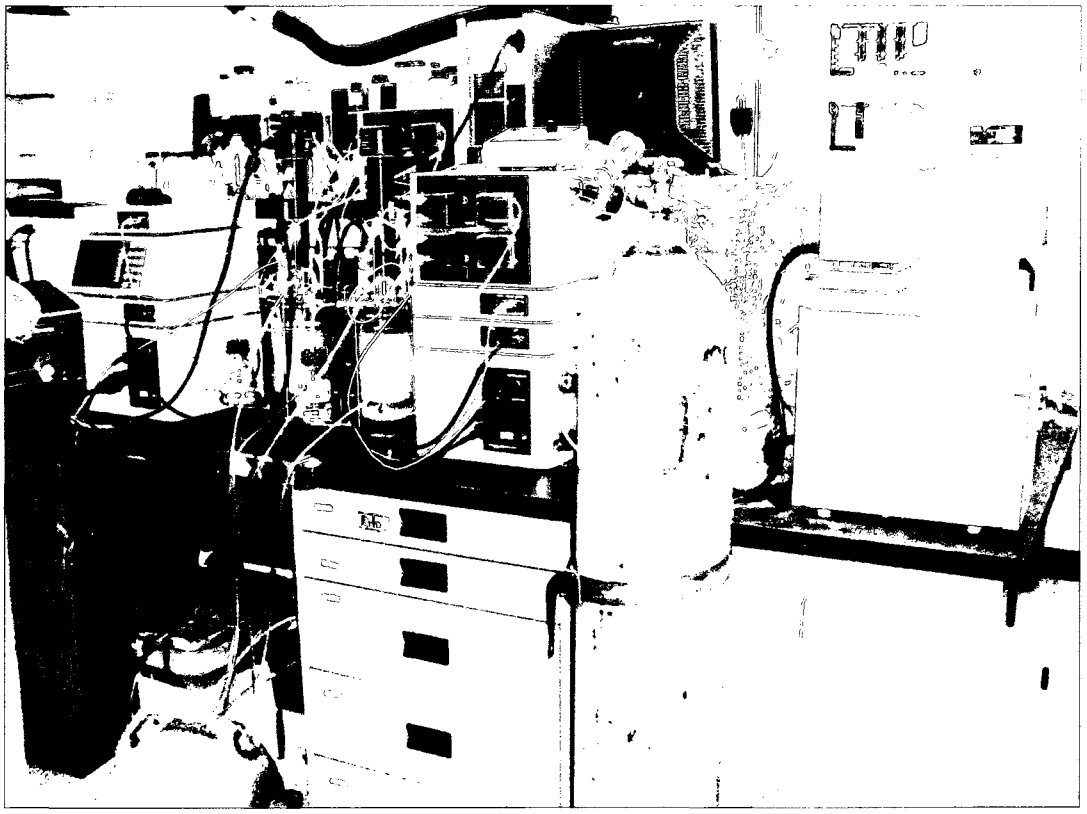


Figure A. 5 A view of the continuous bioreactor setups.

APPENDIX C

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2. **Baghchehsaraee B**, Nakhla G, Karamanev D, Margaritis A. Effect of extrinsic lactic acid on fermentative hydrogen production. *International Journal of Hydrogen Energy*, 2009; 34(6):2573-9.

3. **Baghchehsaraee B**, Nakhla G, Karamanev D, Margaritis A. Fermentative hydrogen production by diverse microflora. Submitted to *International Journal of Hydrogen Energy*.

4. Hafez H, **Baghchehsaraee B**, Nakhla G, Karamanev D, Margaritis A, Neggar H. Comparative assessment of decoupling of biomass and hydraulic retention times in hydrogen production bioreactors. *International Journal of Hydrogen Energy*, August 2009, (online).

5. **Baghchehsaraee B**, Nakhla G, Karamanev D, Margaritis A. Revivability of biological hydrogen producing bioreactors. In progress for submission to *Biotechnology and Bioengineering*.

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- Western Graduate Research Scholarship (WGRS)* 2005-2009
- Western Engineering Scholarship (WES)* 2005-2009
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*These awards have been from The University of Western Ontario