9-11-2019 10:00 AM

Evaluation of Biohydrogen Production from Co-fermentation of Carbohydrates and Proteins

Emmanuel Andrew Tepari Jr,, *The University of Western Ontario*

Supervisor: Nakhla, George, *The University of Western Ontario*

A thesis submitted in partial fulfillment of the requirements for the Master of Engineering Science degree in Chemical and Biochemical Engineering

Abstract

The aim of this study was to examine volatile fatty acid (VFA) production from a proteinaceous substrate, bovine serum albumin (BSA) for a pH range of 5 – 9, and to further assess its impact on hydrogen production in a co-fermentation process using starch and BSA at different ratios. The established optimum conditions for VFA production from BSA were an initial pH of 8, incubation time of 3 days and operation temperature of 37 ℃. Using these fermentation conditions, the stoichiometric reactions that describe the anaerobic degradation of BSA were investigated. A methodology that describes organic acid production from BSA by using a single stoichiometric reaction was developed. With the amino acid content of BSA and by selecting the dominant amino acid fermentation reaction pathways, it was feasible to determine the stoichiometric coefficients of the dominant VFA in the single reaction step. Hydrogen production from the co-fermentation of starch and BSA in batch system was studied for five different ratios (C1 – C5). The co-fermentation process had a synergistic impact on hydrogen production and the optimum ratio occurred at C4 (80% starch + 20% BSA) with a hydrogen yield of 350 $mLH_2/gCOD_{added}$ which was 38% higher than the expected. MINITAB-16 was used for data analysis, 3D contour diagrams and response (VFA, ammonia and hydrogen) optimizations for C4 (80% starch + 20% BSA) were developed. The regression analysis of the responses adequately followed second-order polynomial models. The optimum concentration range for VFA and ammonia at which pH control was not necessary obtained from the Box-Behnken design were respectively 125 – 133 mg/L and 41 – 47 mg/L. Thus, the fermentative hydrogen production process would be feasible without pH control at a carbohydrate-to-protein COD ratio of 4:1.

Keywords: Biohydrogen; protein degradation; co-fermentation; volatile fatty acids; fermentation pathways; starch; BSA

Summary for Lay Audience

Presently, most of the global energy demand is met with fossil fuels which are rapidly depleting. In addition, fossil fuels produce greenhouse gases on combustion and contribute to climate change, global warming, and ozone layer depletion. There have been considerable efforts towards the development of biofuels that will be sustainable to meet the dual challenges of meeting future energy demands and also minimizing adverse environmental impacts. Biohydrogen can provide a solution to the aforementioned concerns as a sustainable and better replacement for fossil fuels. Microorganisms mediate the production of biohydrogen from organic feedstock and carbohydrates are the most preferred organic source. However, microbial breakdown of carbohydrates as the only feedstock produces substantial organic acids which lower the fermentation pH to a level detrimental to the activity of the microorganisms. In this study, as the anaerobic digestion of proteins produces ammonia which has the potential to counteract the abrupt pH drop as a result of the substantial organic acids production, proteins and carbohydrates were fermented at five different ratios to ascertain the optimum ratio at which pH control would not be required. The optimum ratio of 80% carbohydrates + 20% proteins was taken through statistical analyses using Response Surface Methodology and the optimization results showed that the biohydrogen production process would be feasible without pH control at a carbohydrate-to-protein ratio of 4:1.

Co-Authorship Statement

Chapter 3: Effect of pH on the Acidification of a Proteinaceous Substrate

Emmanuel Andrew Tepari, George Nakhla

Emmanuel Andrew Tepari: Design of experiment

Laboratory Work

Data collection and interpretation of findings

Paper writing

Dr. George Nakhla: Supervisory role

Critical data analysis and interpretation

Review of manuscript and corrections

Chapter 4: Co-fermentation of Carbohydrates and Proteins for Biohydrogen Production

Emmanuel Andrew Tepari, George Nakhla

Emmanuel Andrew Tepari: Design of experiment

Laboratory Work

Data collection and interpretation of findings

Paper writing

Dr. George Nakhla: Supervisory role

Critical data analysis and interpretation

Review of manuscript and corrections

Acknowledgments

First, I give glory to the Almighty God for the gift of life and the source of strength during the most difficult times in undertaking this project.

Conducting research and writing a thesis is a bit like gathering some raw materials and through an extensive refining process, shaping them into a finely tuned, high-performance automobile – it takes an experienced and skilled professional to provide adequate guidance and supervision in order to turn a concept into reality. I have been blessed to have Dr. George Nakhla as my supervisor, first for providing me the opportunity to join his research group. Dr. Nakhla, you have been such a delightful and incredible advisor throughout my graduate studies with you, and I have learned a lot from you, and there is still more to learn. I greatly appreciate your guidance and discipline in bringing out the very best in me. Your regular pieces of advice and insight would serve me well in my future endeavors.

