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Hydrogen Production from Molasses Using Chlorinated Alkane-contaminated Groundwater as an Inoculum

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**HYDROGEN PRODUCTION FROM MOLASSES USING CHLORINATED ALKANE-
CONTAMINATED GROUNDWATER AS AN INOCULUM**

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science in Civil Engineering
in

The Department of Civil and Environmental Engineering

By
Hector Narez
B.S., Louisiana State University, 2005
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TABLE OF CONTENTS

ACKNOWLEDGEMENTS	ii
LIST OF TABLES	vi
LIST OF FIGURES	vii
ABSTRACT.....	ix
CHAPTER 1: INTRODUCTION.....	1
1.1 Overview.....	1
1.2 Objectives	3
1.3 Thesis Organization	4
CHAPTER 2: LITERATURE REVIEW.....	5
2.1 Overview.....	5
2.2 Fermentative Hydrogen Production.....	5
2.3 Substrates	6
2.4 Metabolic Pathways and End Products.....	9
2.5 End Product Inhibition.....	10
2.6 Microbial Selection Strategies	11
2.7 Effects of Reactor Operating Conditions.....	12
2.8 Microbial Communities	15
CHAPTER 3: HYDROGEN PRODUCTION CAPACITY OF THE MICROBIAL CONSORTIUM AT FOUR DIFFERENT pH VALUES (BATCH MODE).....	17
3.1 Introduction.....	17
3.2 Materials and Methods.....	18
3.2.1 Reactor Configuration.....	18
3.2.2 Materials Preparation	19
3.2.3 Reactor Startup and Operation.....	21
3.2.4 Analytical Methods.....	23
3.3 Results and Discussion	24
3.3.1 Initial Conditions and pH.....	24
3.3.2 Sugar Concentration.....	27
3.3.3 Biogas Production and H ₂ Composition	29
3.3.4 H ₂ Production and Yield	32
3.3.5 Volatile Fatty Acids and Homoacetogenesis	33
3.3.6 Further Discussion and Implications Related to the PPI Site	35
3.4 Conclusions.....	36
CHAPTER 4: THE EFFECT OF pH VALUE ON THE CONTINUOUS PRODUCTION OF HYDROGEN (CSTR) AS A RENEWABLE ENERGY SOURCE	38
4.1 Introduction.....	38
4.2 Materials and Methods.....	38
4.2.1 Reactor Configuration.....	38
4.2.2 Feed Preparation	39

4.2.3 Reactor Startup and Operation.....	40
4.2.4 Analytical Methods.....	42
4.3 Results.....	42
4.3.1 Temperature and pH.....	42
4.3.2 Organic Loading Rate, Sugar Consumption, and Biomass Concentration.....	43
4.3.3 Biogas Production and Composition.....	43
4.3.4 Volatile Fatty Acids.....	46
4.3.5 Hydrogen Production Rate and Yield.....	48
4.4 Discussion.....	50
4.4.1 Assessment of Capacity in Comparison to Similar Studies.....	51
4.5 Conclusions.....	52

CHAPTER 5: THE EFFECTS OF INCREASED ORGANIC LOADING RATE ON A HYDROGEN PRODUCING CSTR..... 55

5.1 Introduction.....	55
5.2 Materials and Methods.....	55
5.3 Results.....	56
5.3.1 Organic Loading Rate, Sugar Consumption, and Biomass Concentration.....	56
5.3.2 Biogas Production, Biogas Composition, and VFA.....	58
5.3.3 Hydrogen Production and Yield.....	60
5.4 Discussion.....	62
5.5 Conclusions.....	63

CHAPTER 6: THE EFFECT OF ELEVATED 1,2-DICHLOROETHANE CONCENTRATIONS ON H₂ PRODUCTION IN A CSTR 65

6.1 Introduction.....	65
6.2 Materials and Methods.....	65
6.3 Results.....	66
6.3.1 1,2-DCA Concentrations.....	66
6.3.2 Sugar Consumption and Biomass Concentration.....	68
6.3.3 Gas Production, Biogas Composition, and Volatile Fatty Acids.....	70
6.3.4 Hydrogen Production and Yield.....	72
6.4 Discussion.....	73
6.5 Conclusions.....	73

CHAPTER 7: OVERALL CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH 75

REFERENCES..... 78

APPENDIX A: DETERMINATION OF OLR IN TERM OF COD DURING INCREASED OLR EXPERIMENT..... 86

APPENDIX B: CALCULATION OF 1,2-DCA CONCENTRATIONS IN THE FEED SOLUTION FOR EXPERIMENTS DESCRIBED IN CHAPTER 6 87

**APPENDIX C: CALCULATION OF 1,2-DCA CONCENTRATIONS IN THE EFFLUENT
FOR EXPERIMENTS DESCRIBED IN CHAPTER 6 89**

VITA..... 91

LIST OF TABLES

Table 2-1: Properties of molasses.....	7
Table 2-2: H ₂ production rates and yields reported in continuous-flow studies using molasses as substrate.....	8
Table 3-1: Groundwater samples used for inoculation of batch-mode experiments 1-4.....	20
Table 3-2: Chlorinated solvent concentrations measured in groundwater from well MW-01 at the PPI site (data provided by NPC Services, Inc.).....	25
Table 3-3: Geochemical parameters measured in groundwater from well MW-01 at the PPI site (data provided by NPC Services, Inc.).....	26
Table 3-4: Initial pH and sugar concentration for batch-mode experiments 1-4.....	26
Table 3-5: Time required for 90% consumption, time of initial decrease in sugar concentration, and total sugar consumption for batch-mode experiments 1-4.....	28
Table 3-6: Biogas production volume, rate, and yield and H ₂ composition during the production period of batch-mode experiments 1-4.....	31
Table 3-7: Total H ₂ production and yield for batch-mode experiments 1-4.....	32
Table 4-1: Dates when continuous-flow experiments 1-4 commenced and ended.....	40
Table 4-2: H ₂ production, yield, and specific production for continuous-flow experiments.....	49
Table 4-3: H ₂ production rates and yields reported in continuous-flow studies using molasses as substrate.....	54
Table 5-1: Average H ₂ production rate, H ₂ yield, and specific H ₂ production rate before, during, and after increasing the OLR.....	62
Table 6-1: Days on which the reactor was fed with molasses solution containing 1,2-DCA and the concentration of 1,2-DCA in the influent molasses solution immediately before feeding and immediately after feeding.....	67
Table 6-2: H ₂ production rate, H ₂ yield, and specific H ₂ production rate during periods of feeding with 1,2-DCA and without 1,2-DCA.....	72
Table A-1: Data used to construct scatter plot for COD to glucose ratio.....	86

LIST OF FIGURES

Figure 3-1: Reactor configuration during batch-mode operations.....	19
Figure 3-2: pH as a function of time for batch-mode experiments 1-4.....	27
Figure 3-3: Sugar consumption as a function of time for batch-mode experiments 1-4.....	28
Figure 3-4: (A) cumulative biogas production volume, (B) biogas production rate, and (C) H ₂ composition during batch-mode experiments 1-4.....	30
Figure 3-5: Distributions of volatile fatty acids (VFAs) in comparison to biogas production and composition for batch-mode experiments 1-4.....	34
Figure 4-1: Reactor configuration during continuous-flow experiments.....	39
Figure 4-2: pH during continuous-flow experiments 1-4.....	43
Figure 4-3: (A) Organic loading rate, (B) influent and effluent sugar concentration, (C) and biomass concentration during continuous-flow experiments 1-4.....	44
Figure 4-4: (A) Biogas production rate, and (B) biogas composition for continuous-flow experiments 1-4.....	45
Figure 4-5: (A) Biogas production rate, (B) biogas composition, and (C) organic acid concentration for continuous-flow experiments 1-4.....	47
Figure 4-6: Hydrogen production rate for continuous-flow experiments 1-4.....	49
Figure 5-1: (A) Organic loading rate, (B) influent and effluent sugar concentration, and (C) biomass concentration prior to, during, and after the increase in OLR.....	57
Figure 5-2: (A) Biogas production rate, (B) biogas composition, and (C) VFA concentration before, during, and after increasing the OLR.....	59
Figure 5-3: Hydrogen production rate before, during, and after increasing of the OLR.....	61
Figure 6-1: 1,2-DCA concentration as a function of time.....	68
Figure 6-2: The (A) influent and effluent sugar concentrations, (B) effluent biomass concentration, and (C) concentrations of 1,2-DCA.....	69
Figure 6-3: (A) Biogas and hydrogen production rate, (B) biogas composition, and (C) VFA concentrations before, during, and after the intervals of feeding elevated concentrations of 1,2-DCA.....	71

Figure A-1: Scatter plot and linear regression for measured sugar concentration and the corresponding COD concentration.....86

Figure C-1: Theoretical and experimentally determined 1,2-DCA concentrations during feeding of molasses solution containing 1,2-DCA.....90

ABSTRACT

The Scenic Highway area of the PetroProcessors of Louisiana, Inc. (PPI) Superfund site located near Baton Rouge, LA, has groundwater contaminated with a variety of chlorinated alkanes (e.g., 1,2-dichloroethane, 1,2-dichloropropane) and alkenes (e.g., trichloroethene and vinyl chloride). In March 2009, a field-scale pilot test was conducted to evaluate the efficacy of injecting diluted agricultural feed grade molasses into the groundwater in an effort to stimulate *in-situ* reductive dechlorination of these chlorinated contaminants. Because H_2 plays an important role as an electron donor in the metabolism of reductively dechlorinating bacteria, in support of the pilot-scale effort, experiments described in this thesis were conducted to better understand the biological hydrogen production potential of the microbial populations from contaminated groundwater at the site. As a means of evaluating the capacity of microbial populations derived from the site to produce H_2 as a potential renewable energy source, additional experiments were carried out to evaluate the rate and yield of hydrogen production using molasses as a feedstock.

Batch-mode experiments carried out in a laboratory-scale bioreactor inoculated with groundwater from the PPI site revealed that molasses was rapidly fermented with concomitant H_2 production at all pH values tested (ranging from 4.5 to 7.5). In the batch tests, a pH of 5.5 resulted in the highest total production H_2 with a net yield of 1.47 mol- H_2 /g-glucose. H_2 production rates at higher pH values (6.5 and 7.5) decreased over time due to homoacetogenesis and methanogenesis. At a pH of 4.5, H_2 production was characterized by lower production rates lasting for a longer period of time. The microbial consortia developed during batch-mode experiments were subsequently employed in continuous-flow (CSTR) experiments to further evaluate the effects of pH on the rate, yield, and stability of H_2 production. As with batch-mode experiments, the highest H_2 production rate (averaging 8.86 L/L/d over a two-month interval)

and yield ($1.93 \text{ mol-H}_2/\text{mol-glucose}_{\text{consumed}}$) was obtained at pH 5.5. In short-term experiments, supplying elevated concentrations of 1,2-dichloroethane in the bioreactor feed was found to have a slight positive effect on H_2 production rate and yield.

CHAPTER 1: INTRODUCTION

1.1 Overview

Hydrogen (H₂) is attractive as an energy source because when it reacts with oxygen in a fuel cell, electricity is generated with water as a harmless byproduct. This eliminates the direct emission of CO₂ encountered with traditional combustion processes employing petroleum or other fossil fuels for energy generation. While the ability of some bacteria to fermentatively produce hydrogen has been known for many years, questions remain regarding the selective pressures that can favorably increase the rate and yield of hydrogen production by mixed cultures.

Biological hydrogen production is also of interest in the field of bioremediation including in the reductive dechlorination of chlorinated aliphatic ethanes and ethenes which are prevalent groundwater and soil contaminants throughout the world. In recent years, several bacteria capable of reductive dechlorination have been isolated and characterized (Maymó-Gatell *et al.*, 1999; Smidt *et al.*, 2000; Luijten *et al.*, 2003). Many of these, including “Dehalococcoides ethenogenes” and *Dehalogenimonas lykanthroporepellens*, utilize H₂ as their primary electron donor (Maymó-Gatell *et al.*, 1999; Moe *et al.*, 2009). Thus, microbes that anaerobically dehalogenate chlorinated solvents in the environment likely rely on fermentative microorganisms as a source of hydrogen (DiStefano *et al.*, 1992; Ballapragada *et al.*, 1997; Maymó-Gatell *et al.*, 1999; Bowman *et al.*, 2006). Subsurface injection of fermentable substrates has been utilized as a bioremediation strategy at many sites around the world including at the Petro-Processors of Louisiana, Inc. (PPI) Superfund Site.

The Petro-Processors of Louisiana, Inc. (PPI) Superfund site is located about 10 miles north of Baton Rouge. The PPI site is comprised of two areas, referred to as the Brooklawn area

and the Scenic Highway area, where industrial wastes including chlorinated solvents were discharged to the environment resulting in soil and groundwater contamination. Several *Clostridium* strains isolated from the Brooklawn portion of the PPI site were demonstrated to produce hydrogen as a product of glucose fermentation (Bowman *et al.* 2006, 2009, 2010a). Furthermore, the strains were shown to be able to produce hydrogen from glucose even in the presence of high concentrations of the chlorinated solvents tetrachloroethene (PCE), 1,2-dichloroethane (1,2-DCA), and 1,1,2-trichloroethane (1,1,2-TCA). In an effort to stimulate reductive dechlorination of chlorinated alkanes and alkenes in the near source zone area of the Scenic Highway portion of the PPI site, diluted agricultural feed grade molasses was injected into the groundwater in a pilot-scale test in March 2009.

Starting on March 3, 2009, a molasses injection field test was initiated at the PPI site (NPC, 2010). During the field test, groundwater extracted from four wells (well ID numbers ED-01, EN-01, ES-01, and EU-01), was supplemented with molasses and then reintroduced into the subsurface via a centrally located injection well (well ID no. I01). During the test, 437,000 gallons of extracted groundwater was removed and reinjected. The flow rate over the entire substrate injection phase averaged 53 gallons per minute (gpm). A total of 9,500 pounds of molasses was injected over the 6-day test period. Molasses concentrations in the injected water averaged 3.57 g/L. Sodium bicarbonate (NaHCO_3) was injected along with the molasses to increase alkalinity in the groundwater by an additional 100 mg/L (NPC, 2010). Based on tracer tests, the groundwater velocity in the vicinity of the pilot-scale test was estimated to be in the range of 0.6 to 0.7 ft/day both before and after the molasses injection (NPC, 2010).

Approximately 11 months after the initial injection of molasses and bicarbonate, additional groundwater extraction and reinjection was employed in an attempt to adjust the pH of groundwater in the vicinity of the initial molasses addition (NPC, 2010). From January 26, 2010

to February 11, 2010, soda ash (sodium carbonate) was added to the groundwater with injection operations that lasted for approximately ten hours per day, five days per week. In this procedure, groundwater was recirculated for a total of 105 hours, during which time soda ash solution was injected for 97 hours. The average groundwater recirculation rate was 48 gpm; 301,000 gallons of water amended with soda ash were injected. In this procedure, a 60 g/L solution of soda ash was added to the recirculated groundwater at an average rate of 5.65 lb/min, resulting in an average soda ash concentration of 0.79 g/L in the reinjected groundwater. A total of 1,980 pounds of soda ash was injected during this phase of the test (NPC, 2010).

1.2 Objectives

The broad goal of the research described in this thesis was to better understand the biological hydrogen production potential of microbial populations from chlorinated solvent contaminated groundwater at the Scenic Highway portion of the PPI Superfund Site and to determine the conditions which favor high hydrogen production rate and yield. The research was divided into four tasks:

(1) Batch-mode experiments were conducted to characterize the hydrogen production potential of the microbial consortium at four pH values (4.5, 5.5, 6.5, and 7.5) and to provide an estimate of the rate which molasses may be fermented if additional molasses was injected at the site from which the groundwater was collected.

(2) Continuous-flow experiments were conducted to determine the effect of pH value on the rate, yield, and stability of hydrogen production (in a CSTR) as a potential renewable energy source.

(3) A sub-set of the continuous-flow experiments involved a step-increase in the molasses concentration to assess the impact of increasing the organic loading rate (OLR) on the rate and yield of H₂ production.

(4) A sub-set of the continuous-flow experiments involved feed of 1,2-DCA to assess the impact of elevated chlorinated solvent concentration on the fermentative, hydrogen-producing bacterial population.

1.3 Thesis Organization

Chapter 2 of this thesis contains a literature review regarding fermentative hydrogen production. Chapter 3 describes both the experimental methods and the results for batch-mode experiments conducted at different pH values. Chapter 4 contains a description of the experimental methods and the results for continuous-flow experiments conducted at different pH values. Chapter 5 contains a description of the experimental methods and the results for continuous-flow experiments in which the loading rate (OLR) was increased. Chapter 6 contains the methods and results for the experiment that evaluated the effect of 1,2-DCA concentration on fermentative hydrogen-producing bacteria. Lastly, Chapter 7 contains overall conclusions and recommendations for future research.

CHAPTER 2: LITERATURE REVIEW

2.1 Overview

Hydrogen (H_2) is widely considered to be an environmentally-friendly energy source. It has the highest energy value by weight (142 kJ/g) of any fuel which can be used as a fuel source in an internal combustion engine, and it can be used in hydrogen fuel cells to produce electricity with water as the innocuous final product (Guo *et al.*, 2010). Currently, hydrogen is not yet widely used as an energy source; however, it is commercially produced in large quantities for use in the petrochemical industry and for the production of fertilizers and ammonia (Nath & Das, 2003; Guo *et al.*, 2010). Though promising in concept as an environmentally friendly fuel, the vast majority (95-96%) of commercially available hydrogen is currently produced from fossil-fuel sources such as steam reformation of natural gas and gasification of coal (Elan *et al.*, 2003; Ewan & Allen, 2005). Such processes are energy intensive and emit CO_2 during the synthesis process, effectively shifting the time and location of CO_2 emissions rather than eliminating them (Nath & Das, 2004; Kapdan & Kargi, 2006). On the other hand, biological hydrogen production methods may be less energy intensive because they may be carried out at near ambient temperature and pressure (Wang & Wan, 2009; Guo *et al.*, 2010), and they may be carbon neutral because the CO_2 emitted from the process can come from plant-derived, renewable resources. Among the processes for producing hydrogen, non-photosynthetic fermentative production by bacteria (i.e. dark fermentation) has received attention as a promising method of converting organic materials to hydrogen (Zheng & Yu, 2005). The following subsections provide a literature review describing previous research on biological hydrogen production.

2.2 Fermentative Hydrogen Production

Fermentative hydrogen production is a function of many factors including microbial community composition, substrate, reactor configuration, nutrient supply, temperature, and pH

(Wang & Wan, 2009). In the absence of electron acceptors such as O_2 , NO_3^- , SO_4^{2-} , and Fe^{+3} , fermentative bacteria partially oxidize substrates in order to produce building blocks and energy for growth (Das & Veziroglu, 2008). In order to maintain electrical neutrality, electrons derived in the process may be transferred to protons (H^+) resulting in the formation of molecular hydrogen (Westermann *et al.*, 2007; Das & Veziroglu, 2008; Wang & Wan, 2009). As further described in Section 2.4, the maximum theoretical yield of hydrogen from the fermentation of one mole of glucose via known fermentation pathways is 4 moles (544 ml H_2 /g hexose at 25°C and 1 atm) when acetate is the final product, and 2 moles (272 ml H_2 /g hexose at 25°C and 1 atm) when butyrate is the final product (Li & Fang, 2007). Experimentally determined yields using fermentative hydrogen production processes studied to date have been somewhat less than the theoretical maximum, with most researchers reporting maximum yields in the range of 2.0-2.4 mol- H_2 /mol-glucose (Kapdan & Kargi, 2006). Low production yield is widely regarded as a primary obstacle to the economic viability of large-scale production of hydrogen via fermentation (Hallenbeck & Benemann, 2002; Guo *et al.*, 2010).

2.3 Substrates

Batch experiments treating several organic wastewaters indicate that carbohydrates and glycerol have significant potential for hydrogen production, but almost no hydrogen is produced from the fermentation of oils and proteins (Okamoto *et al.*, 2000; Akutsu *et al.*, 2009). The most common substrates used in fermentative hydrogen production studies have been glucose, sucrose, and starch (Wang & Wan, 2009). However, several studies using starch-based materials have also been used. Such include rice slurry, apple and potato processing wastewater, cheese whey, and several others (Chong *et al.*, 2009). Lignocellulosic materials have also been used, however, a complication associated with this latter material is the need for a pretreatment process for conversion of cellulose to simple sugars prior to fermentation (Mosier *et al.*, 2005).

In order for organic materials to be economically viable as feedstock for sustainable biohydrogen production, they must be inexpensive, abundantly available, and readily biodegradable (Guo *et al.*, 2010). Molasses is one such material (Chong *et al.*, 2009; Ren *et al.*, 2006). Because carbohydrate-rich molasses and molasses-containing wastewaters are less expensive than pure substrates (e.g., glucose or sucrose) and contain some nutrients, they can significantly reduce the cost of anaerobic fermentation (Guo *et al.*, 2008). Typical composition of molasses is shown on Table 2-1

Table 2-1: Properties of molasses.

Component	Percentage (%, w/w)	Component	Percentage (%, W/W)
Dried materials	78-85	MgO	0.01-0.1
Total Sugar	48-58	K ₂ O	2.2-4.5
TOC	28-34	SiO ₂	0.1-0.5
TKN	0.2-2.8	Al ₂ O ₃	0.05-0.06
P ₂ O ₅	0.02-0.07	Fe ₂ O ₃	0.001-0.02
CaO	0.15-0.8	Ash content	4-8

References: Ren *et al.* (2006), Li *et al.* (2007), and Guo *et al.* (2008).

Using mixed-cultures at mesophilic temperatures (25-40°C), several continuous-fermentative hydrogen production experiments have been conducted using molasses as the substrate. Table 2-2 lists some of the most recent studies found in literature. The source of inoculum sludge and the organic loading rate (OLR) have varied, the optimum pH has been between 4.0-5.5, and the major product of fermentation from these studies has been ethanol (except by Aceves-Lara *et al.*, 2008). The highest H₂ production rate obtained from these studies was 0.71 L/L/h (17.04 L/L/d equivalent) by Guo *et al.* (2008) under the following conditions: an expanded granular sludge bed process with granular activated carbon, 35° C, 120 kg-COD/m³/d organic loading rate, and without pH regulation. The H₂ yield (3.47 mol-H₂/mol-sucrose) was also the highest obtained among these studies.

Table 2-2: H₂ production rates and yields reported in continuous-flow studies using molasses as substrate.