I owe much gratitude to Dr. Hisham Hafez for co-supervising this project and the funding support from Greenfields Global Inc. is highly acknowledged.

I would also like to express my gratitude to the current and past members of Dr. Nakhla's research group for their support, encouragement, and friendship throughout this journey. Special thanks also go to Dr. Min Gu Kim for his resourcefulness, patience, and time. The laboratory training by Basem Haroun is highly appreciated. I gratefully appreciate and acknowledge the financial support received through the Western Engineering Scholarship.

Table of Contents

List of Tables

List of Figures

List of Appendices

Nomenclature

AD	Anaerobic Digestion
ADS	Anaerobic Digester Sludge
BESA	Bromoethane Sulfonic Acid
BSA	Bovine Serum Albumin
CCD	Central Composite Design
COD	Chemical Oxygen Demand
EBPR	Enhanced Biological Phosphorus Removal
L	Lag time
Pmax	Maximum Cumulative Hydrogen Production
RSM	Response Surface Methodology
Rmax	Maximum Hydrogen Production Rate
TCOD	Total Chemical Oxygen Demand
TSS	Total Suspended Solids
VFA	Volatile Fatty Acids
VSS	Volatile Suspended Solids

Chapter 1
Introduction

1.1Background

Presently, most of the global energy demand is met with fossil fuels which are rapidly depleting. In addition, fossil fuels produce greenhouse gases on combustion and contribute to climate change, global warming, and ozone layer depletion (Bharathiraja et. al. 2016). There have been considerable efforts towards the development of biofuels that will be sustainable to meet the dual challenges of meeting future energy demands and also minimizing adverse environmental impacts. Hydrogen can provide a solution to the aforementioned concerns as a sustainable and better replacement for fossil fuels. Hydrogen upon combustion produces only water vapor and heat and does not contribute to greenhouse gases. It has an energy value of 286 kJ/mol which is at least twice that of fuels generated from fossil fuels (Romão et al., 2014).

There are different methods of hydrogen production which include biomass gasification, electrolysis of water, steam reforming of natural gas among others. Hydrogen production methods through biological routes are the most environmentally friendly (Bharathiraja et al., 2016). Dark fermentation and photo-fermentation are the two most common biological hydrogen production methods. Dark-fermentation is the most preferred because it utilizes a wide range of organic wastes as the substrate, coupled with its independence on light (Silvestre et al., 2015). Additionally, the rates of hydrogen production in dark fermentative environments are significantly greater than those from photo-fermentation (Romão et al., 2014).

The use of naturally mixed anaerobic consortia as inoculum has been reported by Danko et. al. (2008) to have numerous advantages over pure cultures because of operational tractability and diverse microbial community. This also makes mixed culture amenable to a wide range of organic feedstocks and significantly enhances the cost-effectiveness of the operation (Prakasham et al. 2009). Complex organic feedstocks essentially require hydrolytic and catabolic breakdown into simpler substances and this gives mixed anaerobic consortia an edge over pure cultures (Romão et al., 2014).

Renewable carbohydrate-rich substrates are the most preferable carbon source for fermentative hydrogen production (Uyar et al. 2009; Craven & Russell, 1988; Mata-Alvarez et al., 2000). Biomass from industrial effluents, food processing industries, agriculture, and municipal wastewater treatment represent abundant sources of the renewable substrate.

Co-fermentation of different classes of carbohydrates such as glucose and starch has been reported by several researchers to have distinctive positive effects on the hydrogen yields and production rates (Han & Shin, 2004; Zhu & Béland, 2006)

1.2 Problem Statement

Several factors including thermodynamic barriers, nature of the substrate, product inhibition and metabolic pathways limit the production of biological hydrogen (Hallenbeck & Benemann, 2002).

A variety of simple and complex carbohydrates and the co-fermentation of the different classes of carbohydrates for biological hydrogen production have been extensively researched and reported in the literature. Granted, carbohydrate-rich

substrates are the most suitable for biological hydrogen production. However, feeding carbohydrates as the sole carbon source usually resulted in the substantial production of VFAs that cause an abrupt drop in the fermentation medium pH. This leads to gradual losses of the hydrogen-producing microorganisms over the fermentation time, resulting eventually in system failure. Anaerobic fermentation of proteins produces ammonia which has the potential to counterbalance the effects of the accumulated VFAs. Besides, real waste streams are very complex in nature and carbohydrates and proteins combined constitute over two-thirds of the total organic matter (Chong et. al, 2009). Therefore, studying co-fermentation of carbohydrates and proteins for biological hydrogen production is worthy of exploration in order to identify the optimum co-digestion ratio that would ensure the maximum hydrogen yield and process stability.