Reference	Inoculum Source	pH Range/Optimum	OLR Range/Optimum (g-COD/L/d)	Yield	Specific Production Rate	H ₂ Production Rate	Major Metabolite
Li et al., 2009	Secondary settling tank sludge from WWTP	4.0-5.2 / 4.2 ± 2	^A 15 (g-COD/L/d) / —	—	2.96 (mol/kg-MLVSS/d)	^A 2.5 (L/L/d)	Ethanol
Ren et al., 2006	MWWT sludge	—	6.32-85.6 / 35–55 (kg-COD/m ³ -reactor/d)	26.13 (mol/kg COD _{removed})	0.75 (m ³ /kg-MLVSS/d)	5.57 (m ³ -H ₂ / m ³ -reactor/d)	Ethanol
Aceves-Lara et al., 2008	^B Anaerobic Digester Sludge (wine distillery)	—/5.5	—	1.47 (mol/mol-glucose)	—	15.3 (L-H ₂ /L/d)	—
Li et al., 2007	Aerobic Activated Sludge (brewing WWTP)	4.3-4.4	—	0.13 (L/g-COD)	0.13 (L/g-MLVSS/d)	^A 1.18 (L/L/d)	Ethanol
Guo et al., 2008	Activated Sludge & and Acidogenic Reactor Sludge from WWTP	3.9-5.3 / 4.2-4.4	8-192 / 120 (kg-COD/m ³ /d)	3.47 (mol/mol-sucrose)	3.16 (mmol/g-VSS/h)	0.71 (L/L/h)	Ethanol
Aceves-Lara et al., 2008	Anaerobic Digester Sludge (wine distillery)	5.5-6 / 5.5	37.12 (g-COD/L/d)	2.5 (mol/mol-sucrose)	—	5.4 (L/L/d)	Butyrate
Ren et al., 2009	Aerobically Cultivated Sewage Sludge	8.0-4.0 / 4.0	40 (g-COD/L/d)	—	—	9.72 (L/L/d)	Ethanol
Ren et al., 2010	Aerobically Cultivated Sewage Sludge	8.0-4.0 / 4.5	32 (g-COD/L/d)	—	5.13 (L/g-VSS/d)	6.65 (L/L/d)	Ethanol
^C This Experiment	Chlorinated Alkane-Contaminated Groundwater	4.5-7.5 / 5.5	34 (g-glucose/L/d)	1.93(mol/mol-glucose)	4.07 (L/g-MLVSS/d)	8.68 (L/L/d)	Butyrate

^A Calculated based on given data

^B Inoculum was heat-shocked

^C Based on averages values, alcohols were not measured

2.4 Metabolic Pathways and End Products

Using glucose as the substrate, the following pathway describes the evolution of hydrogen during fermentative hydrogen production. During glycolysis, 2 moles of reduced nicotinamide adenine dinucleotide (NADH) are produced in the conversion of one mole of glucose to 2 moles of pyruvate. Pyruvate is then cleaved by pyruvate-ferredoxin oxidoreductase (PFR) to form acetylcoenzyme A (acetyl-CoA), carbon dioxide, and 2 reduced forms of ferredoxin (Fd_{red}) (Li & Fang, 2007; Valdez-Vazquez & Poggi-Varaldo, 2009). If acetyl-CoA is converted to acetate, the enzyme hydrogenase regenerates both oxidized forms of electron carriers (NAD^+ and Fd_{oxd}) by catalyzing the transfer of electrons from NADH and Fd_{red} to protons (H^+); this yields a maximum of 4 moles of hydrogen per mole of glucose (Hawkes *et al.*, 2007). If butyrate is formed, NADH is oxidized in the formation of the metabolite (butyrate), and hydrogenase transfers electrons from Fd_{red} only. Consequently, the net yield is a maximum of 2 moles of hydrogen per mole of glucose (Fand & Li, 2007). In addition, in most reactor conditions, hydrogen partial pressure is not sufficiently low (< 60 Pa) to favor the production of hydrogen from NADH via hydrogenase (Largus *et al.*, 2004). Instead, the formation of other products such as ethanol, butanol, and lactate more favorably allow for reoxidation of NADH for the continuation of glycolysis; these products contain hydrogen atoms that were not liberated as gas (Levin *et al.*, 2004; Fang & Li, 2007; Hallenbeck & Gosh, 2009). The metabolites resulting from fermentative hydrogen production may also depend on the microorganism involved and environmental conditions such as pH and temperature (Fang & Li, 2007; Argun *et al.*, 2009). As previously elucidated, high acetate concentrations in fermentative processes would be expected to be associated with high hydrogen yields. Homoacetogens, however, are versatile anaerobes which can convert a variety of substrates to acetate (Diekert & Wohlfarth, 1994) without yielding any hydrogen. For example, *Clostridium aceticum* can convert hydrogen and carbon

dioxide to acetate, or even directly convert hexose directly to acetate (Drake & Kusel, 2005). The hydrogen yield observed in practice, thus, can be appreciably lower than calculated based on the amount of acetate accumulation and the assumption of 4 moles of H₂ produced per mole of acetate formed (Guo *et al.*, 2010). Overall, hydrogen yields from fermentation of carbohydrates may be lower than the theoretical maximum for several reasons. First, glucose may be metabolized via other than the glycolytic pathway. Second, some glucose may be consumed for biomass growth instead of hydrogen production. Third, maximum yields occur only at equilibrium conditions which require very low hydrogen partial pressure as will be explained in section 2.7 (Woodward *et al.*, 2000; Hallenbeck & Benemann, 2002). Finally, conditions may favor the formation of reduced by-products such as propionate (Vavilin *et al.*, 1995).

2.5 End Product Inhibition

During fermentative hydrogen production, high concentrations of sugars supplied to microbial cultures can result in high concentrations of organic acids which can inhibit bacterial growth and consequently reduce hydrogen production (Van Ginkel & Logan, 2005). Inhibition occurs when, at sufficiently high concentrations, non-polar undissociated acids infiltrate the cell wall at low pH, releasing a proton inside the higher-pH inner cell and disrupting the physiological balance (Jones & Woods, 1986; Bo *et al.*, 2008). The immediate effect is a decrease in the flux of glucose through glycolysis, and the long term result is inhibition of bacterial growth due to the higher energy required to maintain neutrality in the involvement of coenzyme A and phosphate pools (Chong *et al.*, 2009). The adaptive response of cells to high concentrations of organic acids is solventogenesis, which acts as a detoxifying mechanism in order to avoid the inhibitory effects (Jones & Woods, 1986). Solventogenesis results in low hydrogen yields because products like ethanol and butanol contain hydrogen atoms that are not liberated as gas (Levin *et al.*, 2004). If the concentration of dissociated organic acids is

sufficiently high in the medium, inhibition may also occur due to cell lysis (Neil *et al.*, 2003; Wang *et al.*, 2008). The inhibitory effects of the four major metabolites (acetic acid, propionic acid, butyric acid, and ethanol) on fermentative hydrogen production have been investigated in batch tests (Chi *et al.*, 2003; Van Ginkel & Logan, 2005; Zheng & Yu, 2005; Wang *et al.*, 2008). Increasing organic acid concentration of all four metabolites was shown to decrease hydrogen production rate, yield, and substrate degradation efficiency (i.e., glucose utilization efficiency). Wang *et al.* (2008) concluded that the inhibitory effect of high ethanol concentration was significantly less than acetic acid, propionic acid, and butyric acid at equal molar concentrations (Wang *et al.*, 2008). It was determined by van Ginkel & Logan (2005) that hydrogen yields deteriorated at a minimum *undissociated* organic acid concentration of 19 mM and higher. Using *Clostridium acetobutylicum* growing on glucose, Grupe & Gottschalk (1992) found an intracellular acid concentration of 40 mM to provoke solvent formation (Grupe & Gottschalk, 1992).

2.6 Microbial Selection Strategies

The use of mixed cultures, as opposed to pure cultures, for fermentative hydrogen production is widely considered to be more practical in terms of operational simplicity and cost at the industrial scale (Valdez-Vazquez *et al.*, 2005). In mixed-culture systems, however, there is the potential for non-hydrogen producing microorganisms (e.g., methanogens) to consume hydrogen as fast as it is produced. Perhaps the most well-characterized systems in which this occurs are the anaerobic digesters widely employed for digestion of municipal wastewater treatment sludges. Methane rather than hydrogen is the dominant final product observed in the digester off-gas, in large part because hydrogenotrophic and acetotrophic methanogens consume fermentation products synthesized by fermentative bacteria (Metcalf & Eddy, 2003). In previously reported laboratory-scale experiments, a common approach employed to minimize

methanogenic activity has been to use heat pre-treatment (e.g., pasteurization at 80°C) and/or acid pre-treatment of inoculums to inactivate methanogens and other non-H₂-producing bacteria while preserving the viability of spore-forming hydrogen-producing species such as those in the genus *Clostridium* (Lay, 2000; Van Ginkel & Sung, 2001; Lee *et al.*, 2004; Lin & Chou, 2004). In terms of operational strategies, maintaining low hydraulic retention times (< 6 hours) and low pH (5-6) has also been found to minimize methanogenic activity (Valdez-Vazquez & Poggi-Varaldo, 2009). These strategies make use of the fact that most methanogens do not grow well at pH≤6, and they have relatively low maximum specific growth rates (resulting in washout at low hydraulic residence times) (Metcalf & Eddy, 2003).

2.7 Effects of Reactor Operating Conditions

There are a number of reactor operating conditions which have been found to influence the rate and yield of hydrogen production. Among these are pH, hydrogen partial pressure in the reactor headspace, and nutrient concentrations. Each of these is briefly discussed below.

Medium pH has been shown to affect production yields, biogas content, type of metabolites produced, and microbial community structure (Kapdan & Kargi, 2006; Ye *et al.*, 2007). Specifically, regulation of pH is important because it affects hydrogenase activity and metabolic pathways (Dabrock *et al.*, 1992; Lay, 2000). As previously mentioned, maintaining low medium pH in reactors intended to fermentatively produce hydrogen can significantly reduce methanogenic activity. In conventional anaerobic reactors, methane production has been found to significantly decrease or stop at a pH below 6.3 (Chen *et al.*, 2002). Maintaining a low medium pH (5-6) has been used successfully as a strategy for inhibiting growth of methanogens in bioreactors intended to produce hydrogen (Chen *et al.*, 2002; Fang & Lui, 2002; Oh *et al.*, 2003).

Hydrogen partial pressure can affect the ability of hydrogenase to completely transfer electrons from NADH and Fd_{red} to protons (H^+) (Largus *et al.*, 2004). It has been proposed that 2 moles of hydrogen per mole of glucose are produced by oxidation of Fd_{red} (via hydrogenase) if partial pressure is less than 3×10^4 Pa. If hydrogen partial pressure is less than 60 Pa, 2 additional moles of hydrogen per mole of glucose may also be produced via oxidation of NADH via hydrogenase (Largus *et al.*, 2004). Most fermentative hydrogen production predominantly involves oxidation of Fd_{red} and not NADH when hydrogen partial pressure is not sufficiently low (Largus *et al.*, 2004) and the formation of other products (such as ethanol and butyric acid) allow for reoxidation of NADH for the continuation of glycolysis (Hawkes *et al.*, 2007; Hallenbeck & Gosh, 2009). Thus, low hydrogen partial pressure is required in order to minimize or avoid intracellular hydrogen-consuming (i.e. NADH consuming) reactions (Hawkes *et al.*, 2002).

The evolution of hydrogen from degradation of acetate is highly unfavorable, but the reverse reaction is favorable. Thus, high gas-phase partial pressures of hydrogen and carbon dioxide can lead to homoacetogenesis, which can decrease the performance of biohydrogen reactors (Guo *et al.*, 2010). In a batch-mode study carried out by Park *et al.* (2005), it was shown that reducing carbon dioxide concentrations using a chemical scavenger (KOH) prevented hydrogen loss through homoacetogenesis, and increased hydrogen production by 43% (from 1.4 to 2 mol H_2 /mol glucose). Some methods which have been used to lower partial pressure are stirring and extraction through membranes, but the most common method has been purging the system with nitrogen or argon gas (Hawkes *et al.*, 2007). Mizuno *et al.* (2000) proved that sparging nitrogen gas into a CSTR fed with glucose increased the yield from 0.85 to 1.43 mol- H_2 /mol glucose (Mizuno *et al.*, 2000). Although hydrogen production was not measured, a similar study showed that sparging with argon led to an increase in residual NADH (Tashino *et al.*, 1998), which might lead to higher hydrogen production (Mizuno *et al.*, 2000). Two

disadvantages of decreasing the H₂ partial pressure by sparging with inert gases, however, are (1) the cost of the inert gas, and (2) a decrease in the final H₂ concentration which could complicate and increase the cost of downstream H₂ purification (on account of dilution with the gas used for sparging).

Pressure release in fermentative hydrogen production reactors has been mostly achieved by two methods: continuous pressure release or intermittent pressure release. While continuous pressure release has been known to produce higher hydrogen yields by maintaining lower H₂ partial pressure in reactor head space (43% more than intermittent pressure release) (Logan *et al.*, 2002), there is some controversy as some studies have concluded that partial pressure buildup through intermittent pressure release did not inhibit hydrogen production (Gadhamshetty *et al.*, 2009). The contending theory is that hydrogen partial pressure affects hydrogenase oxidation of NADH only and not Fd_{red}, and therefore, it is possible to obtain hydrogen yields even at a partial pressure of 2.32×10^5 Pa through intermittent pressure release (Gadhamshetty *et al.*, 2009).

Nitrogen is an essential nutrient and serves as a building block for of nucleic acid and protein synthesis, and it is considered to be one of the most important factors affecting the growth of hydrogen producing bacteria. Phosphate is also essential as a nutrient and can play an important role as a buffering agent (Wang & Wan, 2009). Metal ions can affect cellular activity of transport processes and hydrogenases, and they are also important enzyme cofactors (Lin & Lay, 2005). Important metal ions in fermentative hydrogen production have been found to include iron, sodium, zinc, and magnesium, which is an important cofactor in the activation of enzymes involved in glycolysis (Lin & Lay, 2005). Iron (Fe⁺²) is also critical in the formation of the protein ferredoxin which is an integral part of hydrogen production through the enzyme hydrogenase (Nicolet *et al.*, 2002).

2.8 Microbial Communities

Several studies have identified rod-shaped, Gram-positive, spore-forming, strict anaerobic *Clostridium* as the most prominent bacteria found in mesophilic hydrogen-producing mixed cultures (Lay, 2000; Fang *et al.*, 2002; Guo *et al.*, 2010). Fang *et al.* (2002) analyzed the microbial composition of a hydrogen producing culture (CSTR, sewage sludge inoculum, pH 5.5, 36°C, 6.6 h HRT, and glucose substrate) by constructing a 16S rRNA gene library (96 clones were selected for plasmid recovery and DNA sequencing). They identified 64% of clones to belong to *Clostridiaceae*, of which 43.8% were most closely related to *Clostridium cellulosi*, 12.5% were most closely related to *Clostridium acetobutylicum*, and 12.5% were most closely related to *Clostridium tyrobutyricum*. They also found that 18.8% and 3.1% were closely associated with *Enterobacteriaceae* and *Streptococcus bovis* (Fang *et al.*, 2002). A separate study by Fang *et al.* (2002) on granular sludge (CSTR, pH 5.5, 26°, 6 h HRT, and sucrose substrate) determined that 69.1% of the clones were associated with four *Clostridium* species including *C. pasteurianum*, *C. acidisoli*, and *C. tyrobutyricum*, and 13.5% of the clones grouped with *Sporolactobacillus racemicus* (Fang *et al.*, 2002). Having a high H₂ yield (1.6-2.4mol/mol-glucose) and high growth rate, *C. butyricum* has been most extensively studied (Minnan *et al.*, 2005).

Rod shaped, Gram-negative, facultative anaerobic *Enterobacter* have also been identified as an abundant species present in fermentative hydrogen producing inoculums (Li & Fang, 2007). Experimental results indicate that *Clostridium* species are more efficient hydrogen producers compared to *Enterobacter* species, generating about 2 mol H₂/mol glucose compared to about 1 mole H₂/mol glucose, respectively (Girbal *et al.*, 1995; Yoki *et al.*, 1995). However, it is known that facultative anaerobes can gain energy (i.e. produce ATP) through aerobic respiration; thus, *Enterobacter* species can play a positive role in fermentative processes by

utilizing any oxygen present and subsequently creating anaerobic conditions favoring fermentative hydrogen production. Yokoi *et al.* (1998) used *C. butyricum* and *E. aerogenes* in a co-culture system as a method of ensuring fermentative conditions and found high hydrogen yields without addition of expensive reducing agents like L-cysteine. In this experiment, hydrogen yield was 2 mol/mol glucose without co-immobilization and 2.6 mol/mol glucose with co-immobilization of both strains on porous glass beads (Yoki *et al.*, 1998).

CHAPTER 3: HYDROGEN PRODUCTION CAPACITY OF THE MICROBIAL CONSORTIUM AT FOUR DIFFERENT pH VALUES (BATCH MODE).

3.1 Introduction

The overall goal of the research described in this chapter was to evaluate the biological hydrogen production potential of microbial populations from chlorinated solvent contaminated groundwater at the Scenic Highway portion of the PPI Superfund Site. This was conducted in support of an on-going effort to better understand microbial processes that may play an important role in enhancing *in-situ* reductive dechlorination rates when subsurface injection of molasses is used as a means of supplying electron donors for driving dechlorination reactions. One question of practical importance in the on-going effort at the PPI site is whether the pH of the groundwater should be controlled (via injection of buffering agents) in the *in-situ* bioremediation effort. The effect of pH on reductive dechlorinating bacteria is being studied in a separate effort. The specific goal of batch-mode experiments described in this chapter was to characterize the hydrogen production potential of the microbial consortium as a function of pH. Four different target pH values (4.5, 5.5, 6.5, and 7.5) were experimentally tested to evaluate the time course over which molasses may be fermented if additional molasses was to be injected at the site from which the groundwater was collected. A secondary goal of these experiments was to serve as the starting point for subsequent efforts (described in Chapter 4) for assessing the rate and yield of H₂ production as a renewable energy source using molasses as a feedstock in conjunction with microorganisms from the chlorinated solvent contaminated groundwater as the inoculum. In the experiments described in this chapter, a total of four experiments were carried out. These were arbitrarily designated as Run 1, Run 2, Run 3, and Run 4, which were operated at target pH values of 4.5, 6.5, 7.5, and 5.5, respectively.

3.2 Materials and Methods

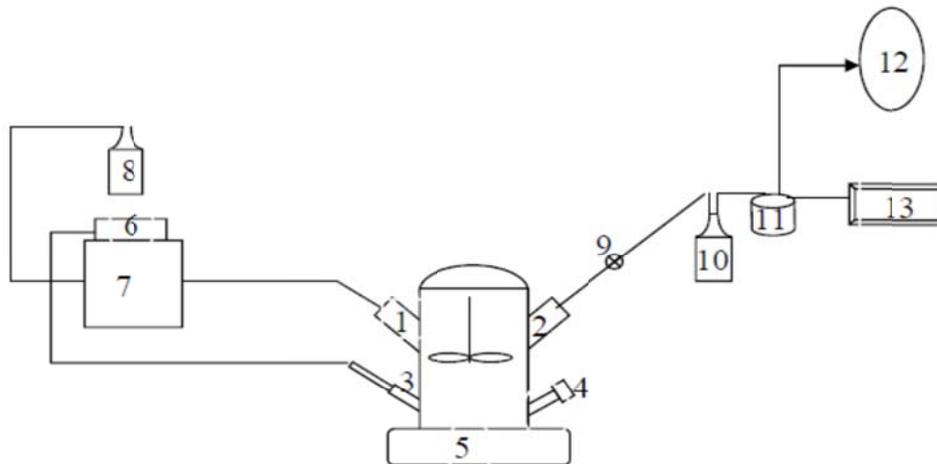
3.2.1 Reactor Configuration

Batch-mode experiments were conducted in a glass bioreactor (Bellco Bio-Probe Complete, nominal 3 L capacity) with a configuration as shown in Figure 3-1. The reactor's total volume (including liquid volume and gas headspace) was 4.3 L. The bioreactor contained two lower (32 mm ID) and two upper (45 mm ID) sidearms openings. The upper sidearms were each fitted with airtight screw caps containing three accessible ports. Each port on each screwcap contained an open/close valve which, depending on functionality assigned, could be used as an inlet, outlet, or sampling port. One of the bottom sidearms was equipped with a stainless steel compression fitting to which an autoclavable pH electrode (Cole-Parmer Sealed pH Electrode, 150 mm) was secured. The pH electrode protruded inside bioreactor without obstructing stirring paddles. The other bottom 32 mm sidearm was equipped with a cap modified to accommodate a single port used for sampling of reactor contents. A variable-speed, heavy-duty magnetic stir plate (Corning model PC-611) in conjunction with a magnetically driven paddle-type mixer (Bellco, Internal Overhead Assembly, 3 L) was used to provide mixing.

The pH electrode mounted inside the reactor was connected to an automatic pH controller (pH190, Eutech Instruments) that activated a peristaltic pump (Masterflex L/S) which added NaOH solution (1 M) until the pH controller set-point was attained. The NaOH solution was delivered to the reactor through Masterflex Precision tubing (Norprene, A 60G, size L/S[®] 14). In cases where the starting pH of the reactor's contents were above the set-point at the start of the experiment, the pH was not externally controlled until the pH decreased to below the set point (i.e., there was no external acid addition).

Biogas produced in the reactor exited through a gas line connected to one of the upper sidearm ports. Biogas production rate and volume was measured using a Milligascounter[®]

(MGC-1, Ritter; Bochum, Germany) which was connected to an online computer installed with Rigamo software (Ritter V 1.30, Bochum, Germany) for data acquisition. Resulting data was stored in the form of a Microsoft Excel file. Biogas exiting the gas flow meter was collected in a 25 liter Tedlar bag (Supelco). A glass sampling port containing a septum-filled sampling port was installed between the bioreactor outlet and the gas flow meter to allow sampling of headspace gas composition. An in-line liquid trap placed between the bioreactor's gas outlet port and the gas flow meter prevented Milligascounter® packing-fluid from entering the bioreactor in case of negative pressure (i.e. vacuum) formation. The liquid trap was used for Run 2 only.



- | | |
|--------------------------------|--------------------------------|
| 1) NaOH inlet port | 8) NaOH solution bottle |
| 2) Gas outlet port | 9) Headspace gas sampling port |
| 3) pH electrode (Autoclavable) | 10) Liquid trap |
| 4) Liquid sampling port | 11) Gas flow meter |
| 5) Heavy-duty stir plate | 12) Tedlar collection bag |
| 6) pH controller | 13) CPU for data acquisition |
| 7) Peristaltic pump | |

Figure 3-1: Reactor configuration during batch-mode operations.

3.2.2 Materials Preparation

Groundwater samples used for inoculation during batch-mode start-up were collected from well ID no. MW-01 (East 3316133, North 760195; State Plane, NAD 83) at the Petro Processors of Louisiana, Inc. (PPI) Superfund site. Groundwater samples were collected in

sterile media bottles (nominal 1 L capacity) leaving little or no air headspace. To maintain anaerobic conditions, bottles were amended with sterile titanium citrate solution (Zehnder & Wuhrmann, 1976) as a reducing agent (final concentration of 1.0 mM Ti(III), 2.0 mM citrate), immediately after collection. After transport to LSU in coolers, groundwater samples were stored in a refrigerator at 8 °C until use for inoculation. Table 3.1 lists the dates of collection and use of each groundwater sample.

Table 3-1: Groundwater samples used for inoculation of batch-mode experiments 1-4.

Run	Date Collected	Date Used	Elapsed Time (d)
1	10/06/09	10/6/09	0
2	12/1/09	12/3/09	2
3	1/12/10	1/13/10	1
4	2/22/10	2/23/10	1

Immediately prior to collection of groundwater samples to use as the inoculum in bioreactor experiments, additional groundwater samples were collected for analysis of volatile organic compounds and various geochemical parameters. These analyses were performed by a commercial laboratory (Gulf Coast Analytical, Baton Rouge, LA). Concentrations of volatile organic compounds were measured using US EPA method 624. Dissolved ethene, ethane, and methane were measured by method RSK 175. Nitrate and nitrite were measured using US EPA method 353.2. Chloride was measured using US EPA method 325.2. Sulfate was measured by ion chromatography using US EPA method 300.0. Sulfide was measured by US EPA method 376.2. Ferrous iron was measured using US EPA method 3500-Fe D. Total organic carbon (TOC) was measured using Standard Method 5310B. Detailed descriptions of US EPA analytical methods referenced above are available elsewhere (National Environmental Index, <http://www.nemi.gov/>). The pH, oxidation reduction potential (ORP), temperature, conductivity, and turbidity of groundwater samples were measured using on-line probes (In Situ, Inc., Ft. Collins, CO) immediately prior to groundwater sample collection.

Agricultural feed-grade cane molasses (Westway Feed Products LCC, Port Allen, LA) obtained on two separate dates was utilized in the experiments. The first batch was obtained from Westway Feed Products LLC on August 19, 2009 and was used for batch-mode experiment Run 1. A second batch of molasses, delivered on October 21, 2009, was used for batch-mode experiments Run 2, Run 3, and Run 4. Molasses was stored in a laboratory refrigerator at 8°C prior to use. The aliquot of diluted molasses used for each batch-mode experiment was prepared one day prior to reactor startup. The solution was prepared by adding 25 mL of molasses and 500 mL of deionized water into a 1 L media bottle. The solution was subsequently autoclaved for 15 minutes at 115°C and 15 psi, and stored at room temperature until reactor startup. Additional nutrients were not added in any of the batch-mode experiments.

At the start of each batch operation, the bioreactor flask and components were autoclaved in order to ensure sterilized conditions during the inoculation process. Autoclaving was achieved by placing reactor flask (with stirring paddles inside, top lid tightened, and sidearm openings covered with aluminum foil) inside an autoclave along with aluminum foil wrapped reactor caps and pH electrode. The bioreactor flask and components were autoclaved for 15 minutes at 115°C and 15 psi. They were subsequently assembled on a clean bench which had been previously disinfected by surface treatment with 90% alcohol and UV light (5 minute exposure).

3.2.3 Reactor Startup and Operation

At the time of startup for each experimental run, 2.3 L of site groundwater and 0.5 L of sterile diluted molasses solution were added to the bioreactor resulting in a total liquid volume of 2.8 L. Prior to and during addition of these components to the bioreactor, filter-sterilized N₂ gas (100%, Capitol Welders Co.) was purged through the bioreactor. After completing addition of substrate and groundwater, the top lid was tightened, the gas outlet line was opened, and the bioreactor headspace was purged with filter-sterilized N₂ gas for an additional 30 minutes at the

same flow rate. At the end of the 30 minute purging period, the digital counter on the gas flow meter was reset, the Rigamo acquisition software was started, and stir plate was turned on. Immediately after, initial gas composition and pH values were recorded, a pristine sample of reactor contents was collected in a 15 mL sterile centrifuge tube (about $\frac{3}{4}$ full) and stored in a freezer, and two additional samples (about 1.5 mL each) were similarly collected and frozen in micro-centrifuge tubes which were later used to determine initial total sugar and COD concentration. After collection of samples for determination of initial parameters, the pH controller (programmed to activate at a specific set-point) was activated; this marked time zero of the experiment. All experimental runs were carried out at ambient laboratory temperature ($\approx 22^{\circ}\text{C}$) without temperature control.

During the first two days of operation, liquid samples (approximately 1.5 mL) were collected (using sterile syringes) approximately every 2-6 hours and stored in a freezer, and less frequent thereafter. Every two or three days, accumulated samples were thawed, centrifuged, and tested for total sugar concentrations. Biogas composition and pH were similarly measured every 2-6 hours for the first two days, and less frequent thereafter. Biogas production volume was recorded for each day as measured by the gas flow meter (and displayed by corresponding data acquisition software). Base addition rate was determined by observing the volume of NaOH added between consecutive days and dividing by the time interval. Daily operation of batch-mode continued until terminal production of biogas was reached.

As described in Section 3.3.3, negative pressure (i.e. vacuum) developed in the reactor headspace during two of the experiments. On these occasions, a 1 L Tedlar bag filled with N_2 gas was attached to one of the upper sidearm ports via autoclaved tubing and a $0.22\ \mu\text{m}$ syringe filter (Nalgene). The port's valve was opened to allow sufficient N_2 to flow into the reactor and equalize pressure (i.e., eliminate the vacuum). This procedure was periodically repeated (2-3

times per day), each time using a with freshly prepared Tedlar bag containing N₂ gas, until vacuum formation was no longer observed.

3.2.4 Analytical Methods

The pH of the reactor's contents was measured using an internal pH electrode (Cole-Parmer Sealed pH Electrode, 150 mm) as described in the reactor design section. The electrode was initially calibrated (two-point calibration), and its accuracy periodically tested with an external pH probe (Denver Instruments).

For analysis of sugar and COD concentrations, 1.5 mL aliquots removed from the bioreactor were centrifuged at 13,000×g for 5 minutes and the supernatant was subject to analysis. Total sugar concentrations were measured in duplicate using the phenol-sulfuric acid reaction method as described by Dubois *et al.* (1956) with absorbance measured at a wavelength of 485 nm with a spectrophotometer (Evolution 60, Thermo Scientific). Samples were diluted as needed to obtain final absorbance values in the range of a calibration curve prepared with standards comprised of D-glucose in deionized water over the concentration range of 15-75 mg/L. Total COD was also measured in duplicate using HACH Digestion Vials (COD2, Ultra High Range 200-15000 ppm); absorbance was measured at a wavelength of 620 nm with a spectrophotometer (Evolution 60, Thermo Scientific). Samples were diluted by a factor of 2 prior to analysis. A calibration curve prepared with known COD standards (using potassium hydrogen phthalate) was used to determine the COD concentration.

Organic acids were measured using an ion chromatograph (Dionex ICS-2000, Sunnyvale, CA) equipped with an IonPac AS11-HC column, ASRS-ULTRA II (4-mm), and conductivity detector. Sample injection volume was 100 µL, with elution performed with KOH (1.5 mM for 10 min then ramping to 30 mM at a rate of 1.6 mM min⁻¹) at a flow rate of 1.5 mL min⁻¹ and temperature of 30°C. Retention times and peak areas of fermentation products were compared to

those of standards which included acetate, butyrate, formate, lactate, propionate, and succinate. Alcohols were not measured for any experiment.

Carbon dioxide, methane, and nitrogen concentrations were measured with a gas chromatograph (SRI 310C; SRI Instruments, Torrance, CA) equipped with a thermal conductivity detector and an Alltech Poropak Q 80/100 column (6'×1/8"×0.085"). Hydrogen concentration was measured using a gas chromatograph (SRI 310C; SRI Instruments, Torrance, CA) equipped with a thermal conductivity detector and molecular sieve column (Alltech Molecular Sieve 5A 80/100) as described by van Ginkel *et al.* (2001).

Biogas production rate (mL/hr) and cumulative production volume (L) were measured and recorded using a gas flow meter (Milligascounter®, Ritter MGC-1) and software (Rigamo). Hydrogen production was calculated by multiplying the volume of biogas produced during a short time interval by the average H₂ concentration during the same time interval; the iterative summation of every time interval yielded the cumulative produced volume. The net production of H₂ was obtained by adding the cumulative produced volume and the volume remaining in the reactor headspace at the end of production (i.e., volume times headspace composition).

For the two experiments described in Section 3.3.3 during which net consumption of gas created negative pressure in the reactor headspace, net H₂ production and total gas production volume were calculated based on the maximum gas volume produced (i.e., subsequent consumption within the reactor after net gas production in the reactor ceased was not included in calculating the net gas production or net H₂ production).

3.3 Results and Discussion

3.3.1 Initial Conditions and pH

The results of analysis to determine organic contaminants in the groundwater at the time of collection are summarized in Table 3-2, and geochemical constituents are summarized in

Table 3-3 (data supplied by NPC Services, Inc.). As shown in Table 3-2, the groundwater contained a variety of chlorinated alkanes and alkenes, and the contaminant concentrations changed somewhat during the course of the study. The temperature of the groundwater was near 20°C on all days samples collected for bioreactor inoculation. The pH differed between sample collection dates but in all cases was moderately acidic (pH range 5.28-5.68). The increase in ferrous iron and sulfide concentrations over time, the decrease in sulfate concentration over time, and consistently low ORP are indicative of anaerobic conditions in the subsurface aquifer.

The initial pH, sugar concentration, COD concentration, and the dates of experiment commencement are listed on Table 3-4. Parameter values listed in the table were measured from the homogenized reactor contents after adding groundwater and diluted molasses to the reactor at the time of startup but prior to activation of the pH controller at time zero. As shown in the table, the initial pH, sugar concentration, and COD concentration were relatively consistent between the different experimental runs. The initial pH differed by a maximum of 0.1 pH unit, sugar concentrations differed by a maximum of 0.67 g/L, and COD concentrations differed by a maximum of 1.0 g/L.

Table 3-2: Chlorinated solvent concentrations measured in groundwater from well MW-01 at the PPI site (data provided by NPC Services, Inc.).

Analyte	Groundwater collection date			
	10/06/2009	12/01/2009	01/12/10	02/22/10
1,1,2,2-Tetrachloroethane (µg/L)	54.2	32.1J	ND (<50)	ND (<50)
1,1,2-Trichloroethane (µg/L)	158	39.9J	ND (<50)	ND (<50)
1,1-Dichloroethane (µg/L)	54.8	67.6	53.4	ND (<50)
1,1-Dichloroethene (µg/L)	235	156	63.7	119
1,2-Dichloroethane (µg/L)	410	281	296	236
1,2-Dichloropropane (µg/L)	3120	3360	3220	2240
Chloroform (µg/L)	122	89.3	160	53.9
Tetrachloroethene (µg/L)	125	43.4	ND (<50)	ND (<50)
Trichloroethene (µg/L)	469	230	126	87.1
Vinyl chloride(µg/L)	3600	4980	5320	3300
cis-1,2-Dichloroethene (µg/L)	3570	4600	3780	4030
trans-1,2-Dichloroethene (µg/L)	371	256	192	204

ND = not detected (Reporting detection limit listed in parentheses)

J = Estimated concentration for compound detected in analysis but at concentration between the reporting detection limit (RDL) and method detection limit (MDL).

Table 3-3: Geochemical parameters measured in groundwater from well MW-01 at the PPI site (data provided by NPC Services, Inc.).

Analyte	Groundwater collection date			
	10/06/2009	12/01/2009	01/12/10	02/22/10
Ethane (µg/L)	ND (<900)	ND (<900)	ND (<900)	ND (<600)
Ethene (µg/L)	4460	6650	6070	7360
Methane (µg/L)	ND (<4500)	ND (<4500)	210J	ND (<4500)
Nitrate (mg/L-N)	ND (<0.01)	ND (<0.01)	ND (<0.01)	ND (<0.01)
Nitrite (mg/L-N)	ND (<0.01)	0.014	0.009B	0.02
Ferrous Iron (mg/L)	24.0	28.2	35.4	45.9
Bromide (mg/L)	27.8	35.0	34.8	14.2
Sulfate (mg/L)	5.4	5.27	3.18	0.53
Chloride (mg/L)	206	164	184	179
Total organic carbon (mg/L)	158	184	220	320
Sulfide (mg/L)	5.21	3.52	3.05	3.15
pH	5.65	5.61	5.28	5.45
ORP (mV)	-195	-185	-179	-215
Temperature (C)	20.4	19.7	20.2	19.9
Conductivity (µS)	898	1007	1130	1410
Turbidity (NTU)	1.26	2.00	0.88	3.52

ND = not detected (Reporting detection limit listed in parentheses)

J = Estimated concentration for compound detected in analysis but at concentration between the reporting detection limit (RDL) and method detection limit (MDL).

Table 3-4: Initial pH and sugar concentration for batch-mode experiments 1-4.

Run ID	Target pH	Start/End Date	Duration (hours/days)	¹ Initial pH	² Initial Sugar Concentration (g/L)	Initial COD Concentration (g/L)
1	4.5	10/06/2009 - 10/19/2009	317 / 13.2	5.6	7.15	10.2
2	6.5	12/3/2009 - 12/16/2009	312 / 13	5.7	6.84	9.39
3	7.5	1/13/2010 - 1/22/2010	216 / 9	5.6	6.48	9.21
4	5.5	2/23/2010 - 3/5/2010	240 / 10	5.7	6.79	9.48

¹pH measured prior to activation of pH controller

²Sugar concentrations as glucose

For Run 2 (target pH=6.5) and Run 3 (target pH=7.5), activation of the pH controller at time zero resulted in immediate addition of NaOH solution to the bioreactor. The pH increased to near the target pH within the first hour of operation where it remained essentially constant for the duration of the experiment (Figure 3-2). For Run 1 (target pH=4.5) and Run 4 pH (target pH=5.5) the initial pH in the bioreactor at the time of startup was above the pH controller set point (Table 3-4). Thus, during both Run 1 and Run 4 there was an initial interval during which the reactor was functionally operated without pH control (the pH control system added base to

maintain pH above the set point but it did not add acid to bring the pH down to the set point). Over time, however, the pH decreased in both experimental Runs 1 and 4, and the pH reached near the target values (within 0.1 pH unit) after 2.33 days and 2.35 days for Run 1 and Run 4, respectively. Thereafter, the pH remained relatively constant near the target values for the remainder of the experiment (Figure 3-2).

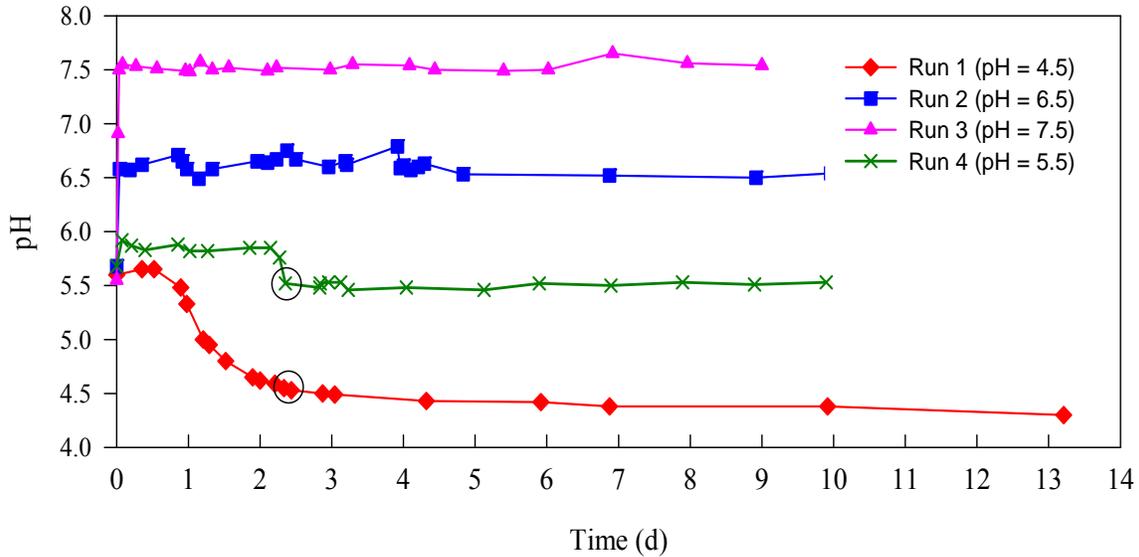


Figure 3-2: pH as a function of time for batch-mode experiments 1-4. Circles denote times when the pH reached within 0.1 pH unit of the target values in runs 1 and 4.

3.3.2 Sugar Concentration

Figure 3-3 depicts the sugar concentration (measured in glucose equivalents) as a function of time for all four experiments. For Runs 1, 3, and 4, there was a small but readily apparent increase in sugar concentration from the initially measured concentration at time zero. For Run 2, the first liquid sample was not collected until 11 hours of operation; thus, an increase may have occurred but was not captured. The small increase in sugar concentrations observed near the start of runs 1, 3, and 4 may have been due to hydrolysis/solubilization of sugar associated with particulate matter. Regardless, it should be noted that in all subsequent calculations (when initial sugar concentration required), the calculations were carried out using the initial sugar concentration measured at time zero.

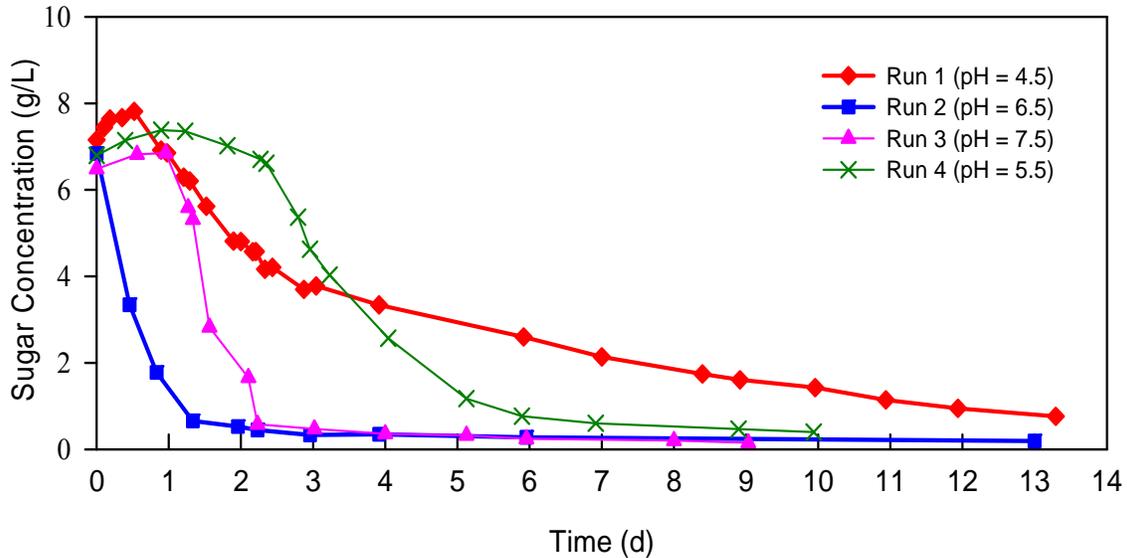


Figure 3-3: Sugar consumption as a function of time for batch-mode experiments 1-4.

From the data depicted in Figure 3-3, it is evident that sugar consumption occurred much faster during Run 2 (target pH=6.5) and Run 3 (target pH=7.5) than in Run 4 (target pH=5.5) or Run 1 (target pH=4.5). Table 3-5 lists the time that it took for 90% of the initial sugars to be consumed, the time when an initial decrease in sugar concentrations was observed, and the total percentage of initial sugar consumption for the entire experiment.

Table 3-5: Time required for 90% consumption, time of initial decrease in sugar concentration, and total sugar consumption for batch-mode experiments 1-4.

Target pH	Run ID	¹ Time When 90% Sugars Consumed (days)	Final Sugar Concentration (g/L)	Total Sugar Consumption (%)	Time of Initial Decrease in Sugar Concentration (hours)
4.5	1	13	0.76	89	20
5.5	4	7	0.40	94	48
6.5	2	1	0.19	97	1
7.5	3	2	0.15	98	28

¹Days rounded to nearest whole number

Higher pH (i.e., pH 6.5 or 7.5) resulted in a shorter time for 90% of sugars to be consumed than lower pH (i.e., pH 4.5 or 5.5). Higher pH also resulted in larger percent sugar consumption. There was no clear correlation, however, between the reactor pH and the time when an initial decrease in sugar concentration was observed.

3.3.3 Biogas Production and H₂ Composition

Figure 3-4 shows the cumulative gas production volume, biogas production rate, and gas hydrogen content during the course of the four batch mode experimental runs. As shown in the figure, gas production was observed in all four experimental runs, and in all cases, H₂ comprised a sizeable fraction of the gas produced. Methane was not detected in any of the four batch-mode experiments.

In Run 2 (target pH=6.5) and Run 3 (target pH=7.5), gas production began after 0.69 and 1.61 days, respectively. The gas production rates increased rapidly thereafter, reaching maximal levels of 140 mL/h and 169 mL/h, respectively. After 3.69 and 2.27 days of operation (72 and 16 hours after gas production was first measured), however, the gas ceased to exit the reactor, and a negative pressure (i.e. vacuum) was observed in the gas headspace (visually observed by the liquid in the gas flow measuring device entering the tube connected to the reactor at a level that increased over time). Arrows in Figure 3-4 denote the times when a vacuum was first detected. This corresponds to times when the sugar concentration had decreased to less than 0.4 and 0.5 g/L, during Runs 2 and 3, respectively (see Figure 3-3). Subsequently, pressure in the gas headspace was increased by introduction of N₂ gas as described in Section 3.2.3. This addition of N₂ resulted in a progressive decrease in H₂ composition in the reactor headspace. As further discussed in Section 3.3.4, consumption H₂ and CO₂ due to homoacetogenic activity occurring within the reactor likely caused the negative pressure to develop and also contributed to further decrease in H₂ concentration over time. Because the volumes of N₂ added to the reactor headspace during Runs 2 and 3 were not measured, the magnitude of the H₂ concentration decrease to dilution with N₂ cannot be precisely determined from the data collected.

The gas production rate during Run 1 (target pH=4.5) was distinctly characterized by two separate phases. The first was characterized by relatively higher production rates during the

interval during which the reactor was effectively operated without pH regulation (time zero to 2.3 days), and the second phase was characterized by a long tail of lower production rates during the interval in which the reactor pH subsequently decreased and was maintained near the target pH (day 2.3 onward).

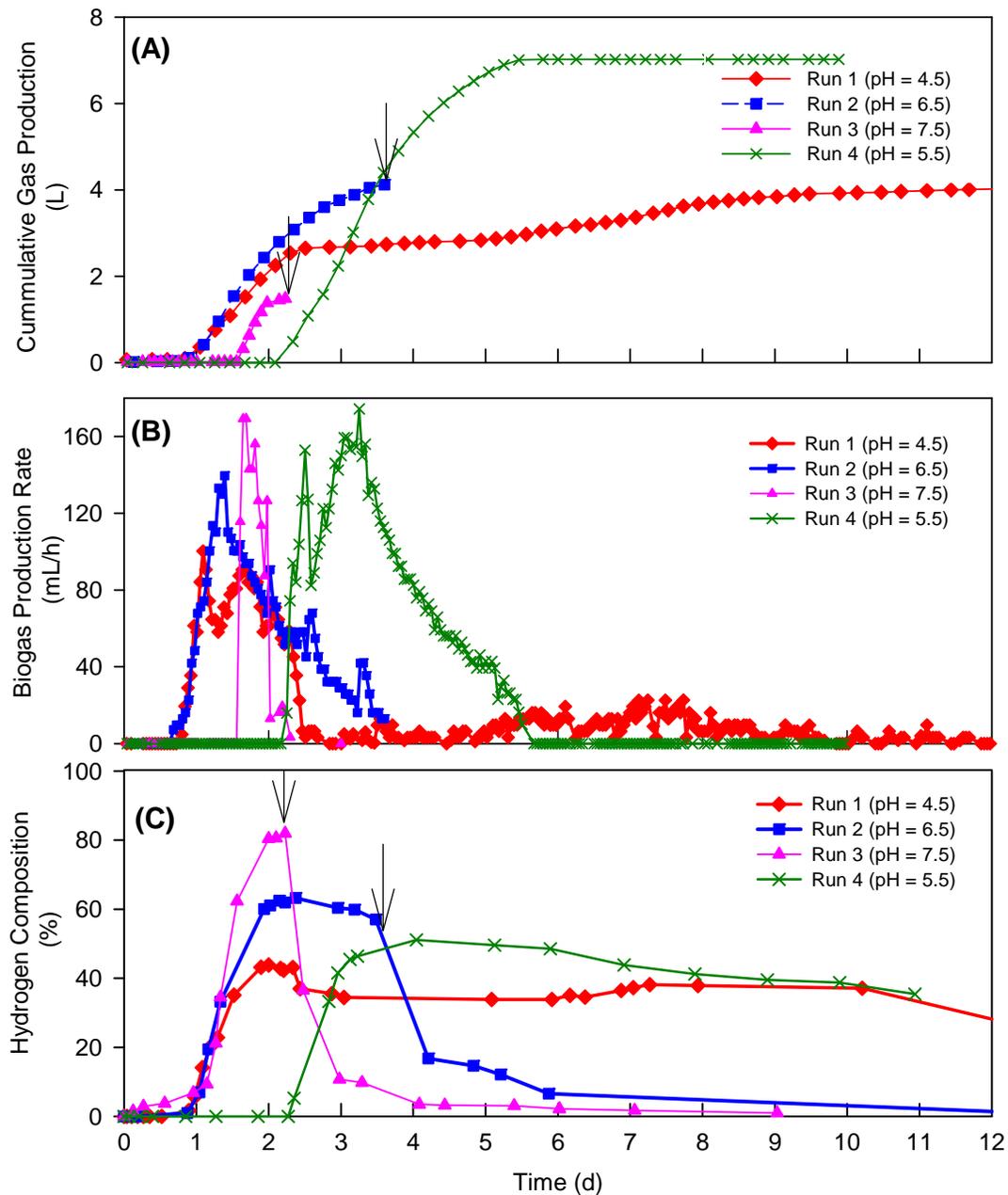


Figure 3-4: (A) cumulative biogas production volume, (B) biogas production rate, and (C) H₂ composition during batch-mode experiments 1-4. Arrows indicate time of vacuum formation and subsequent introduction of N₂ gas for reactor pressure equalization during Run 2 (target pH=6.5) and Run 3 (target pH=7.5).