1.3 Research objectives

The main objective of this research was to study the acidification of proteins and to further evaluate the impact of co-digestion of carbohydrates and proteins on the hydrogen production process. The following are the specific objectives:

- Assess the effect of pH on volatile fatty acids production from a proteinaceous substrate, bovine serum albumin, as a model protein
- Investigate the stoichiometric reactions that describe the anaerobic fermentation of proteins
- Assess the synergy of co-fermentation of carbohydrates and proteins for biohydrogen production

1.4 Thesis Organization

This thesis covers five chapters and complies to the "integrated article" format as dictated by the Thesis Regulation Guide by the School of Graduate and Postdoctoral Studies (SGPS) of the University of Western Ontario. The chapters covered are as follows:

Chapter	Scope
1	General introduction and objectives of the research
2	A review of the literature on biological hydrogen production
3	Effect of pH on volatile fatty acid production from bovine serum albumin (BSA)
4	Co-fermentation of carbohydrates and proteins for biohydrogen production
5	Summarizes the major findings of this research and also provides recommendations for further studies.

1.5 References

Bharathiraja, B., Sudharsanaa, T., Bharghavi, A., Jayamuthunagai, J., & Praveenkumar, R. (2016). Biohydrogen and Biogas – An overview on feedstocks and enhancement process. *Fuel*, *185*, 810–828. https://doi.org/10.1016/j.fuel.2016.08.030

Chong, M. L., Sabaratnam, V., Shirai, Y., & Hassan, M. A. (2009). Biohydrogen production from biomass and industrial wastes by dark fermentation. *International Journal of Hydrogen Energy*, *34*(8), 3277–3287. https://doi.org/10.1016/j.ijhydene.2009.02.010

Craven, S. E., & Russell, R. B. (1988). Increased Sporulation of Clostridium perfringens in a Medium Prepared with the Prereduced Anaerobically Sterilized Tech- nique or with Carbon Dioxide or Carbonate, *51*(9), 700–706.

Danko, A. S., Abreu, Â. A., & Alves, M. M. (2008). Effect of arabinose concentration on dark fermentation hydrogen production using different mixed cultures. *International Journal of Hydrogen Energy*, *33*(17), 4527–4532. https://doi.org/10.1016/j.ijhydene.2008.05.060

Hallenbeck, P. C., & Benemann, J. R. (2002). Biological hydrogen production; Fundamentals and limiting processes. *International Journal of Hydrogen Energy*, *27*(11–12), 1185–1193. https://doi.org/10.1016/S0360-3199(02)00131-3

Han, S. K., & Shin, H. S. (2004). Biohydrogen production by anaerobic fermentation of food waste. *International Journal of Hydrogen Energy*, *29*(6), 569–577. https://doi.org/10.1016/j.ijhydene.2003.09.001

Mata-Alvarez, J., Macé, S., & Llabrés, P. (2000). Anaerobic digestion of organic solid wastes. An overview of research achievements and perspectives. *Bioresource Technology*, *74*(1), 3–16. https://doi.org/10.1016/S0960-8524(00)00023-7

Prakasham, R. S., Brahmaiah, P., Sathish, T., & Sambasiva Rao, K. R. S. (2009). Fermentative biohydrogen production by mixed anaerobic consortia: Impact of glucose to xylose ratio. *International Journal of Hydrogen Energy*, *34*(23), 9354–9361. https://doi.org/10.1016/j.ijhydene.2009.09.104

Romão, B. B., Batista, F. R. X., Ferreira, J. S., Costa, H. C. B., Resende, M. M., & Cardoso, V. L. (2014). Biohydrogen production through dark fermentation by a microbial consortium using whey permeate as substrate. *Applied Biochemistry and Biotechnology*, *172*(7), 3670–3685. https://doi.org/10.1007/s12010-014-0778-5

Silvestre, G., Fernández, B., & Bonmatí, A. (2015). Significance of anaerobic digestion as a source of clean energy in wastewater treatment plants. *Energy Conversion and Management*, *101*, 255–262. https://doi.org/10.1016/j.enconman.2015.05.033

Uyar, B., Eroglu, I., Yücel, M., & Gündüz, U. (2009). Photofermentative hydrogen production from volatile fatty acids present in dark fermentation effluents. *International Journal of Hydrogen Energy*, *34*(10), 4517–4523. https://doi.org/10.1016/j.ijhydene.2008.07.057