In Run 4 (target pH=5.5), there was an initial increase in gas production rate followed by a decrease to zero gas production by day 5.5 corresponding to the time at which the sugar concentration had decreased to 0.98 g/L as glucose. In contrast to Runs 2 and 3, however, negative pressure did not develop inside the reactor headspace during Run 4.

The following summary applies only to the interval during which biogas was produced (hereafter referred to as the biogas production period). Higher pH resulted in higher H₂ composition within the reactor during the biogas production period. Higher pH, however, resulted in a shorter duration of the biogas production period. Run 3 (target pH=7.5) had the highest average and peak H₂ concentration (76% and 82% respectively), but the duration of biogas production was short-lived (0.67 days). Conversely, Run 1 (target pH=4.5) had the longest biogas production period (11.5 days) but had the lowest average and peak hydrogen composition (32% and 4.3% respectively) among the four experimental runs.

In terms of biogas yield, there was no clear trend with respect to pH. Results show that Run 4 (target pH=5.5) had the highest biogas production and yield, followed by Run 2 (target pH=6.5), then Run 1 (target pH=4.5), and Run 3 (target pH=7.5) resulted in the lowest biogas yield. Table 3-6 summarizes total biogas produced, biogas yield, and average and peak H₂ composition for each experiment.

Table 3-6: Biogas production volume, rate, and yield and H₂ composition during the production period of batch-mode experiments 1-4.

Target pH	Run ID	Biogas Production Duration (days)	Biogas Production Rate	Total Biogas Produced (L)	Biogas Yield (mL/g-glucose _{consumed})	H ₂ Concentration
			Average/Peak (mL/h)			Average / Peak (%)
4.5	1	11.5	14.9 / 100	4.06	220.8	32 / 43
5.5	4	3.3	86.5 / 174	6.75	377.3	40 / 51
6.5	2	3.0	57.2 / 133	4.12	221.3	46 / 63
7.5	3	0.7	91.9 / 169	1.47	82.9	76 / 82

*A reactor liquid volume of 2.8 L and initial sugar concentration equal to that measured at time zero (see Table 3-4) were used for all calculations. All calculations are based on pressure of 1.0 atm and temperature of 22°C.

Consistent with the negative pressure that developed in the reactor gas headspace of Runs 2 and 3 of the present batch-mode experiments (operated at target pH values of 6.5 and 7.5, respectively), a similar trend of decreasing H₂ and CO₂ composition within reactor headspace at the end of biogas production was found by Oh *et al.* (2003) in experiments using heat-treated inoculums at pH of 6.2 and 7.5.

3.3.4 H₂ Production and Yield

Using the calculation approach described in the Analytical Methods section of this chapter (Section 3.2.4), H₂ production and yield were calculated for each of the batch mode experimental runs. Results are summarized in Table 3-7. Run 4 (target pH=5.5) displayed the highest H₂ production volume (3.51 L-H₂) and yield (1.47 mol-H₂/mol-glucose_{consumed}). Run 1 (target pH=4.5) and Run 2 (target pH=6.5) resulted in nearly equal H₂ volume production and yield. Run 3 (target pH=7.5) had the lowest H₂ production volume (1.51 L) and yield (0.64 mol-H₂/mol-glucose_{consumed}). The ratio of total H₂ to CO₂ produced increased with pH, with the ratio for Run 3 (target pH=7.5) being substantially higher than the rest of the batch-mode experiments (e.g., 12.5 times higher than the Run 1).

Table 3-7: Total H₂ production and yield for batch-mode experiments 1-4.

Target pH	Run ID	Total CO ₂ volume Produced (L)	Total H ₂ volume Produced (L)	Ratio of H ₂ /CO ₂ Produced	¹ H ₂ Yield (mol-H ₂ /mol-glucose _{consumed})
4.5	1	2.61	2.62	1	1.09
5.5	4	3.08	3.51	1.1	1.47
6.5	2	1.17	2.75	2.4	1.11
7.5	3	0.12	1.51	12.5	0.64

* A reactor liquid volume of 2.8 L and initial sugar concentration equal to that measured at time zero (see Table 3-4) were used for all calculations where needed.

All calculations are based on pressure of 1 atm and temperature of 22°.

The H₂ yield obtained for Run 4 (target pH=5.5) in this study (1.47 mol-H₂/mol-glucose_{consumed}) is comparable to values reported in literature. The conversion efficiency in this

case was 37% based on a maximum theoretical yield of 4 mol-H₂/mol-glucose. A similar study by Oh *et al.* (2003) using glucose (3 g-COD/L initial concentration) resulted in 24.2% conversion efficiency using heat-treated mixed inocula (pH of 6.2 and 25°C). Kuromoto *et al.* (1998) achieved a H₂ yield (mol-H₂/mol-hexose) of 0.52 and 1.58, under normal operation and with purging with argon respectively, via fermentation of molasses with *Enterobacter aerogenes* (pH of 6 and 38°C). Li *et al.* (2008) used heat-treated natural sludge from a river and an operating temperature of 38°C to conduct a similar study investigating variable constant pH values (5-7) in batch-mode, and they obtained a maximum yield of 1.83 mol-H₂/mol-glucose at a pH of 6.

3.3.5 Volatile Fatty Acids and Homoacetogenesis

The volatile fatty acids (VFAs) measured for all four experimental runs were lactate, acetate, propionate, formate, butyrate, and succinate. Alcohols were not measured in the study described here. The concentration of VFAs during each experimental run, along with gas production rate and composition, are graphically depicted in Figure 3-5. For every experiment, acetate and butyrate concentrations increased rapidly during the biogas production period.

For Run 1 (target pH=4.5) and Run 4 (target pH=5.5), butyrate concentrations remained higher than acetate throughout the experiment. For Run 2 (target pH=6.5) and Run 3 (target pH=7.5), acetate and butyrate concentrations were nearly equal during the time interval of net biogas production. During Run 2, acetate concentration surpassed butyrate concentration shortly after biogas production ceased and H₂ and CO₂ consumption initiated. Similarly for Run 3, terminal biogas production and consumption of H₂ and CO₂ resulted in a sharp increase in acetate concentration. Although metabolic pathway associated with the production of acetate favors H₂ production, the absence of methane and an inverse relationship between biogas consumption and acetate concentration indicates that homoacetogenesis was occurring during Run

2 and Run 3. Some studies have reported that H₂ production estimated from accumulation of acetate was lower than expected, and inconsistencies have also been attributed to homoacetogenesis. (Kotsopoulos *et al.*, 2009; Oh *et al.*, 2003). A decrease in H₂ and CO₂ was also observed during later stages of Run 1 and Run 4; however, acetate concentrations did not remarkably increase.

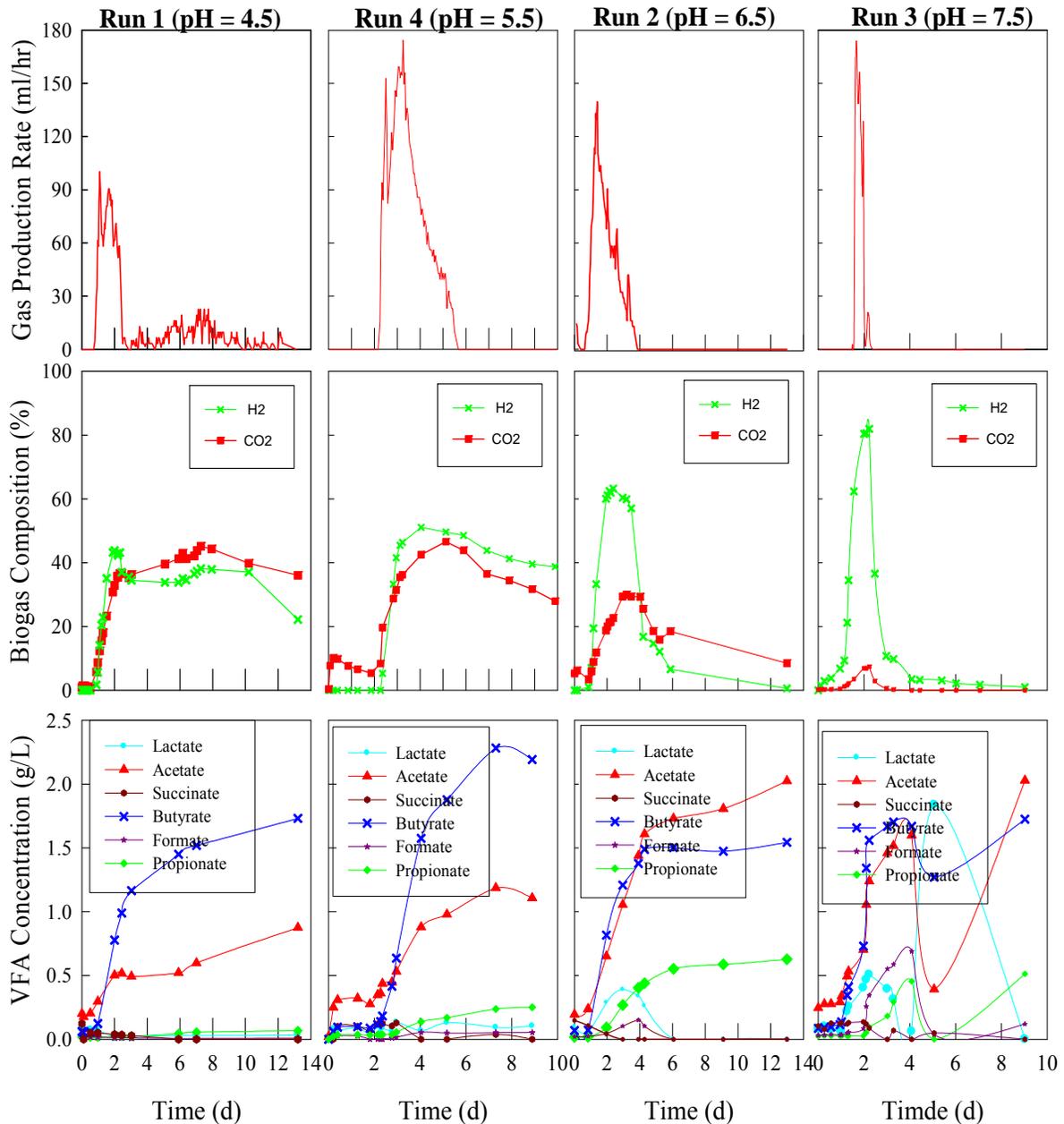


Figure 3-5: Distributions of volatile fatty acids (VFAs) in comparison to biogas production and composition for batch-mode experiments 1-4. Black arrows indicate vacuum formation.

Lactate and propionate were not detected at a pH of 4.5, and they were generally measured at higher concentrations with an increase in pH. Average concentrations of lactate for Runs 4 (target pH=5.5), 2 (target pH=6.5), and 3 (target pH=7.5) were 0.08 g/L, 0.16 g/L, and 0.32 g/L respectively, and average concentration of propionate were 0.1 g/L, 0.33 g/L, and 0.12 g/L respectively. Formate was generally measured at very low concentrations (< 0.1 g/L) for Run 1 and Run 2, and the highest concentration measured during Run 2 and Run 3 (target pH=7.5) was 0.15 g/L and 0.6 g/L respectively. Concentrations of succinate remained low (< 0.15 g/L) during all four batch-mode experiments; the highest succinate concentration measured was 0.13 g/L during Run 3.

3.3.6 Further Discussion and Implications Related to the PPI Site

Because the four batch-mode experiments described in this chapter were initiated using groundwater collected at different times (during which the groundwater contaminant concentrations and geochemistry were changing – see Tables 3-2 and 3-3), drawing unequivocal conclusions about differences in reactor performance due only to differences in the operating pH is complicated or impossible. Differences in sugar consumption rates and gas production rates may have been at least partially due to differences in microbial community structure and concentrations in the starting groundwater inoculum. Indeed, 16S rRNA gene libraries constructed using tag pyrosequencing indicate that the microbial community structure in the groundwater changed during the time of the experiments described in this thesis (Bowman et al., 2010b). Nevertheless, it is possible to develop some generalizations with relevance to the cleanup operations at the PPI site. With an average initial sugar concentration of 6.82 g-glucose/L and ambient laboratory temperature ($\approx 22^{\circ}\text{C}$) only slightly higher than in-situ conditions (temperature approximately 20°C , see Table 3-3), at least of 90% of the initial sugar concentrations were consumed within 13 days for all pH values tested (see Table 3-5). At target

pH values of 6.5 (Run 2) and 7.5 (Run 3) the vast majority of the sugars (>90%) were consumed within two days. The average movement of groundwater at the PPI site has been estimated at 0.6 to 0.7 ft/day (NPC, 2010). Thus, if an equivalent sugar concentration were to result from the injection of additional molasses, it is likely that the readily fermentable substrates will not travel far via advection in the groundwater flow field. This may have serious implications because the availability of fermentable substrate, and consequently H₂ production, may not be available for reductive dechlorination downstream of the injection site. Furthermore, if the pH was between 6.5 and 7.5, it is expected that H₂ consumption (via homoacetogenesis) would likely occur, limiting the amount of H₂ ultimately available to dechlorinating bacteria.

3.4 Conclusions

Under ambient laboratory temperature and at initial molasses concentrations equivalent to 6.5-7.0 g-glucose/L, the microbial consortium contained in the groundwater collected from well MW-01 at the PPI Site produced H₂ via fermentation at all pH values tested over the range from 4.5 to 7.5. A pH of 5.5 resulted in the highest H₂ yield of 1.47 g-H₂/g-glucose and total production of 3.51 L. Thus, it may be concluded that additional injection of molasses at the site from which the groundwater was collected may result in the highest H₂ production at a pH of 5.5. Moreover, the H₂ consuming processes of homoacetogenesis and methanogenesis were not noticeable. Despite having high H₂ compositions in the reactor headspace for a limited time period, the experiments operated at target pH values of 6.5 and 7.5 resulted in lower hydrogen yields. This likely resulted from H₂ consumption via homoactogenesis as indicated by relatively higher acetate production especially during the interval in which biogas consumption (as indicated from negative headspace pressure) was observed. The experiment operated at the lowest pH (Run 1, target pH=4.5) had low H₂ production rates; however, the period of hydrogen production was the longest and substrate was consumed at a slower rate. Although the H₂ yield

was lowest at this pH, it is important to note that H₂ was still formed at this low pH value. A low but long-lived H₂ production rate may be advantageous for full-scale molasses injection because it has the potential for larger spread of electron donors in the subsurface.

CHAPTER 4: THE EFFECT OF pH VALUE ON THE CONTINUOUS PRODUCTION OF HYDROGEN (CSTR) AS A RENEWABLE ENERGY SOURCE

4.1 Introduction

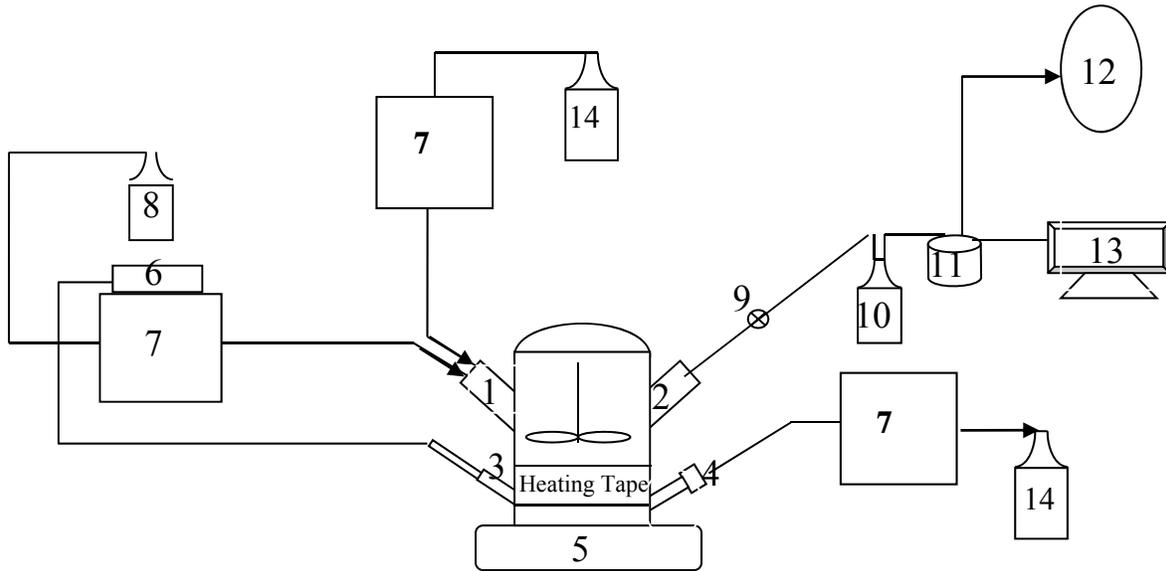
Previous studies have demonstrated that the rate and yield of hydrogen production via non-photosynthetic fermentation is a function of many factors including microbial community composition, substrate, reactor configuration, nutrient supply, temperature, and pH (Wang & Wan, 2009; Kapdan & Kargi, 2006; Ye *et al.*, 2007). Initial batch-mode experiments described in Chapter 3 of this thesis demonstrated that the microbial community present in the chlorinated solvent contaminated groundwater in the Scenic Highway area of the PPI site was able to produce H₂ over a wide range of pH values. The overall objective of continuous-flow experiments described in this chapter was to further assess the potential of the microbial community from this source to produce H₂ as a renewable energy source using molasses as a substrate. To achieve this objective, the microbial consortia developed in batch-mode experiments described in Chapter 3 were employed in continuous-flow (CSTR) experiments. In each experiment, the hydrogen production capacity was assessed in terms of production rate, yield, and stability. Continuous-flow experiments were designated in the same manner as batch-mode experiments: Run 1, Run 2, Run 3, and Run 4, were operated at target pH values of 4.5, 6.5, 7.5, and 5.5, respectively.

4.2 Materials and Methods

4.2.1 Reactor Configuration

The reactor configuration employed during continuous-flow experiments (Figure 4-1) was essentially the same as the configuration described in Chapter 3 except for the incorporation of additional components for temperature regulation, feeding, and wasting. Temperature control was accomplished using electrical heating tape (Thermolyne® silicone rubber-encapsulated)

attached to the exterior surface of the bioreactor in conjunction with a variable transformer (Variac, Staco Energy Products, Type 3PN1010). Inflow and outflow were achieved using variable speed positive displacement pumps (Masterflex).



- | | |
|--------------------------------|------------------------------------|
| 1) Feed and base inlet ports | 8) NaOH solution bottle |
| 2) Gas outlet port | 9) Headspace gas sampling port |
| 3) pH electrode (Autoclavable) | 10) Liquid trap |
| 4) Waste effluent port | 11) Gas flow meter |
| 5) Heavy-duty stirrer | 12) Tedlar collection bag |
| 6) pH controller | 13) CPU for data acquisition |
| 7) Peristaltic pump | 14) Effluent/Influent Amber bottle |

Figure 4-1: Reactor configuration during continuous-flow experiments.

4.2.2 Feed Preparation

The type of molasses and source from which it was obtained were the same as described in Chapter 3. All continuous-flow experiments were fed with the second batch of agricultural feed grade molasses delivered on October 21, 2009. Influent feed was prepared by adding 80 mL of undiluted molasses and 3.5 L of deionized water into a one gallon nominal amber glass bottle. The feed was supplemented with 2.68 g of NH_4Cl and 0.31 g of KH_2PO_4 , equivalent to concentrations of 200 mg/L-N and 20 mg/L-P. The feed solution was subsequently autoclaved

for 15 minutes at 115°C and 15 psi, and stored at room temperature ($\approx 22^\circ\text{C}$) for approximately 12-24 hours prior to use.

4.2.3 Reactor Startup and Operation

Prior to reactor start-up in continuous flow mode, a Tedlar bag filled with high purity N_2 gas was attached to one of the upper sidearm ports via autoclaved tubing and a $0.22\ \mu\text{m}$ syringe filter (Nalgene). The reactor working liquid volume was decreased from 2.8 L (during batch-mode) to 1.35 L by switching on the effluent pump and opening the port to allow N_2 gas from the 1 L Tedlar bag to fill the vacuum. After decreasing the working liquid volume, the port was closed, and both influent and effluent pumps were switched on to pump at the same flow rate ($\approx 2.97\ \text{L/d}$). The resulting hydraulic retention time (HRT) and solids residence time (SRT) was 10.9 hours. Finally, the variable transformer attached to the external heating tape was turned on to increase the target temperature to 35°C . Time zero of the experiment corresponds to the time when the temperature controller and influent and effluent pumps were turned on. Table 4-1 lists the dates when experiments were started and the duration of each experiment.

Table 4-1: Dates when continuous-flow experiments 1-4 commenced and ended.

Run ID	Target pH	Start/End Date	Duration (d)
1	4.5	10/19/09 – 11/29/09	41
2	6.5	12/16/09 – 12/26/09	10
3	7.5	1/22/10 – 2/12/10	21
4	5.5	3/9/10 – 5/20/10	72

On a daily basis, feed and effluent samples (1.5 mL) were collected in microcentrifuge tubes and stored in a freezer until analysis. Once per week, a larger sample of reactor contents was collected in a 15 mL sterile centrifuge tubes (about $\frac{3}{4}$ full) and stored in a freezer. Total suspended solid (TSS), volatile suspended solid (VSS), and biogas composition were measured on a daily basis. Total sugars and organic acids (VFA) were measured on a less frequent basis (every 2-3 days), and COD concentration was measured at 1-2 week intervals. Except for Run 4

(target pH=5.5), which was subsequently used for further experiments described in Chapters 5 and 6, reactor liquid temperature was only measured at the end of each experimental run. This was accomplished by submerging a temperature probe (Oakton, 0-200°C) into the reactor medium with top lid closed and allowing the reading to stabilize.

On day 13 of Run 3 (target pH=7.5) and on day 24 of Run 4 (target pH=5.5), the internal mixing paddles were found to be static (i.e., not mixing). In both cases, at the time static paddles were noticed and before mixing was restarted, the pH measured by the internal probe was at the target pH value. Immediately after mixing was restarted, however, automatic addition of NaOH initiated and the pH value read by the probe decreased to lower than pH target; this is the value that was recorded for that time. For both experiments, pH increased to the target value within the following 30 minutes. The total time of no mixing for Run 3 and Run 4 was estimated to be 8 hours and 16 hours, respectively.

On day 6 of Run 3 (target pH=7.5), negative pressure formed in the reactor headspace due to biogas consumption as further described in Section 4.3.3. The vacuum was eliminated by the same method as described in Chapter 3 Section 3.2.3 of batch-mode experiments (addition of N₂ gas to the reactor headspace). On the day 45 of Run 4 (target pH=5.5), the influent line was found to be clogged, and the reactor's liquid volume had decreased to 1 L. Due to technical issues with autoclaving of the replacement line, the reactor was not fed the following 52 hours (2.2 days) while the reactor was temporarily operated in batch-mode. During this 2.2 day interval, a vacuum formed in the system, and the method used to eliminate negative pressure was the same as described in Chapter 3 Section 3.2.3. Biogas composition was not measured for days 46 and 47. Feeding was restarted on day 48, and working liquid volume was increased to 1.35 within the following 6 hours by temporarily slowing the rate of the effluent pump. Once the

target working liquid volume inside the reactor was reached (1.35 L), both pumps were operated at flow rates of 2.97 L/d.

4.2.4 Analytical Methods

Total Sugars (i.e., measured in glucose equivalents), COD, organic acids, biogas composition, and pH were measured as described in Chapter 3. Total suspended solids (TSS) and volatile suspended solids (VSS) concentrations were measured according to Standard Method 2540 D and 2540 E, respectively (APHA, 1998). The cumulative biogas production volume and flow rate were measured and recorded using a gas meter (Milligascounter®, Ritter MGC-1) and software (Rigamo). Daily production of biogas was calculated by taking the volume produced between consecutive days, dividing by the number of hours within the time interval, and multiplying by 24 to obtain a standardized production rate (L/d). H₂ and CO₂ production rates were determined by averaging the headspace composition between consecutive days and multiplying by the volume of biogas produced during the time interval.

4.3 Results

4.3.1 Temperature and pH

The reactor liquid temperature at the end of each experiment (measured as described in Section 4.2.3) was 35°C. The temperature during each experiment was presumably maintained at 35±1°C since ambient laboratory temperature (≈22°C) varied by ±1°C. During Run 1 (target pH=4.5) and Run 2 (target pH =6.5), the pH was essentially maintained at target value as shown in Figure 4-2. During Run 3 (target pH=7.5) and Run 4 (target pH=5.5), the pH was also maintained at target value except during the time when the reactor was not completely mixed due internal stirring paddle failure as described in Section 4.2.3. On these days (day 13 of Run 3 and day 24 of Run 4), the pH was found to be 6.5 and 4.7 for Run 3 and Run 4, respectively, and was brought to target value within the following 30 minutes (Figure 4-2).

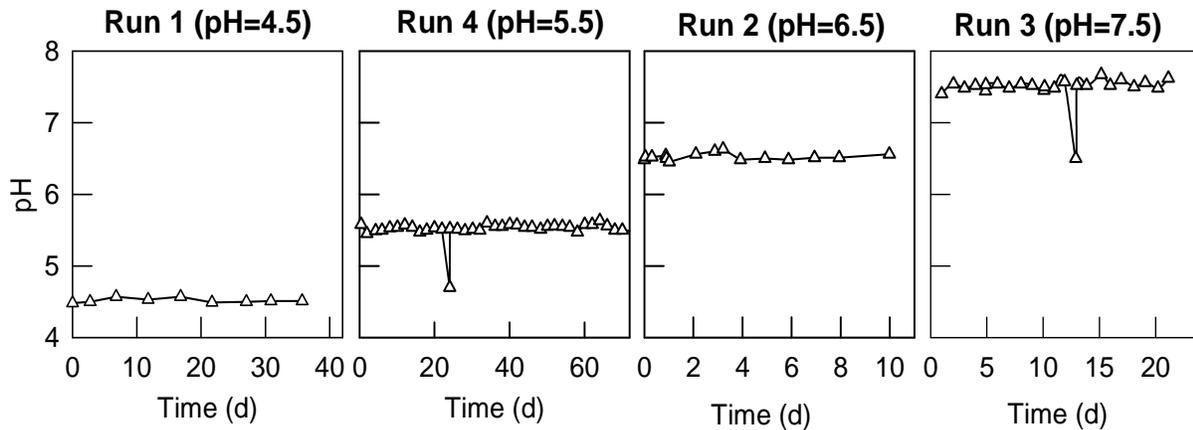


Figure 4-2: pH during continuous-flow experiments 1-4.

4.3.2 Organic Loading Rate, Sugar Consumption, and Biomass Concentration

The organic loading rate (OLR), influent and effluent sugar concentration, and biomass concentration during continuous-flow experiments 1-4 are shown in Figure 4-3. Although the target OLR was $37 \text{ g-glucoseL}^{-1}\text{d}^{-1}$, the actual value varied during each experiment. It varied notably during Run 1 (target pH=4.5, much less during Run 2 (target pH=6.5), and remained relatively constant during Run 3 (target pH=7.5) and Run 4 (target pH=5.5).

As shown on Figure 4-3 B, sugar consumption was significantly lower during Run 1 (target pH=4.4) with only 48.3% of sugars fed consumed. For Run 2, 3, and 4, the sugar consumption was 94%, 93%, and, 94% respectively. The average biomass concentration was also significantly lower for Run 1 (Figure 4-3 C), with a concentration 0.85 g-VSS/L compared to 2.13 g-VSS/L and 2.45 g-VSS/L for Run 4 and Run 3, respectively. Biomass concentration was not measured during Run 2. The biomass yield ($\text{mg-VSS/g-glucose}_{\text{consumed}}$) for Run 1, Run 3, and Run 4 were 122.48, 155.75, and 138.76, respectively.

4.3.3 Biogas Production and Composition

The average biogas production rate during Run 1 (target pH=4.5) was 5.4 L/d . During the experiment, there was a sharp drop to 0.15 L/d on day 13; the average production before and after the plunge was 4.42 L/d and 6.03 L/d respectively.

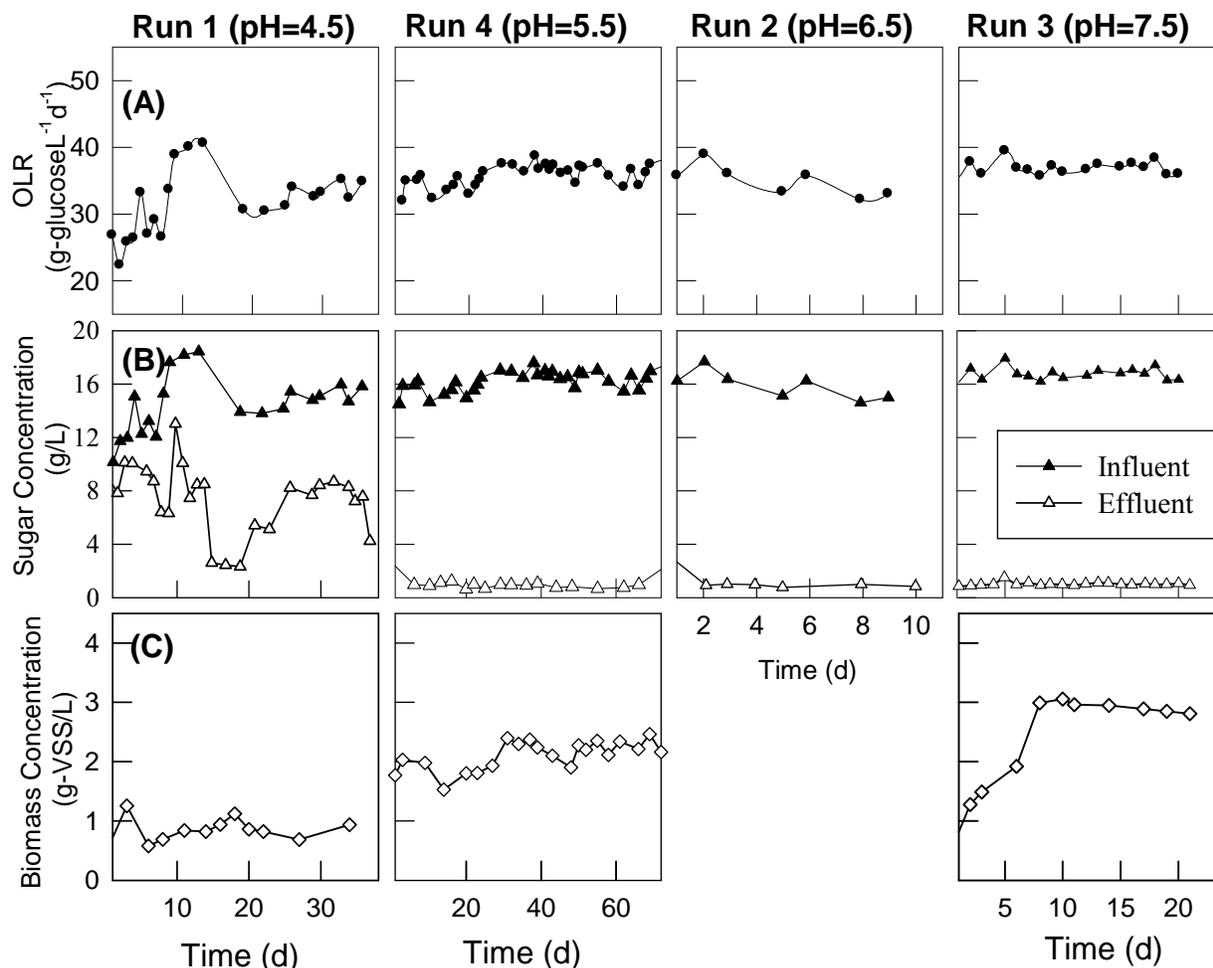


Figure 4-3: (A) Organic loading rate, (B) influent and effluent sugar concentration, (C) and biomass concentration during continuous-flow experiments 1-4.

The biogas production rate was the highest during Run 4 (target pH=5.5) despite the two incidents in which the pH decreased below target value and when the reactor was not fed, as described in Section 4.2.3 and indicated on Figure 4-4 A and B by the red arrow and black circle. On each occasion, the reactor was able to recover to a stable biogas production rate. Excluding days on which the reactor was not completely mixed or was not fed (day 24, 46, and 47), the average biogas production was 22.7 L/d.

The average biogas production rate during Run 2 (target pH=6.5) was 16.24 L/d. From Figure 4-4, it can be seen that there was a downward trend in biogas production with time. The highest production rate was 21.9 L/d which was produced on the second day. However, the final

biogas production rate was down to 10.3 L/d. During Run 3 (target pH=7.5), biogas was only produced on the following three days: day 5, day 6, and day 13 at 1.06 L/d and 2.4 L/d 0.57 L/d, respectively. After day 6, biogas consumption initiated (Figure 4-4 by black circles) and continuously formed; vacuum was eliminated by adding N₂ to headspace as described in Chapter 3 Section 3.2.3. On day 13 however, a small amount of biogas was produced due to internal paddle failure and a subsequent decrease of pH below target value as described in Section 4.2.3.

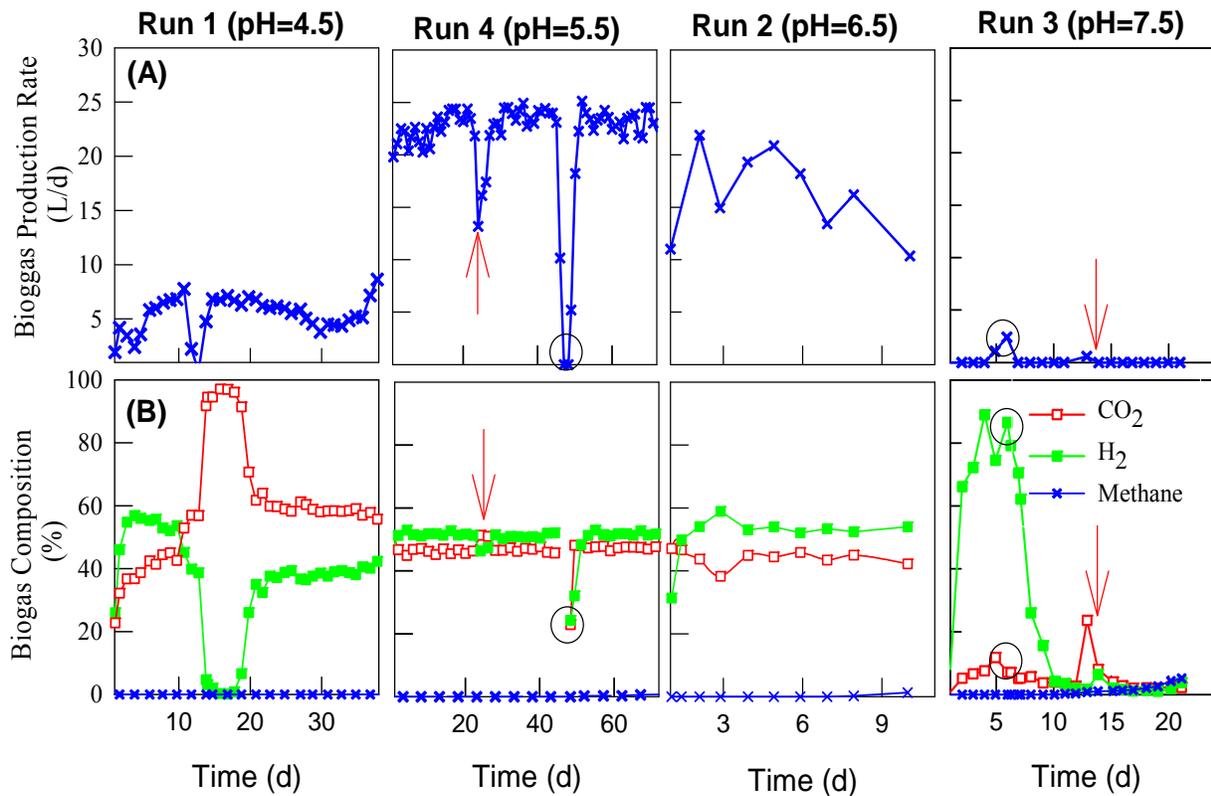


Figure 4-4: (A) Biogas production rate, and (B) biogas composition for continuous-flow experiments 1-4. Black circle indicates when vacuum formation was detected and N₂ gas was subsequently added for negative pressure elimination. Red arrows indicate when pH dropped due to internal mixing paddle failure. Biogas composition was not measured during day 46 and 47 of Run 4.

There were drastic changes in biogas composition during Run 1 (target pH=4.5). For the first thirteen days, H₂ composition remained above that of CO₂, at 47% and 42% respectively. However, when biogas production dropped to 0.15 L/d on day 13, CO₂ composition increased to 92% and H₂ decreased to 5%; CO₂ and H₂ and composition remained at an average of 90% and

7% until day 19. After day 19, CO₂ concentration decreased but remained above that of H₂ for the rest of the experiment, at an average of 59% and 38 % respectively. Methane was not detected during Run 1. For Run 4 (target pH=5.5), excluding days in which the reactor was not fed on day 46 and on day 47, H₂ composition was maintained at an average of 50.42%. On day 24, when pH dropped below target value, CO₂ concentration increased above that of H₂, but it dropped below that of H₂ after pH was adjusted back to the target value. The highest methane concentration was 1% on day 69, and it was measured at 0.78% at the end of the experiment.

For Run 2 (target pH=6.5), H₂ and CO₂ concentration increased during the first two days and was maintained at an average of 53% and 42% percent, respectively. Methane composition was measured at 0.2% and 1.2 % on day 8 and at the end of the experiment (day 10), respectively. For Run 3 (target pH=7.5), H₂ composition increased up to 66% on day 2, and was maintained at an average of 79% until day 6. After that, the H₂ concentration steadily decreased, reaching 4% on day 10 and remained at less than 4% until the end of operation (day 21). CO₂ composition reached a maximum concentration of 23.6% during the time when the pH dropped below target value (on day 13) but decreased again thereafter. Methane was first detected on day 11 at 0.24% and increased to a final concentration of 5.16 % at the end of the experiment (day 21).

4.3.4 Volatile Fatty Acids

Figure 4-5 shows the VFA concentration as well as the biogas production rate and biogas composition during Runs 1-4. The average concentration of acetate, butyrate, and lactate during Run 1 (target pH=4.5) was 0.6 g/L, 0.93 g/L 1.06 g/L, respectively. During the first nine days of the experiment, butyrate concentration and acetate concentration increased to 1 g/L and 0.8 g/L respectively. Lactate concentration was maintained at a low level (average 0.14 g/L) during this period. However, when there was a drastic increase in CO₂/H₂ ratio and a decrease in biogas

production, there was a substantial increase in lactate and a decrease in butyrate concentration. After H_2 increased again on day 20, butyrate concentration increased as well as lactate concentration.

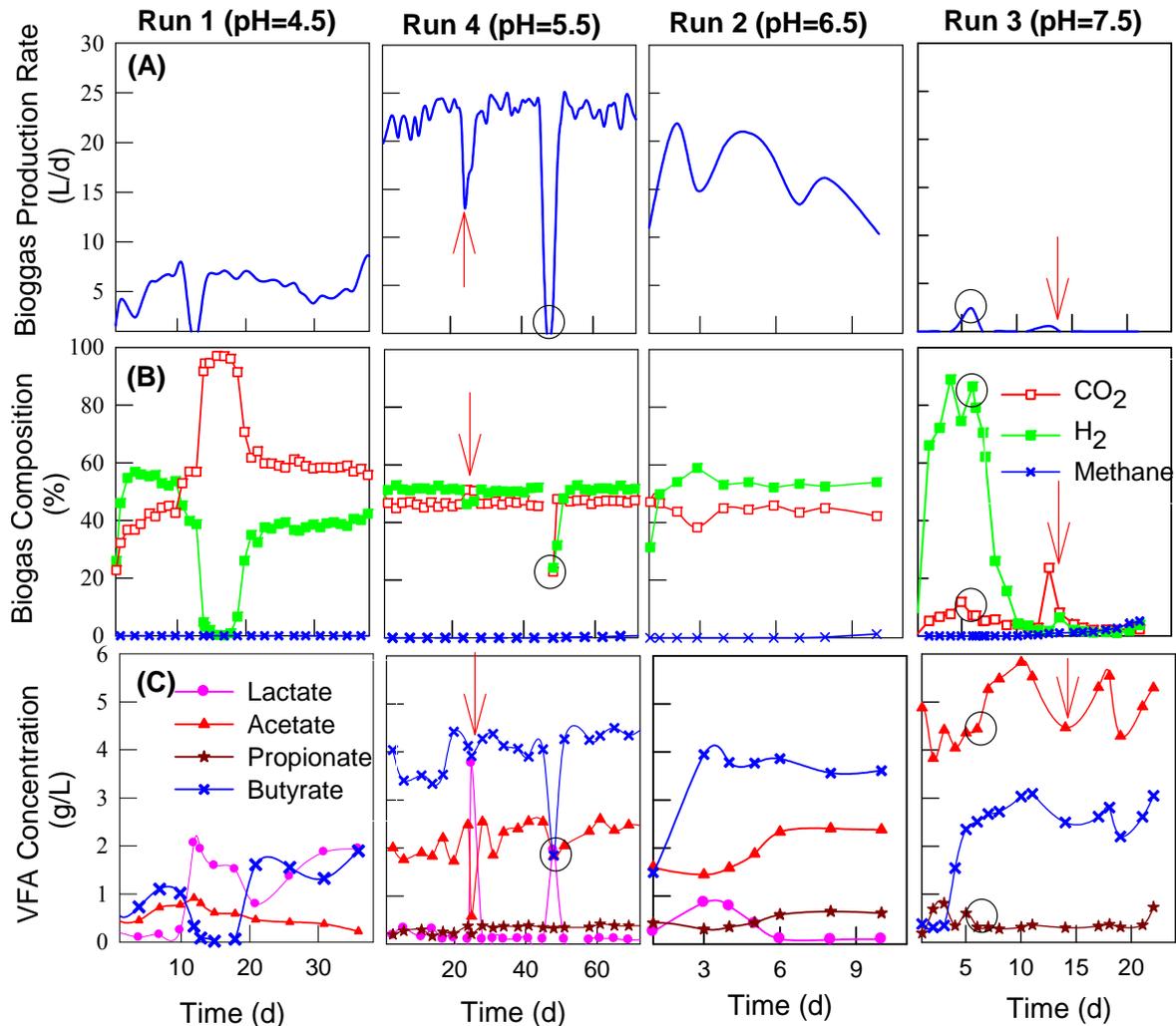


Figure 4-5: (A) Biogas production rate, (B) biogas composition, and (C) organic acid concentration for continuous-flow experiments 1-4. Biogas composition was not measured during day 46 and 47 of Run 4. Black circle indicates when reactor a negative pressure formed in the system and N_2 gas was added to headspace to the vacuum. Red arrows indicate when pH dropped due to internal mixing paddle failure during Run 3 and Run 4.

During Run 4 (target pH=5.5), the average acetate and butyrate concentrations were 2.15 g/L and 4.06 g/L respectively. The ratio of average acetate to average butyrate concentration was 0.53. Lactate generally remained less than 0.3 g/L except on the days when the reactor was not mixed or fed, during which lactate reached peak concentrations of 3.76 g/L and 1.96 g/L

respectively (days 24 and 47). Propionate remained relatively constant at an average concentration of 0.33 g/L. Formate and succinate concentrations were consistently low (< 0.1 g/L). The VFA concentrations varied during the first three days of Run 2 (target pH=6.5) but stabilized after day 6. The average butyrate concentration was 3.20 g/L, and the average acetate concentration was 1.96 g/L. The ratio of average acetate to average butyrate concentration was 0.61. Lactate concentration increased to 0.90 g/L on day 4, but decreased thereafter; the final concentration was 0.8 g/L. Propionate and formate concentrations remained at less than 1.0 g/L, and their respective average concentration was 0.44 g/L, and 0.50 g/L.

For Run 3 (target pH=7.5), the average acetate and butyrate concentration was 4.70 g/L and 2.14 g/L respectively. As seen from Figure 4-5, Run 3 is the only experiment in which acetate concentration was maintained higher than butyrate. The ratio of average acetate to average butyrate concentration during the experiment was 2.2. Also from Figure 4-5, it can be seen that acetate concentration increased when a vacuum was formed in the reactor headspace. During the period when the pH dropped to 6.5 as a result of internal impeller failure, both acetate and butyrate concentrations decreased. The average formate and succinate concentrations were 1.35 and 0.88 respectively during the first six days, but only trace amounts were detected thereafter (<0.10 g/L). Lactate was not detected during the experiment.

4.3.5 Hydrogen Production Rate and Yield

The H₂ production rate (expressed in units of liters H₂ (T=22°C, P=1.0atm) per liter reactor liquid volume per day, L/L/d) for all four experiments is compared in Figure 4-6. Results indicate that Run 4 (pH=5.5) resulted in the highest H₂ production rate, 8.68 L/L/d, and the highest yield, 1.93 mol-H₂/mol-glucose. Also, Run 4 displayed the highest stability; production rates were relatively constant throughout the 72 days of operation and recovered quickly after being unintentionally interrupted on two separate occasions as described in Section 4.2.3. In

comparison, Run 2 (target pH=6.5) produced H₂ at moderate rates for the first four days, but rates slowly decreased with time. The average production rate for the entire experiment was 6.58 L/L/d, and the yield was 1.03 mol-H₂/mol-glucose. Run 1 (target pH=4.5) had low H₂ production rates (average of 1.39 L/L/d), and Run 3 (target pH=7.5) showed no net production except for an initially small amount. Table 4-2 summarizes the H₂ production rate, yield, and the specific production rate for all four experiments based on *average* values. It is important to note that the first two days of data were not included in calculations. This was intended to exclude any lag-phase due to prior operation of the reactor in batch-mode. Also, for Run 4, data on the days on which the reactor was accidentally disturbed were excluded from calculations (day 24, 46, and 47).

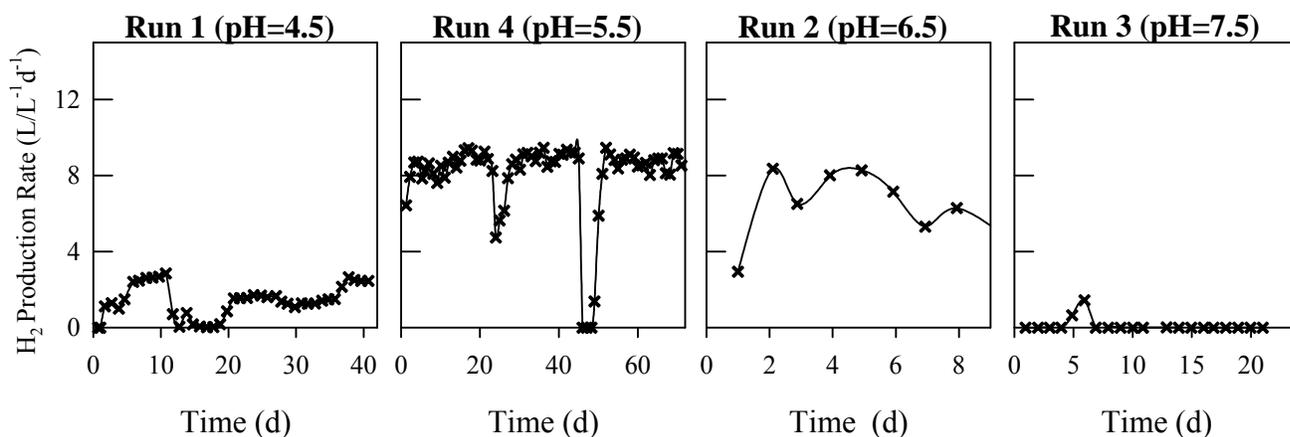


Figure 4-6: Hydrogen production rate for continuous-flow experiments 1-4.

Table 4-2: H₂ production, yield, and specific production for continuous-flow experiments.

Experiment/ target pH	Production Rate (L-H ₂ /L/d)	Yield (mole-H ₂ /mole-glucose _{consumed})	Specific Production Rate (L-H ₂ /g-MLVSS/d)
1 / 4.5	1.39	0.68	1.64
2 / 6.5	6.68	1.03	² N/A
3 / 7.5	0.12	0.02	0.05
¹ 4 / 5.5	8.68	1.93	4.07

¹Base on average values and excluding the first two days of operation and all calculations at temp of 22°C and pressure of 1 atm.

²Days on which reactor was not completely mixed and fed are not included in calculations (day 24, 46, and 47).

³VSS were not measured during Run 2.

4.4 Discussion

As indicated by the low substrate utilization efficiency (48.3%) and low biomass concentration (0.85 g/L), results of Run 1 (target pH=4.5) indicate that fermentative bacteria grew at low specific rates which resulted in low H₂ production rates. At this pH, there was a relatively higher average concentration of lactate (relative to acetate and butyrate) and CO₂ concentration was higher H₂. The low H₂ content and high CO₂ content during periods with appreciable lactate production is consistent with known metabolic pathways; lactic acid producing pathways are involved in a zero-hydrogen balance and the production of CO₂ (Guo *et al.*, 2010). Tanisho and Ishiwata (1995) found that lactate negatively correlated with H₂ production when using molasses and *Enterobacter aerogenes* for continuous H₂ production. A similar increase in CO₂ concentration in conjunction with an increase in lactate concentration was also noted for Run 4 when the pH was found at 4.7.

Run 3 resulted in the lowest biogas production rate. After a small amount produced, biogas consumption initiated and acetate increased during day 6 of the experiment. Run 3 is the only experiment in which average acetate concentration was found to be higher than average butyrate concentration (4.7 g/L compared to 2.14 g/L respectively). The average ratio of acetate to butyrate concentration was 2.2, compared to 0.65, 0.53, and 0.61 for Runs 1, 4, and 2 respectively. Thus, it is reasonable to conclude that the low H₂ yield was largely a result of homoacetogenesis occurring inside the reactor. Also, at the end of the experiment, methane composition was measured at 5.15% which suggests methanogenic activity would eventually contribute to a larger degree to low H₂ consumption.

The highest H₂ production rate was obtained for Run 4 (pH=5.5). At this pH, H₂ production remained relatively stable at an average rate of 8.68 L-H₂/L⁻¹d⁻¹. The yield was 1.93 mol-H₂/mol-glucose, which is 48.3% percent of the theoretical maximum (based on the

maximum of 4 mol-H₂/mol-glucose). Run 4 also displayed the highest stability because H₂ production rates recovered despite being interrupted with the two unintended occurrences previously discussed in Section 4.2.3. H₂ composition remained stable at 50.4%, and only trace concentrations of methane were detected. It is reasonable, therefore, to conclude that H₂ consumption via homoacetogenesis was minimal at pH of 5.5 in comparison to other pH values tested. Except for lactate during the days when the reactor not mixed or fed (days 24, 46, 47), the only VFAs measured at appreciable concentrations were butyrate and acetate. This is an indication of the predominance of metabolic pathways that favor H₂ production (Antonopoulou *et al.*, 2008; Koskinen *et al.*, 2008).

In comparison to Run 4, Run 2 (target pH=6.5) resulted in moderate H₂ production rates during the first six days, but production slowly decreased over time. Methane concentration was measured at 1.2% in the gas headspace at the end of the experiment which could account for the slow decrease in H₂ production. Propionate concentrations were relatively higher than in the other three experiments. Propionate is associated with metabolic pathways which consume hydrogen (Li & Fang, 2007; Guo *et al.*, 2010), which may have contributed to the slow decrease in H₂ production over time.

4.4.1 Assessment of Capacity in Comparison to Similar Studies

Table 4-3 lists H₂ production rates and yields observed in previous studies using molasses as a substrate. The inoculum source, pH, HRT, and OLR varied among these studies, and the optimum pH has been between 4.0-5.5 (see Table 4-3). The highest average H₂ production rate (8.68 L/L/d) found in the present study at a pH of 5.5 is lower than that obtained by Ren *et al.* (2009), Aceves-Lara *et al.* (2008) and Guo *et al.* (2008) who reported H₂ production rates of 9.72, 15.7, and 17.0 L/L/d, respectively. However, the yields reported by Aceves-Lara *et al.* (2008) and Guo *et al.* (2008) were 36% and 43.9% of the theoretical maximum. These are both

lower than the maximum (48.3%) obtained in this study. A yield was not reported by Ren *et al.* (2009). The specific H₂ production rate (L-H₂/g-VSS/d) obtained in the present study was relatively high (4.07), with only Ren *et al.* (2009) obtaining a higher value (5.13)

A considerable challenge in the development of an economically attractive fermentative hydrogen production has been low production yields (Hallenbeck & Benemann, 2002; Guo *et al.*, 2010). The highest yields obtained have been around 2.0-2.4 mol-H₂/mol-glucose (Kapdan & Kargi, 2006), and the highest H₂ yield obtained in this study (1.93 mol-H₂/mol-glucose) is on the low end of that range. Compared to the studies listed in Table 4-3, the yield in this study (48.3%) was higher than those reported in terms of sugar (i.e. glucose and sucrose), with Guo *et al.* (2008) reporting the highest (43.4 %). A comparison with those studies which reported the yield in terms of COD is not possible because the effluent COD concentration was not measured in this study. Li *et al.* (2009) and Ren *et al.* (2009) did not report a yield.

4.5 Conclusions

The results of the four experiments conducted at different pH values (in a CSTR with no recycle) showed that the groundwater inoculum from the Scenic Highway area of the PPI Site is best suited for continuous H₂-production using at a pH of 5.5 (Run 4). At this pH, homoacetogenic and methanogenic activity were minimal in comparison to observations at higher pH values, and the net production of H₂ was higher than at other pH values. The main VFA products were butyrate and acetate, indicative of metabolic pathways which favor the production of H₂. The experiment conducted at a pH of 7.5 (Run 3) was not able continuously produce H₂ for the duration of the experiment due to homoacetogenesis, and acetate concentration more than doubled that of butyrate during the experiment. A slow increase in methane composition was also observed within the reactor at pH 7.5. The experiment conducted at a pH of 6.5 (Run 2) showed moderate H₂ production during the first six days but slowly

decreased over time. Acetate and butyrate were the main products of fermentation, and a slow increase in methane composition was measured over time. The experiment operated at a pH of 4.5 (Run 1) produced H₂ at very slow rates. H₂ consumption was inhibited, but low substrate utilization efficiency and low biomass concentration indicated that fermentative bacteria grew slowly.

Table 4-3: H₂ production rates and yields reported in continuous-flow studies using molasses as substrate.

Reference	Inoculum Source	pH Range/Optimum	OLR Range/Optimum (g-COD/L/d)	Yield	Specific Production Rate	H ₂ Production Rate	Major Metabolite
Li et al., 2009	Secondary settling tank sludge from WWTP	4.0-5.2 / 4.2 ± 2	^A 15 (g-COD/L/d) / —	—	2.96 (mol/kg-MLVSS/d)	^A 2.5 (L/L/d)	Ethanol
Ren et al., 2006	MWWT sludge	—	6.32-85.6 / 35–55 (kg-COD/m ³ -reactor/d)	26.13 (mol/kg COD _{removed})	0.75 (m ³ /kg-MLVSS/d)	5.57 (m ³ -H ₂ /m ³ -reactor/d)	Ethanol
Aceves-Lara et al., 2008	^B Anaerobic Digester Sludge (wine distillery)	—/5.5	—	1.47 (mol/mol-glucose)	—	15.3 (L-H ₂ /L/d)	—
Li et al., 2007	Aerobic Activated Sludge (brewing WWTP)	4.3-4.4	—	0.13 (L/g-COD)	0.13 (L/g-MLVSS/d)	^A 1.18 (L/L/d)	Ethanol
Guo et al., 2008	Activated Sludge & Acidogenic Reactor Sludge from WWTP	3.9-5.3 / 4.2-4.4	8-192 / 120 (kg-COD/m ³ /d)	3.47 (mol/mol-sucrose)	3.16 (mmol/g-VSS/h)	0.71 (L/L/h)	Ethanol
Aceves-Lara et al., 2008	Anaerobic Digester Sludge (wine distillery)	5.5-6 / 5.5	37.12 (g-COD/L/d)	2.5 (mol/mol-sucrose)	—	5.4 (L/L/d)	Butyrate
Ren et al., 2009	Aerobically Cultivated Sewage Sludge	8.0-4.0 / 4.0	40 (g-COD/L/d)	—	—	9.72 (L/L/d)	Ethanol
Ren et al., 2010	Aerobically Cultivated Sewage Sludge	8.0-4.0 / 4.5	32 (g-COD/L/d)	—	5.13 (L/g-VSS/d)	6.65 (L/L/d)	Ethanol
^C This Experiment	Chlorinated Alkane-Contaminated Groundwater	4.5-7.5 / 5.5	34 (g-glucose/L/d)	1.93 (mol/mol-glucose)	4.07 (L/g-MLVSS/d)	8.68 (L/L/d)	Butyrate

^A Calculated based on given data

^B Inoculum was heat-shocked

^C Based on averages values, alcohols were not measured

CHAPTER 5: THE EFFECTS OF INCREASED ORGANIC LOADING RATE ON A HYDROGEN PRODUCING CSTR

5.1 Introduction

The research results presented in Chapter 4 demonstrated that at pH of 5.5, H₂ could be produced at a reasonably high rate and yield using molasses as a substrate in conjunction with microbial populations originating from contaminated groundwater at the PPI site as the inoculum. The experiments described in chapter 4, however, were conducted at a single organic loading rate (i.e., the influent molasses concentration was maintained at a single target level). The objective of research presented in this chapter was to evaluate the impact of increasing the organic loading rate (OLR) on the rate and yield of H₂ production in the continuous flow reactor operated at pH 5.5. Thus, at the conclusion of Run 4 described in chapter 4, the bioreactor operation was extended and operated under identical conditions as previously while increasing the molasses concentration to a target OLR of 74 g-glucose/L/d.

5.2 Materials and Methods

The reactor configuration for this experiment was identical to that employed for the continuous-flow experiments described previously in Chapter 4. The analytical methods used for this experiment were also identical to those described in Chapter 4.

Preparation of the sterile molasses feed solution was the same as that described in Chapter 4 except for the doubling of target influent molasses concentration (160 mL molasses added per 3.5 L of feed solution) and nutrient concentrations (amendments were increased to 400 mg/L nitrogen and 40 mg/L phosphorus). Feed of the higher molasses concentration commenced on May 22, 2010, corresponding to day 74 of continuous feed operation (with the start of operation in continuous-flow mode designated as time zero as described in chapter 4). Other operating conditions for this experiment were identical to those described for Run 4 in Chapter 4

(e.g., temperature control remained at 35°C, influent and effluent flow rates remained 2.97 L/d, and pH control was provided by NaOH addition from a pH controller with a set point of pH=5.5).

On day 87 (June 4, 2010), the effluent line was found clogged and the reactor liquid volume had increased to 2.5 L (versus a target reactor liquid volume of 1.35 L). To clear the blockage, the feed pump's flow direction was briefly reversed for a very short time interval (a few seconds) and then restored to normal feeding. The flow-rate on the effluent pump was temporarily increased until the reactor's target liquid volume (1.35 L) was attained, and the flow rate was adjusted back to "normal" flow-rate of 2.97 L/d. On day 88 (June 5, 2010), the reactor's effluent line was found clogged again. The reactor was operated in batch-mode (no feed or withdrawl) until the following day (day 89) when a new autoclaved effluent line was installed and the reactor operation was resumed. When continuous feed/withdrawl resumed on day 89, the influent molasses and nutrient concentrations were returned to the "base-line" levels described in chapter 4 (target OLR \approx 34 g-glucose/L/d). The operation at this OLR was continued from day 89 (June 6, 2010) to day 108 (June 25, 2010).

Because of the irregularities in bioreactor operation that arose from problems associated with clogged effluent tubing (on days 87 and 88), for purposes of analysis, the performance of the bioreactor during the period of increased loading is discussed in the Results section for the 12-day interval from day 74 (when the increased OLR began) to day 86 (the last day the reactor was operated at the higher OLR without interruption).

5.3 Results

5.3.1 Organic Loading Rate, Sugar Consumption, and Biomass Concentration

Figure 5-1 shows the OLR, influent and effluent sugar concentrations, and the biomass concentration (measured in terms of VSS) before, during, and after increasing the OLR. The

arrows pointing upward denote when the OLR was increased (day 74), and the arrows pointing downward denote when the OLR was decreased (day 89) back to the previous range. The experimentally determined average OLR before, during, and after the OLR was increased was 36 g-glucose/L/d, 76.4 g-glucose/L/d, and 38 glucose/L/d, respectively.

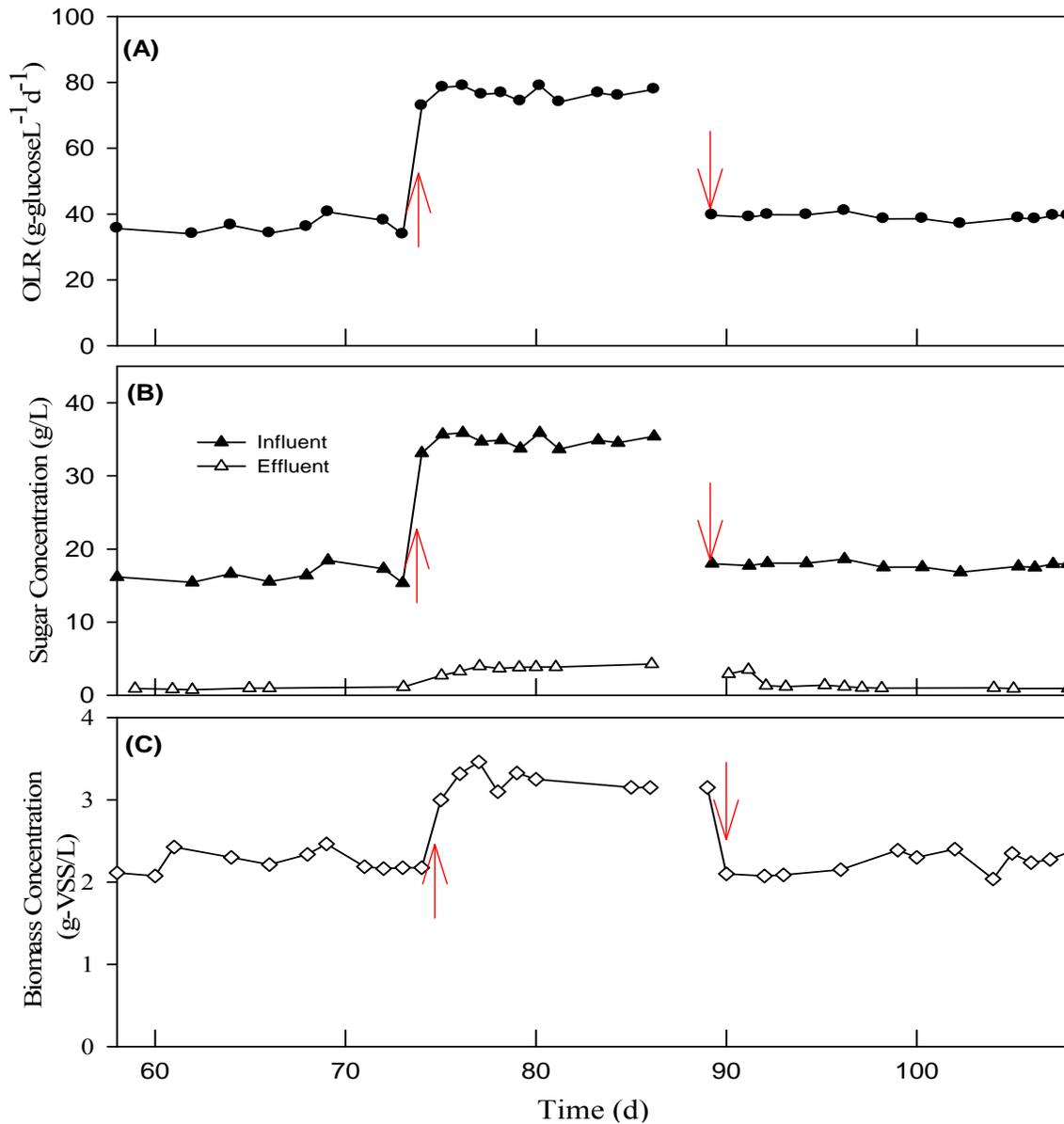


Figure 5-1: (A) Organic loading rate, (B) influent and effluent sugar concentration, and (C) biomass concentration prior to, during, and after the increase in OLR. Arrows pointing up indicate when the OLR was increased, and arrows pointing down indicate when the OLR was decreased back to previous range. Data for day 88 and 89 are not available due to variably changing of working liquid volume as a result of effluent line clogging.

As shown on Figure 5-1 B, the effluent sugar concentration increased when the OLR was increased and decreased when the OLR was subsequently decreased. Sugar consumption before (day 58-74), during (day 74-86), and after (day 89-108) the OLR was increased was 94%, 89.7%, and 92%, respectively. During the last 7 days of operation after the OLR was decreased (day 101-108), the sugar consumption was 94%. The average biomass concentration (g-VSS/L) before, during, and after the OLR was increased was 2.24, 3.22, and 2.37, respectively. The biomass yield (mg-VSS/g-glucose_{consumed}) during these intervals was 160, 103, and 134, respectively.

5.3.2 Biogas Production, Biogas Composition, and VFA

Figure 5-2 shows the biogas production rate, biogas composition, and VFA concentrations during the experiment. As shown in the figure, during the interval prior to the increase in OLR, the biogas production rate was relatively constant, averaging 23.1 L/d from days 58 to 74. During the first two days after the influent molasses concentration was increased (day 74-76), the biogas production rate rapidly increased, reaching a peak of 47 L/d H₂ on day 76. During this period, the H₂ concentration remained unchanged (\approx 50%). After day 76, however, the biogas production rate progressively decreased over the next 10 days and reached a production rate of 28.3 L/d on day 86. During the period of decreasing biogas production rates, H₂ concentration decreased by a small amount and CO₂ concentration increased by a small amount with an average concentration of 47%, and 51% respectively.

Methane concentration was stable at a low concentration (0.8% average) before the OLR was increased, but it was not detected after increasing the OLR. Considering the 12-day interval of increased organic loading rate when the reactor did not experience any irregularities in operation (i.e., days 74 to 86) the biogas production rate averaged 38.2 L/d. After the reactor was returned to operation at the “base-line” OLR (average 38 g-glucose/L/d), following a period of

operation in batch-mode as described in Section 5.2, biogas production rate recovered quickly (0 L/d to 17 L/d from day 89 to day 91) and stabilized after day 95.

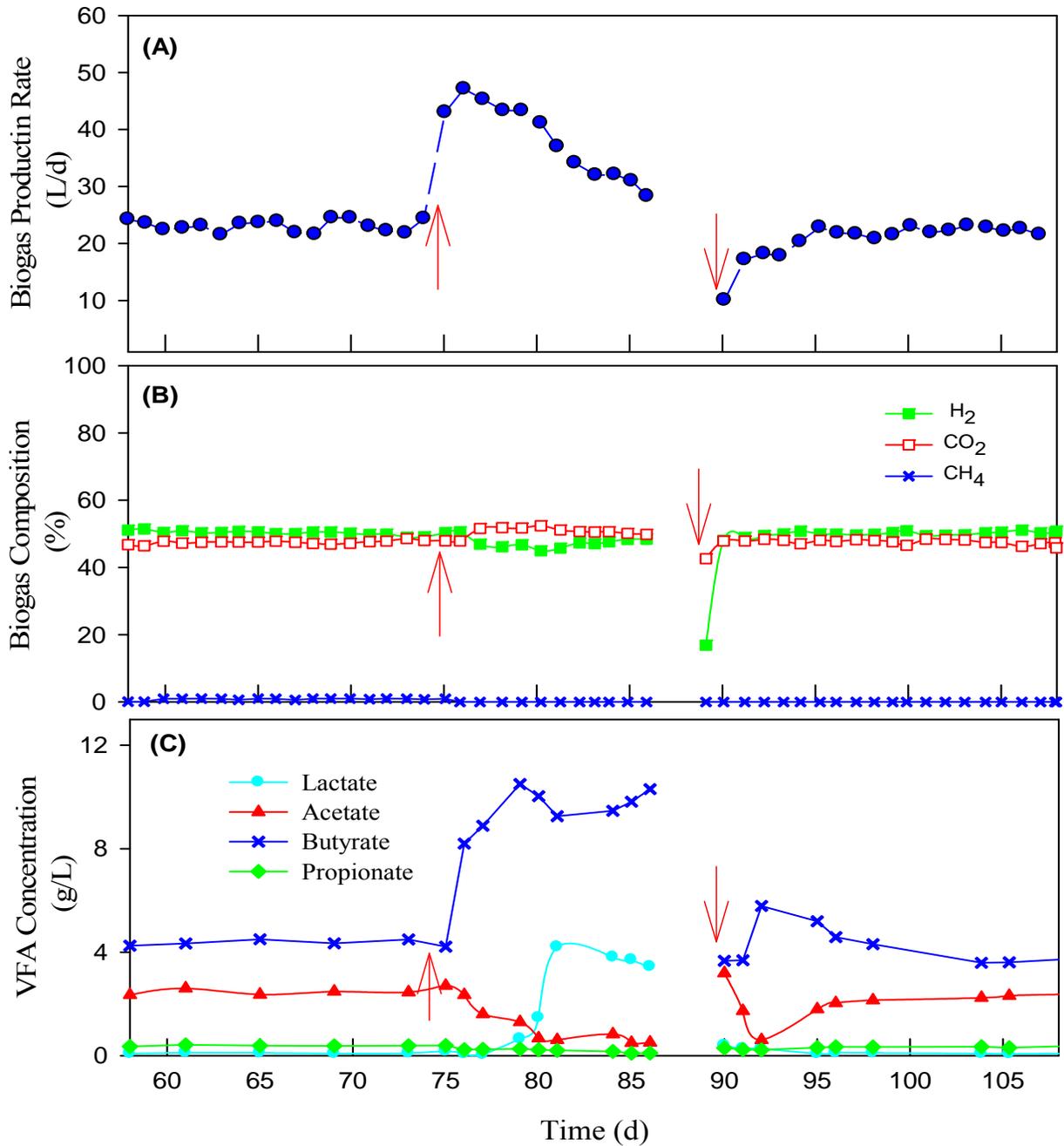


Figure 5-2: (A) Biogas production rate, (B) biogas composition, and (C) VFA concentration before, during, and after increasing the OLR. Arrows pointing up indicate when the OLR was increased, and arrows pointing down indicate when the OLR was decreased back to the previous range. Data for day 88 and 89 are not available due to variably changing of working liquid volume as a result of effluent line clogging.

The average biogas production rate after day 95 was 22.2 L/d which is close to the production rate (23.1 L/d) before the OLR was increased. H₂ concentration rapidly increased from a concentration of 17% on day 89 to a concentration of 48% on day 90. This was followed by a gradual increase to 51% on day 108. As shown in Figure 5-2 C, prior to doubling of the OLR on day 74, butyrate and acetate remained stable at average concentrations of 4.40 g/L and 2.50 g/L, respectively. At the same time, the propionate concentration was also stable at an average concentration of 0.40 g/L, and lactate concentration remained low (< 0.20 g/L). Succinate and formate were not detected. During the first two days after the OLR was increased, the butyrate concentration increased sharply from 4.20 g/L on day 74 to 10.50 g/L on day 76, and remained high at an average concentration of 9.80 g/L until the OLR was subsequently decreased (day 86). The acetate concentration gradually decreased after the OLR was increased, reaching a level of 0.50 g/L by day 86. During the increased OLR period, propionate concentration remained low (<0.30 g/L), and formate and succinate were not detected. As shown in Figure 5-2 C, acetate and butyrate concentration were nearly equal on day 90 (one day after reactor was operated at the “normal” OLR). Subsequently, the butyrate concentration increased and acetate concentration decreased, reaching stable concentrations near those observed prior to the increase in OLR by day 104 (at which time butyrate and acetate concentrations were 4.21 and 2.24 g/L respectively). The low hydrogen concentration and high acetate concentration observed on the day 89 likely reflects the occurrence of homoacetogenesis during the interval when the bioreactor was temporarily operated in batch mode.

5.3.3 Hydrogen Production and Yield

The H₂ production rates before, during, and after the OLR was increased are shown in Figure 5-3. During the first two days after the organic rate was increased (i.e., days 74-76), the

H₂ production rate increased rapidly reaching a peak of 17.63 L/L/d on day 76. This is more than double the average production rate (8.60 L-H₂/L/d) prior to the increase. However, the H₂ production rate progressively decreased over the next 10 days, reaching a rate of 10.84 L/L/d on day 86. As with the biogas production rate (Figure 5-2A), it is evident that H₂ production rate (Figure 5-3) recovered fairly quickly after the reactor was restarted in continuous flow following a period of operation in batch-mode (see Section 5.2). The H₂ production rate reached 6.17 L/L/d on day 91 (two days after continuous flow operation was resumed at the original OLR). It is also evident from Figure 5-3 that H₂ production stabilized after day 111. The average H₂ production rate during the last seven days shown in Figure 5-3 was 8.4 L/L/d which is comparable to the rate prior to increasing the OLR (average of 8.6 L/L/d on days 101 to 107).

The average specific H₂ production rate (L-H₂/g-MLVSS/d) increased from 3.84 to 4.20 after doubling of the OLR, but H₂ yield decreased by 25% from 1.91 mol-H₂/mol-glucose_{consumed} to 1.48 mol-H₂/mol-glucose_{consumed}. Table 5-1 lists the average values for H₂ production rate, specific H₂ production rate, and H₂ yield before, during, and after the increase in OLR.

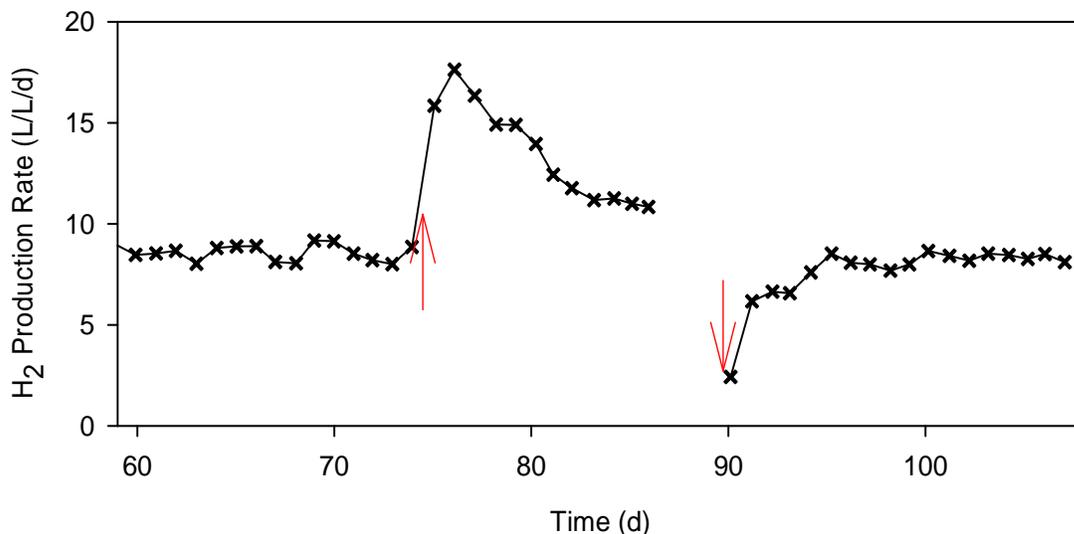


Figure 5-3: Hydrogen production rate before, during, and after increasing of the OLR. Arrows pointing up indicate when the OLR was increased, and arrows pointing down indicate when the OLR was decreased back to previous range. Data for day 88 and 89 is not available due to variably changing of working liquid volume as a result of effluent line clogging.

Table 5-1: Average H₂ production rate, H₂ yield, and specific H₂ production rate before, during, and after increasing the OLR.

Experiment Days Considered	Organic Loading Rate (g-glucose/L/d)	H ₂ Production Rate (L/L/d)	H ₂ Yield (mol-H ₂ /mol-glucose _{consumed})	Specific H ₂ Production Rate (L-H ₂ /g-MLVSS/d)
58-74 (Before Increase)	36	8.60	1.91	3.84
74-86 (During Increase)	76	13.51	1.48	4.20
89-108 (After Increase)	38	8.40	1.64	3.70

*Base on average values

All calculations based on pressure of 1.0 atm and temperature of 22°C.

5.4 Discussion

Overall, increasing the influent molasses and nutrient concentrations, thereby increasing the OLR, increased the H₂ production rate to a maximum of 17.63 L/L/d which is higher than those obtained for similar studies using molasses as a substrate (see Table 4-3). The reactor performance was somewhat unstable, however, and the H₂ production rate decreased over time. Additionally, the percentage of the influent sugar consumed in the bioreactor decreased from 94% prior to the increase in OLR to 89.7% during the interval of the higher OLR. The high concentrations of butyrate (see Figure 5-2 C) in the reactor at the elevated OLR may have substantially affected H₂ production bacteria. The emergence of higher concentration of lactate is indicative of a metabolic shift and/or a change in microbial community structure. Van Ginkel and Logan (2005) tested the effect of butyrate and acetate concentration in a continuous-flow reactor. They found that a butyric acid concentration of 25 mM (pH=5) in the feed decreased H₂ yield by 22% and a concentration of 60 mM (pH=5) in the feed decreased H₂ yield by >93% (undissociated acid concentrations). They also reported that a concentration of 19 mM of self-produced acids [through fermentation] significantly decreased H₂ yield and caused solventogenesis (i.e., production of acetone, butanol, and other alcohols). Because acetone, ethanol, butanol, and other alcohols were not measured, it is impossible to determine if that was the case in the in the study reported herein. Nevertheless, it is plausible that the high butyrate

concentration measured during the experiment (peak of 10.50 g/L on day 79 and average of 9.80 g/L thereafter, corresponding to 111.36 mM) had an inhibitory effect on H₂ production and caused subsequent solvent and lactate formation. Moreover, methanogenic activity was also affected by the increase in OLR; methane decreased from an average of 0.8% prior to increasing OLR to undetectable levels after the OLR was increased.

In terms of COD (calculated using a regression equation from the scatter-plot of COD vs. glucose concentration for this experiment, see Appendix A), the average OLR was increased from 51.53 g-COD/L/d to 109.36 g-COD/L/d which corresponded to an increase in glucose loading rate from 36 g-glucose/L/d and 76 g-glucose/L/d, respectively (Table 5-1). Ren *et al.* (2006) found that H₂ yield increased with an increase in OLR rate within the range of 3.11-68.21 kg-COD/m³/d, but H₂ yield decreased at higher OLRs (68.21-85.57 kg-COD/m³/d). The elevated OLR in the present experiment (109.36 kg-COD/m³/d) was above the threshold OLR for which H₂ yield was adversely affected in the Ren *et al.* (2006) study.

5.5 Conclusions

The results from this experiment indicate that increasing the target OLR from 36 g-glucose/L/d to 76 g-glucose/L/d in a previously functionally-stable H₂-producing reactor fed with molasses resulted in a H₂ production rate (17.6 L/L/d) which is higher than that obtained in previous studies using molasses as a substrate. The peak H₂ production rate was relatively short-lived, however, and after reaching the maximum production rate within two days of the start of the higher OLR, production rates progressively decreased. Experimental results indicate that increasing the OLR led to 25% decrease in H₂ yield. The potential benefits of elevated OLR in full-scale H₂ production facilities, thus, will likely depend on whether it is preferable to maximize the rate of H₂ production or the H₂ yield. As a final observation, despite not feeding the bioreactor for two days (during which time the reactor was operated in batch-mode),

subsequent operation of the reactor after resuming the base-line OLR resulted in stable H₂ production rates comparable to those observed prior to the increase in OLR. This provides a general indication that the process is relatively robust and able to recover from minor disruptions in substrate supply.

CHAPTER 6: THE EFFECT OF ELEVATED 1,2-DICHLOROETHANE CONCENTRATIONS ON H₂ PRODUCTION IN A CSTR

6.1 Introduction

It has been reported previously that some bacterial species in the genus *Clostridium* are able to produce H₂ even in the presence of high concentrations of 1,2-dichloroethane (1,2-DCA) (Bowman *et al.*, 2009; Bowman *et al.*, 2010a). Experiments to assess the ability of mixed cultures to fermentatively produce H₂ in the presence of high concentrations of 1,2-dichloroethane (1,2-DCA), however, have not been reported previously. The objective of the study described in this chapter was to determine the effects of 1,2-DCA concentrations on the rate and yield of H₂ production by the mixed-culture present in the H₂-producing CSTR fed with molasses solution operated at pH 5.5. Thus, after conclusion of the experiments described in Chapter 5, operation of the pH 5.5 bioreactor was continued but with 1,2-DCA supplied in the influent feed.

6.2 Materials and Methods

The bioreactor configuration for this experiment was identical to that employed for the continuous-flow experiments described in Chapter 4. Preparation of the nutrient-amended molasses feed solution was as described in Chapter 4 (i.e., same molasses and nutrient concentrations), with the exception that after preparation, a sterile magnetic stir bar was placed inside the feed bottle, and 1,2-dichloroethane (Sigma Aldrich, ≥ 99.8 %) was added using a sterile syringe and needle. The feed solution bottle was then tightly capped, placed on top of a magnetic stir plate, and mixed for 12-24 hours prior to use.

The experiment to evaluate effects of 1,2-DCA on the bioreactor's performance was performed in two phases. During first phase of testing, the volume of 1,2-DCA added to 3.5 L of feed solution on a daily basis was 5.6 mL (added to 3.9 L bottle containing 0.4 L gas headspace).

The resulting target aqueous-phase 1,2-DCA concentration was 1,996 mg/L (20.25 mM), calculated as described in Appendix B. Feed of this solution to the bioreactor commenced on day 108 (June 25, 2020) and ended on day 111 (June 28, 2010). The duration of the interval during which this 1,2-DCA amended feed was supplied in the reactor influent was 3.29 days (79 hours). From day 111 (June 28, 2010) to day 114 (July 1, 2010), the reactor was fed with nutrient-amended molasses feed solution as described in Chapter 4 (i.e., same molasses and nutrient concentrations) but without any 1,2-DCA.

During second phase of testing, the volume of 1,2-DCA added to 3.5 L of feed solution on a daily basis was 11.2 mL (added to 3.9 L bottle containing 0.4 L gas headspace). The resulting target aqueous-phase 1,2-DCA concentration was 3,992 mg/L (40.5 mM), calculated as described in Appendix B. Feed of this solution to the bioreactor commenced on day 114 (July 1, 2010) and ended on day 116 (July 3, 2010), at which time the experiment was terminated. The total duration of feeding 1,2-DCA in the second phase of testing was 2.17 days (52 hours).

Concentrations of 1,2-DCA in the liquid influent and effluent were measured via gas chromatography as described by Yan *et al.* (2009). Concentrations of ethene, ethane, and chloroethane (potential products of reductive dechlorination of 1,2-DCA), were measured in gas headspace samples using gas chromatography as described by Yan *et al.* (2009). All other analytical methods (e.g., sugar concentration, pH, biogas composition, and biogas production rate) were identical to those described in Chapter 4.

6.3 Results

6.3.1 1,2-DCA Concentrations

Table 6-1 lists the days on which the reactor was fed with molasses solution containing 1,2-DCA and the experimentally determined concentrations of 1,2-DCA in the molasses solution immediately prior to the start of feeding and immediately after the bottle was replaced with a

freshly prepared bottle of feed solution on a daily basis. As shown in the table, the 1,2-DCA concentration in the feed during phase 1 testing was close to but slightly less than the target aqueous-phase concentration of 1,996 mg/L. The average 1,2-DCA concentration in the feed, calculated as the average of the start and end concentration for the three days that the 1,2-DCA was supplied during phase 1 testing, was 1797 mg/L. The average 1,2-DCA concentration in the feed during phase 2, calculated in the same manner, was 3742 mg/L, close to but slightly less than the target concentration of 3,992 mg/L.

Figure 6-1 shows the experimentally measured 1,2-DCA concentrations in the bioreactor’s effluent during the phase 1 and phase 2 experiment. The red arrows denote the beginning of the first and second periods of feeding the reactor with 1,2-DCA. The blue arrow denotes the beginning of the period during which 1,2-DCA was not fed to the reactor. Also shown in Figure 6-1 (green line) is the 1,2-DCA concentration predicted from mass balance calculations (see Appendix C for equations and assumptions used to construct mass balance).

Table 6-1: Days on which the reactor was fed with molasses solution containing 1,2-DCA and the concentration of 1,2-DCA in the influent molasses solution immediately before feeding and immediately after feeding.

Experimental Phase	Day Fed with 1,2-DCA	Date Fed with 1,2-DCA	1,2-DCA Concentration (mg/L)	
			Start	End
1	108	6/25/2010	1888	1905
	109	6/26/2010	1611	1870
	110	6/27/2010	1912	1595
2	114	7/1/2010	4119	3444
	116	7/2/2010	4058	3346

For the first and second 1,2-DCA addition periods, it can be seen from Figure 6-1 that there was an initial rapid increase in 1,2-DCA concentration in the bioreactor’s effluent after the feed of 1,2-DCA-containing solution began. The effluent 1,2-DCA concentrations reached maximum levels of 1520 mg/L and 3107 after 24 and 22 hours after the commencement of the

first and second period, respectively. After the first 24 hours, the effluent 1,2-DCA concentrations did not increase but were measured at variable concentrations. The cause for variable concentrations in the effluent cannot be conclusively determined from the data collected. A likely cause, however, is that a fraction of the 1,2-DCA likely volatilized from the liquid phase to gas phase prior to introduction into the bioreactor (resulting in a decrease in the feed-bottle aqueous-phase concentration over time throughout each day). Furthermore, the model concentration shown in Figure 6-1 also did not account for partitioning of 1,2-DCA into the reactor's gas headspace or biogas exiting the reactor (see Appendix C). The concentrations of potential dechlorination products (ethene, ethane, and chloroethane) were measured in the gas-phase two times during the experiment. In both cases, ethene, ethane, and chloroethane were not detected, suggesting that reductive dechlorination was not occurring in the bioreactor.

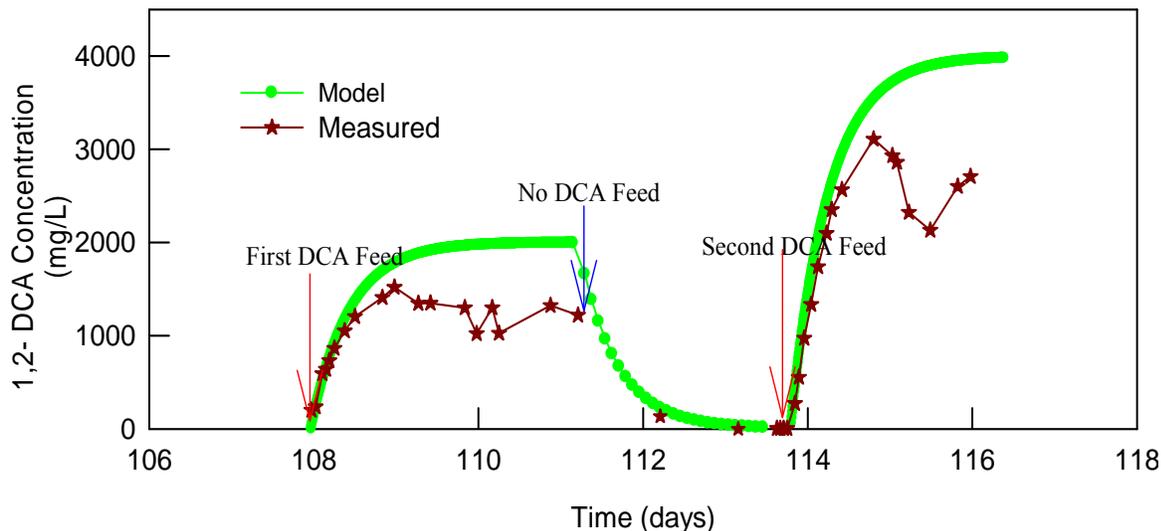


Figure 6-1: 1,2-DCA concentration as a function of time. Red arrows denote the beginning of the first and second period of feeding with molasses solution containing 1,2-DCA. The blue arrow indicates when the reactor was fed with molasses solution without 1,2-DCA.

6.3.2 Sugar Consumption and Biomass Concentration

Figure 6-2 shows the influent and effluent sugar concentrations and the effluent biomass concentration (measured as VSS) during the experiment along with the effluent 1,2-DCA concentration. During the entire experiment, the influent sugar concentration remained stable

between 17-18 g-glucose/L, averaging 17.6 g-glucose/L. As shown in the figure, sugar consumption remained relatively constant in spite of the high 1,2-DCA concentrations. The effluent sugar concentration ranged from 0.94 to 1.11 g-glucose/L, averaging .098 g-glucose/L.

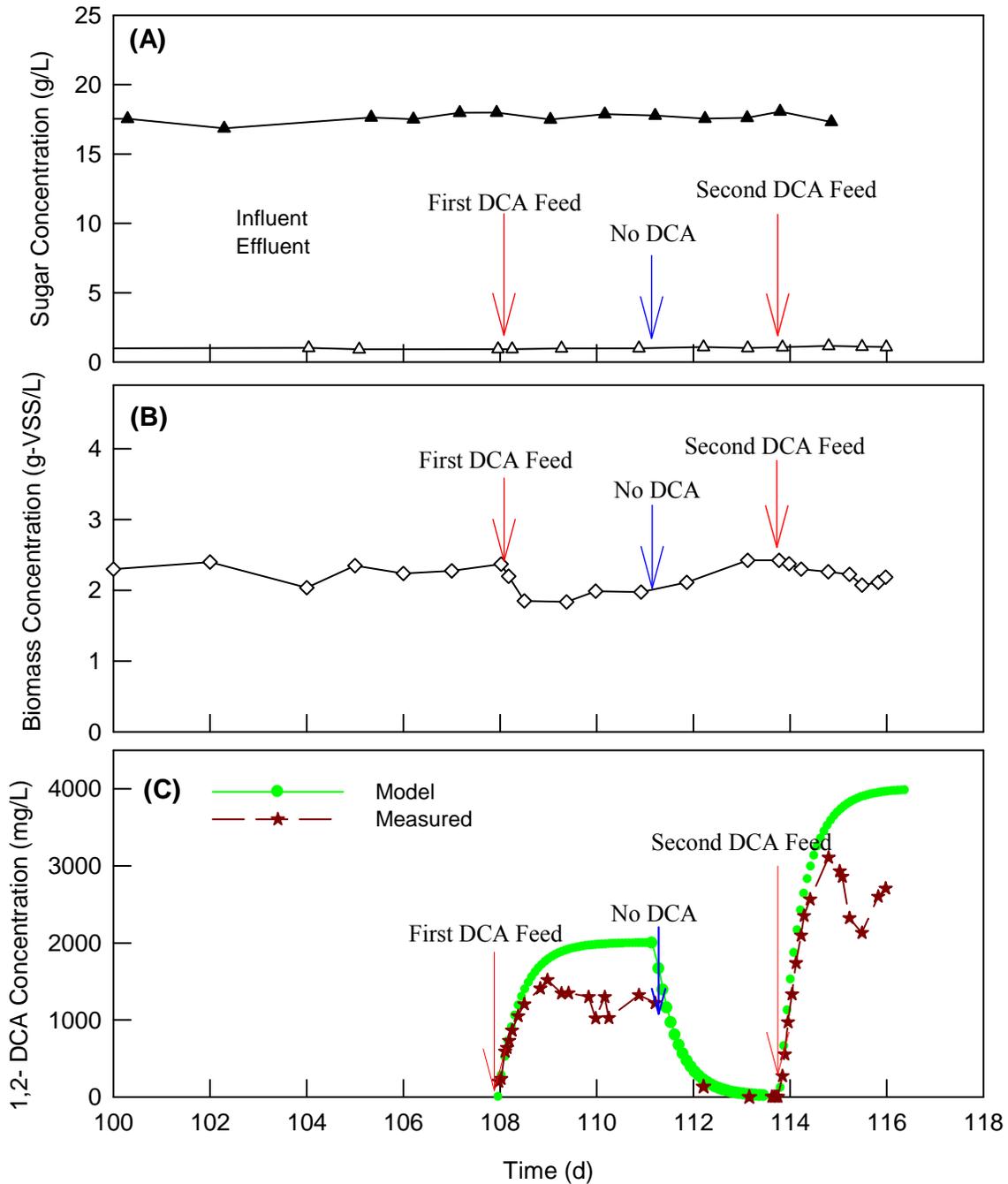


Figure 6-2: The (A) influent and effluent sugar concentrations, (B) effluent biomass concentration, and (C) concentrations of 1,2-DCA. Red arrows denote the beginning of the first and second period of feeding with molasses solution containing 1,2-DCA. The blue arrow indicates when the reactor was fed with molasses solution without 1,2-DCA.

During the first 1,2-DCA addition period, the biomass concentration decreased from 2.37 g-VSS/L to 1.85 g-VSS/L over the time interval when the 1,2-DCA concentration increased from 0 to 1200 mg/L (12 hours after commencement of first period). Thereafter, the biomass concentration increased slightly and reached 2.00 g-VSS/L by the end of the first 1,2-DCA addition period. After 1,2-DCA was temporarily omitted from the feed, the biomass concentration slowly increased and reached 2.43 g-VSS/L before the reactor was fed for a second time with molasses solution containing 1,2-DCA. During the second 1,2-DCA addition period, a decrease in biomass concentration was observed but to a lesser degree than what was observed during the first period. During the first 48 hours of the second 1,2-DCA addition period, the biomass concentration decreased from 2.43 g-VSS/L to a low of 2.10 g-VSS/L, and it remained nearly the same the following 12 hours when the experiment ended.

6.3.3 Gas Production, Biogas Composition, and Volatile Fatty Acids

Figure 6-3 shows the biogas production rate, biogas composition, VFA concentration, and the concentration of 1,2-DCA during the experiment. The biogas production rate increased during the first period of feeding with 1,2-DCA, it decreased during the interval when 1,2-DCA was omitted from the feed, and subsequently increased during the second period of feeding with 1,2-DCA at a higher concentration. As shown in Figure 6-3 B, the H₂ and CO₂ concentrations in the effluent gas remained stable during the experiment at average concentrations of 52% and 47%, respectively. Methane was not detected prior to or during the experiment.

As shown in Figure 6-3 C, the distribution of VFAs remained unchanged from the first period of 1,2-DCA addition to the end of the period in which 1,2-DCA was temporarily omitted from the influent (indicated by the second red arrow). During this interval, the average butyrate, acetate, propionate, and lactate concentrations were 4.01 g/L, 2.4 g/L, and 0.33 g/L, and 0.1 g/L, respectively. Formate and succinate were measured at very low concentrations (< 0.05 g/L).

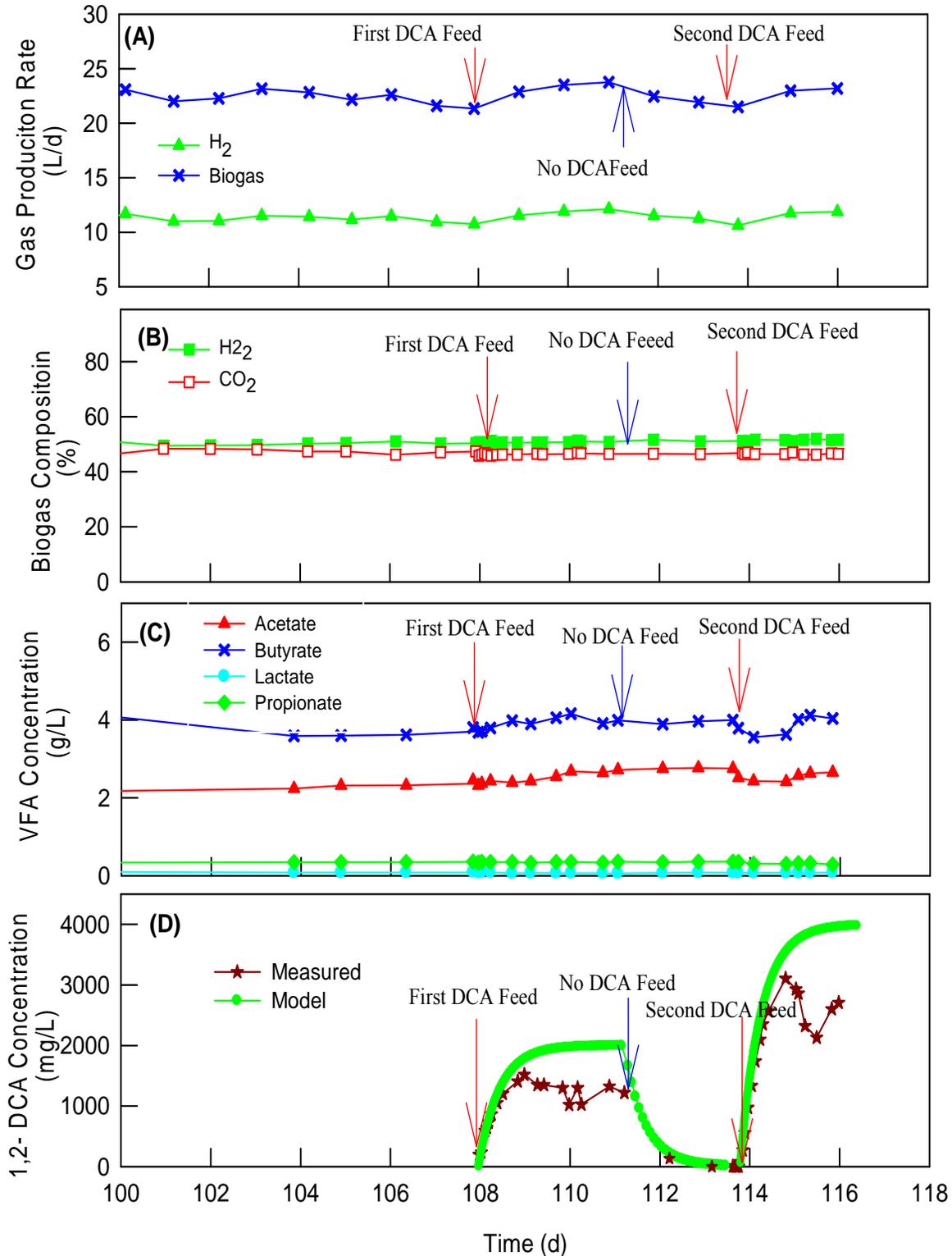


Figure 6-3: (A) Biogas and hydrogen production rate, (B) biogas composition, and (C) VFA concentrations before, during, and after the intervals of feeding elevated concentrations of 1,2-DCA. Red arrows denote the beginning of the first and second period of feeding with molasses solution containing 1,2-DCA. The blue arrow indicates when the reactor was fed with molasses solution without 1,2-DCA.

Following the first six hours of the second 1,2-DCA addition period, VFAs also remained unchanged. However, when the 1,2-DCA concentration inside the reactor (i.e., as measured in the effluent) reached approximately 1500 mg/L, there was a noticeable decrease in butyrate concentration from 4.04 g/L to 3.56 g/L and a decrease in acetate concentration from 2.76 g/L to 2.43 g/L. All other VFAs remained essentially unchanged. Butyrate and acetate concentrations remained at the lower concentration the following 17 hours during which the 1,2-DCA concentration increased from 1500 mg/L to peak a concentration for the experiment at 3107 mg/L. After dropping below peak concentration, butyrate and acetate concentrations increased back to previous concentrations.

6.3.4 Hydrogen Production and Yield

The H₂ production rate followed a trend identical to that of biogas production rate since biogas composition remained unchanged as shown on Figure 6-3 A. Table 6-2 lists the H₂ production rate, H₂ yield, and the specific H₂ production rate prior to and during the 1,2-DCA addition experiments. The H₂ yield remained unchanged, and H₂ production increased during the periods of feeding with 1,2-DCA. The specific production during the second period was less than the first period of feeding with 1,2-DCA, but it was higher than the period during which 1,2-DCA was not fed (i.e. before first period) and the interval between 1,2-DCA additions.

Table 6-2: H₂ production rate, H₂ yield, and specific H₂ production rate during periods of feeding with 1,2-DCA and without 1,2-DCA.

Period of 1,2-DCA Feeding	Time (d)	Production Rate (L-H ₂ /L/d)	Yield (mole-H ₂ /mole-glucose _{consumed})	Specific Production Rate (L-H ₂ /g-MLVSS/d)
¹ No 1,2-DCA feeding	105-108	8.22	1.7	3.57
First Period	108 - 111.21	8.82	1.8	4.25
Purging Period	111.21 - 113.8	8.22	1.6	3.54
Second Period	113.8 – 116	8.74	1.8	3.97

*All calculations based on pressure of 1 atm and temperature of 22°C.

¹Calculations are from the previous three days prior to first period of feeding with 1,2-DCA.

6.4 Discussion

During the periods of feeding with 1,2-DCA, it was observed that the sugar consumption and gas H₂ concentration remained essentially constant while the average H₂ production rate slightly increased and biomass concentration decreased. Consequently, the specific H₂ production rate (H₂ production rate divided by VSS concentration) increased, especially during the first 1,2-DCA addition period. A likely explanation for these observations is that H₂-producing bacteria may be unaffected or at least less adversely affected by high concentrations of 1,2-DCA than non-H₂-producing bacteria. An alternative explanation for the decrease in biomass concentration is that the microbial population may have had a lower net yield of biomass at high 1,2-DCA concentrations. Regardless of the exact reason, for the first period of feeding 1,2-DCA, an appreciable decrease in biomass concentration was observed when 1,2-DCA concentration reached ≥ 1200 mg/L. This may be due to the fact that bacteria unable to survive at high 1,2-DCA concentrations died, were inhibited, or were washed out of the system, leaving a smaller bacterial population better adapted for survival at high 1,2-DCA concentrations. Consequently, during the second period of feeding with 1,2-DCA at nearly double the 1,2-DCA concentration, a less drastic decrease in biomass concentration was observed, and biomass concentration during this period did not reach levels as low as during the first period of feeding with 1,2-DCA. Volatile fatty acids remained stable during the experiment except during a short period (17 hours) when butyrate and acetate concentrations decreased temporarily but subsequently increased. This observation cannot be explained as there was no clear correlation with 1,2-DCA concentration.

6.5 Conclusions

It was demonstrated that introducing elevated concentrations of 1,2-DCA on a functionally-stable H₂-producing CSTR fed with molasses and which was previously inoculated chlorinated-solvent containing groundwater did not negatively affect H₂ production rate or yield.

In fact, the results obtained in this study showed that H₂ production rate and yield may have positively been affected by introduction of 1,2-DCA into the reactor medium. At least a portion of the H₂-producing population was able to produce H₂ at elevated concentrations of 1,2-DCA.

CHAPTER 7: OVERALL CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH

The objective of batch-mode experiments in this study was to characterize the hydrogen production potential of the microbial consortium contained in the groundwater collected from the Scenic Highway portion of the PPI Site and to gain insights into the rate at which molasses may be fermented if additional molasses were to be injected into the subsurface. Under ambient laboratory temperature ($\approx 22^{\circ}\text{C}$) and at initial molasses concentrations equivalent to 6.5-7.0 g-glucose/L, results showed that a target pH of 5.5 produced the highest volume of H_2 (3.51 L) and the highest H_2 yield (1.47 mol- H_2 /mol-glucose_{consumed}). Homoacetogenic and methanogenic activity was not noticeable at this pH. It is therefore concluded that if additional molasses were injected at the Scenic Highway portion of the PPI Site, the highest H_2 would likely be produced at a pH 5.5. In bioreactor experiments at higher pH values (6.5 and 7.5), lower H_2 production volumes and yields were observed. Increasing acetate concentrations concomitant with decreasing H_2 concentrations and gas headspace pressure suggests that this was due to homoacetogenesis. At the lowest experimentally tested pH (pH 4.5), the H_2 production rate was slow, the period biogas production was the longest, and the rate of sugar consumption was remarkably slower. Slow degradation of substrate (i.e., molasses) and long-lasting H_2 production at low rates, however, could be advantageous for full-scale injection of molasses since substrate would be available over a larger area, and consequently, H_2 required to drive dechlorination reactions would also be available over a larger area. The utility of this approach, however, depends on the ability of reductive dechlorinating bacteria to function at much lower pH values.

The objective of the first set of continuous-flow experiments presented in this thesis (chapter 4) was to assess the potential of using the microbial communities contained in Scenic Highway portion of the PPI Site in a CSTR for production of H_2 as a renewable energy source.

Batch-mode experiments described in the first part of this study were extended for operation in a CSTR at a temperature of 35°C and with the same pH target value. The H₂ production capacity for each experiment was assessed in terms of production rate, yield, specific yield, and stability. The experiment operated at a pH of 5.5 displayed the highest H₂ yield (1.93 mol-H₂/mol-glucose), production rate (8.68 L-H₂/L⁻¹d⁻¹), and stability. Bioreactor performance rapidly recovered following a period during which the reactor pH temporarily decreased and a period during which the reactor was not fed for over two days. At this pH value (5.5), the H₂ concentration in the reactor headspace remained essentially constant near 50% (v/v), and only trace levels of methane were detected (< 1%). At the higher pH values (i.e., 6.5 and 7.5), the initially observed H₂ production rates decreased with time due net consumption of H₂ via homoacetogenesis, and methane concentrations were observed to increase with time. At a pH of 4.5, H₂ production rates did not decrease with time but production rates were relatively low. At this pH, fermentative bacteria grew at comparatively slow rates as indicated by the low sugar consumption rate and biomass concentration.

The organic loading rate (OLR) was increased on the stable H₂-producing CSTR operated at a pH of 5.5 (presented in the first part of the continuous-flow experiments) and the effects were assessed in terms of H₂ production rate, yield, and stability. After increasing the target OLR from 36 g-glucose/L/d to 76 g-glucose/L/d, the H₂ production rate increased during the first two days to a maximum of 17.6 L/L/d which is among the highest observed for continuous-flow studies using molasses as a substrate (see Table 2-2). This high H₂ production rate was not sustained, however, and a gradual decrease in H₂ production rates was observed, reaching 10.8 L/L/d on the final day of the elevated OLR experiment. The decrease in H₂ production rate likely resulted from inhibition caused by accumulation of high concentration of butyrate (10.5 g/L peak and 9.8 g/L average).

The final objective of this study was to assess the impact of feeding elevated concentrations of 1,2-DCA on a functionally-stable H₂-producing CSTR. The effects of elevated 1,2-DCA were tested in two phases with an average 1,2-DCA concentration of 1,797 mg/L and 3,472 mg/L for Phase 1 and Phase 2, respectively. For both phases, there was no observable negative effect on H₂ production and yield. In fact, the results showed that high concentrations of 1,2-DCA may have positively affected H₂ yield and rate (see Table 6-2). This implies that at least a portion H₂-producing population was able to produce H₂ at such elevated 1,2-DCA concentrations.

During the first set of continuous-flow experiments, a pH of 5.5 was determined to favor high H₂ production and yield. However, other parameters like temperature and hydraulic retention time, which were not experimentally varied in the present studies, have been shown to affect H₂ production rate and yield. Thus, using the same substrate and inoculum as in this study, it is possible that variation of such parameters in a CSTR at a pH of 5.5 could result in even higher H₂ production rates and yield than were experimentally observed here. Moreover, a more modest incremental increase in OLR, rather than a large increase as tested in experiments described in chapter 5, may also result in higher H₂ production rates and yield.

Because geochemical properties and contaminant concentrations differed among the inocula used for each experiment, at least to some degree, it would be valuable to conduct experiments identical to those described in this study but with multiple replicates and multiple pH values. Also, microbial analysis of samples collected during the experiments may give insight as to which bacteria are prevalent under the various bioreactor operating conditions tested.

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APPENDIX A: DETERMINATION OF OLR IN TERM OF COD DURING INCREASED OLR EXPERIMENT

Data from days on which both COD and sugar concentrations were measured in the molasses feed solution (Table A-1) were used to construct a scatter plot. The dependent variable (y) was COD concentration, and the independent variable (x) was sugar concentration. Figure A-1 shows the resulting scatter plot and the equation of the regression line. As shown on the figure, the average OLR in terms of glucose concentration was multiplied by 1.431 to obtain the OLR in terms of COD concentration.

Table A-1: Data used to construct scatter plot for COD to glucose ratio.

Date	Experiment Day	COD (g/L)	Sugar (g-glucose/L)
5/12/2010	64	24.69	16.65
5/21/2010	73	26.89	15.37
5/24/2010	76	53.59	35.89
5/28/2010	80	52.95	35.89
6/1/2010	84	51.98	34.53
6/6/2010	89	26.59	18.01
6/13/2010	96	27.08	18.63
6/17/2010	100	27.60	17.53
6/22/2010	105	26.38	17.64
6/24/2010	107	26.87	19.85

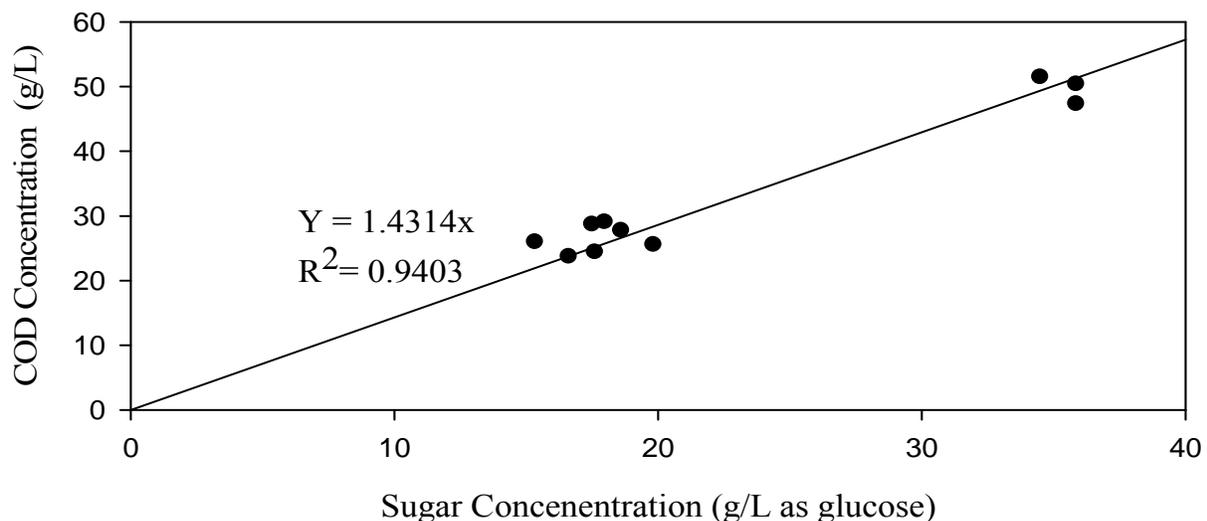


Figure A-1: Scatter plot and linear regression for measured sugar concentration and the corresponding COD concentration.

APPENDIX B: CALCULATION OF 1,2-DCA CONCENTRATIONS IN THE FEED SOLUTION FOR EXPERIMENTS DESCRIBED IN CHAPTER 6

The total mass of 1,2-DCA added to each feed bottle can be calculated using eqn. B-1.

$$\dot{M}_{1,2-DCA} = \rho_{1,2-DCA} \times V_{1,2-DCA} \quad (\text{B-1})$$

Where: $\dot{M}_{1,2-DCA}$ = mass of 1,2-DCA added to the bottle

$\rho_{1,2-DCA}$ = density 1,2-DCA (1.253 g/mL),

V_{DCA} = volume of 1,2-DCA added (5.6 mL for Phase 1, and 11.2 mL for Phase 2)

Substituting the measured volumes of 1,2-DCA added per feed bottle (5.6 mL in Phase 1 and 11.2 mL in Phase 2) into eqn. B-1, the calculated mass of 1,2-DCA added per bottle of feed was 7.02 g for Phase 1 and 14.03 g for Phase 2. Based on the principle of mass balance, assuming no other sources or sinks for 1,2-DCA, the total mass 1,2-DCA in the feed bottle would be equal to the mass of 1,2-DCA in the gas-phase plus the mass 1,2-DCA in the aqueous-phase as indicated by eqn. B-2.

$$\dot{M}_{1,2-DCA} = C_{aq}V_{aq} + C_gV_g \quad (\text{B-2})$$

Where: C_{aq} = concentration of 1,2-DCA in the aqueous-phase

V_{aq} = volume of the aqueous-phase (3.5 L)

C_g = concentration of 1,2-DCA in the gas-phase

V_g = volume of the gas-phase (0.4 L)

There are two unknowns in eqn. B-2 (C_{aq} and C_g). To solve, a second equation is needed to relate the gas-phase and aqueous-phase concentrations. Assuming that equilibrium was achieved between the gas-phase and aqueous-phase and assuming that the partitioning between the two phases is the same as the partitioning between air and water, the gas-phase concentration can be calculated from Henry's Law (eqn. B-3).

$$K_H = \frac{C_g}{C_{aq}} \quad (\text{B-3})$$

Where: K_H = Dimensionless Henry's Law Constant (dimensionless)

C_{aq} = concentration of 1,2-DCA in the aqueous-phase (mg/L)

C_g = concentration of 1,2-DCA in the gas-phase (mg/L)

Assuming the value of K_H at 22°C to be 0.0349 (from the U.S. EPA Office of Solid Waste and Emergency Response method, <http://www.epa.gov/ATHENS/learn2model/part-two/onsite/esthenry.html>), and rearranging eqn. B-3 to solve for the gas-phase concentration results in eqn. B-4.

$$C_g = K_H \times C_{aq} = 0.0349 C_{aq} \quad (\text{B-4})$$

Substituting this expression into the overall mass balance equation (eqn. B-2) and solving for C_{aq} results in eqn. B-5.

$$C_{aq} = \frac{\dot{M}_{1,2-DCA}}{K_H V_g + V_{aq}} = \frac{\rho_{1,2-DCA} \times V_{1,2-DCA}}{K_H V_g + V_{aq}} \quad (\text{B-5})$$

Substituting parameter values into this expression results in calculated target aqueous-phase concentrations of 1,996 and 3,992 mg/L 1,2-DCA in Phase 1 and Phase 2 experiments, respectively. In the alternative units of millimoles per liter, the target 1,2-DCA concentrations were 20.2 and 40.3 mM for Phases 1 and 2, respectively.

APPENDIX C: CALCULATION OF 1,2-DCA CONCENTRATIONS IN THE EFFLUENT FOR EXPERIMENTS DESCRIBED IN CHAPTER 6

As an estimate of the 1,2-DCA concentration expected in the bioreactor's effluent during experiments described in chapter 6, the bioreactor was modeled as an ideal continuous flow stirred tank reactor (CSTR) without recycle. It was assumed that 1,2-DCA did not undergo any reactions or sorb to the bioreactor components or biomass, and it was assumed that 1,2-DCA did not transfer to the gas-phase of the bioreactor's headspace. During the period immediately after the start of feed of molasses solution amended with 1,2-DCA, the general form of the mass balance equation for 1,2-DCA is shown by eqn C-1.

$$\frac{dC}{dt}V + C_{out}Q_{out} = C_{in}Q_{out} \quad C-1$$

Where: C = concentration of 1,2-DCA inside the reactor

C_{out} = concentrations of 1,2-DCA in the liquid effluent

C_{in} = concentration of 1,2-DCA in the molasses feed solution

$Q_{out} = Q_{in}$ = flow rate in and out of the reactor (2.97 L/d)

V = reactor liquid volume (1.35 L)

Solving for concentration inside the reactor as a function of time yields eqn. C-2:

$$C(t) = C_{in} - \frac{C_{in}}{e^{(Q/V)t}} \quad C-2$$

Substituting for the calculated concentration of 1,2-DCA in the molasses feed solution (C_{in}) during Phase 1 and Phase 2 as determined in Appendix B (1,996 mg/L in Phase 1, 3,992 mg/L in Phase 2), the change in 1,2-DCA concentration during Phase 1 and Phase 2 are represented by eqn. C-3 and C-4, respectively. The resulting curve is shown in Figure C-1.

$$C(t) = 1,996 \frac{mg}{L} - \frac{1,996 \frac{mg}{L}}{e^{\left(\frac{2.97 \frac{L}{d}}{1.35 L}\right)t}} \quad C-3$$

$$C(t) = 3,992 \frac{mg}{L} - \frac{3,992 \frac{mg}{L}}{\left(\frac{2.97 \frac{L}{d}}{1.35 L} \right) t} \quad C-4$$

During the period of feeding with molasses solution without 1,2-DCA (i.e., the interval between Phase 1 and Phase 2 1,2-DCA additions), the general form of the mass balance equation for 1,2-DCA as shown by eqn C-1. Given that $C_{in} = 0$ and solving for the concentration of 1,2-DCA inside the reactor with respect to time results in eqn. C-5.

$$C(t) = C_0 e^{-\left(\frac{Q}{V}\right)t} \quad C-5$$

Where: C_0 = concentration of 1,2,-DCA in the bioreactor immediately prior to the start of feeding with molasses solution without 1,2,-DCA (i.e., the end of Phase 1). Using eqn. C-3 formulated for Phase 1, the value of C_0 is 1,993 mg/L. Substituting in for C_0 in eqn. C-5 yields eqn. C-6. The resulting model curve is shown on Figure C-1 along with the experimentally determined measurements.

$$C(t) = 1,993 \frac{mg}{L} e^{-\left(\frac{2.97L/d}{1.35L}\right)t} \quad C-6$$

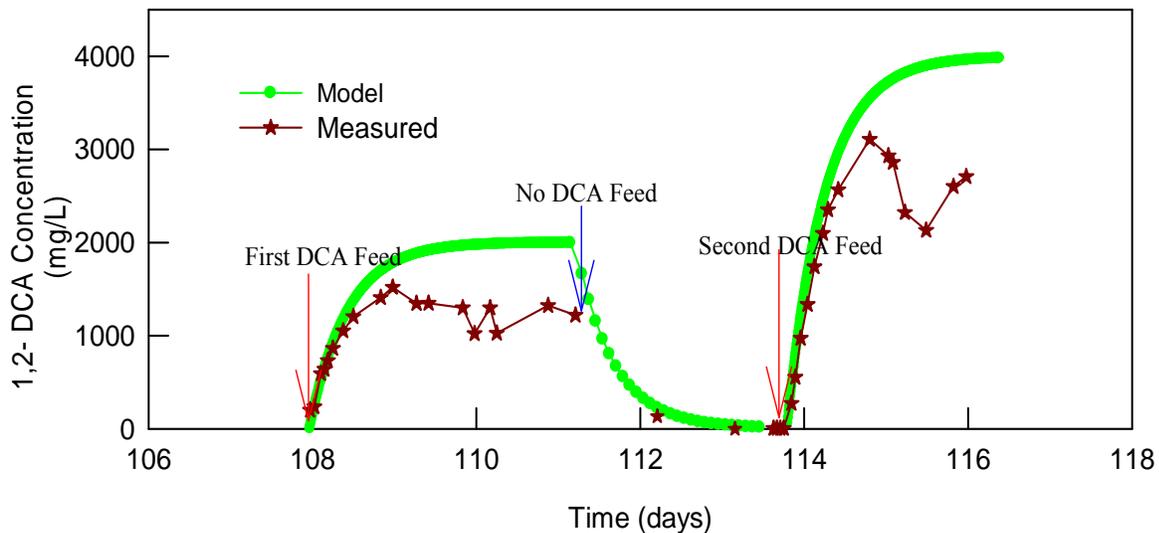


Figure C-1: Theoretical and experimentally determined 1,2-DCA concentrations during feeding of molasses solution containing 1,2-DCA.

VITA

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