Zhu, H., & Béland, M. (2006). Evaluation of alternative methods of preparing hydrogen producing seeds from digested wastewater sludge. *International Journal of Hydrogen Energy*, *31*(14), 1980–1988. https://doi.org/10.1016/j.ijhydene.2006.01.019

Chapter 2

Literature Review

2.1 Introduction

Fossil fuels are the most common sources of energy in the world today and are dwindling rapidly. The International Energy Agency has reported that the consumption of energy globally is expected to rise by 56% by the year 2040 (International Energy Agency, 2013). The combustion of fossil fuels to provide energy is often accompanied by the release of greenhouse gases with their attendant climate change issues, and this has heightened interest in global environmental protection (Ramachandran et al., 2008). The development of an alternative renewable energy source which is carbon-neutral has become very imperative to meet the ever-increasing energy demand as a result of rapid population growth (Bharathiraja et al., 2016). Hydrogen upon combustion produces only heat and water and does not contribute to greenhouse gases. It has an energy value of 286 kJ/mol which is at least twice that of fuels generated from fossil fuels and has therefore been deemed to have a major futuristic role (Cai et al., 2004).

rapid industrialization and high human population growth rate has resulted in enormous quantities of waste emanating from agriculture, industry and the domestic setting and as a result, improper handling of these waste poses a major threat to the quality of the environment (Ren et al., 2007). Utilization of the organic matter within

the waste for biological hydrogen production is a promising technique to effectively manage and transform waste into clean energy generation (Elbeshbishy, 2011).

2.2 Hydrogen Production through Biological means

Microorganisms mediate the production of biological hydrogen from organic waste. The four known mechanisms for biological hydrogen production include dark-fermentation, direct and indirect biophotolysis, and photo-fermentation.

2.2.1 Photo-Fermentation

Purple non-sulfur bacteria mediate organic acids conversion to hydrogen in the presence of light, nitrogenase and under limited nitrogen environment (Das & Veziroglu, 2008).

$$CH_3COOH + 2H_2 \xrightarrow{light\ energy} 4H_2 + 2CO_2 \quad \text{.....(1)}$$

The major drawbacks of this approach are oxygen inhibition of nitrogenase and extremely low (< 6%) efficiency of conversion of light energy (Das & Veziroglu, 2008).

2.2.2 Direct Biophotolysis

Cyanobacteria and green algae have the capability of extracting electrons and protons from water by directly using energy from the sun. This phenomenon results in the release of hydrogen and oxygen (Benemann et al., 1980).

$$2H_2O + light\ energy \rightarrow 2H_2 + O_2 \quad \text{.....(2)}$$

The main limitation of this process is the high light intensity requirement and the low efficiency of the photochemical reaction as well as the inhibitory effects of oxygen (Das & Veziroglu, 2008).

2.2.3 Indirect Biophotolysis

Cyanobacteria produce hydrogen through photosynthetic means in a two-step water splitting process (Levin et al., 2004).

$$12H_2O + 6CO_2 + light\ energy \rightarrow C_6H_{12}O_6 + 6O_2 \quad \text{.....(3)}$$

$$C_6H_{12}O_6 + 12H_2O + light\ energy \rightarrow 12H_2 + 6CO_2 \quad \text{.....(4)}$$

In the aerobic stage (first step), carbohydrates are produced through the photosynthetic process. In the anaerobic stage (second step), there is a breakdown of carbohydrates to release hydrogen. Due to the series of steps involved in the indirect biophotolysis process, it is not as effective as the direct biophotolysis (Levin et al., 2004). The major limitation of indirect biophotolysis is the need to remove hydrogenase enzymes (Das & Veziroglu, 2008).

2.2.4 Dark-Fermentation

Dark fermentation occurs under anoxic or environments devoid of oxygen. Fermentative bacteria oxidizes organic matter to generate electrons and as a result of the anoxic environment, there is no oxygen availability, therefore, protons are reduced to molecular hydrogen which functions as an electron acceptor (Das & Veziroglu, 2008). Anaerobic processes are inexpensive, simpler to handle and generate higher hydrogen production rates than photosynthetic processes. A major limitation, however, is the existence of a thermodynamic barrier for the hydrogen-producing

bacteria to overcome which limits the complete utilization of the substrate (Hallenbeck et al., 2009). The end products of carbohydrates as a source of carbon for fermentation include but not limited to ethanol, acetate, lactic acid, propionate, and butyrate (Guo et al., 2010).

Mixed biogas is essentially produced during dark fermentation with hydrogen and carbon dioxide as the primary gases and may contain other gases like methane, hydrogen sulfide, and carbon monoxide (Ren et al., 2008).

The hydrogen yield by glucose is different and is usually dictated by the end products and the fermentation pathway. Hydrogen-producing bacteria are mostly either strict anaerobes, facultative bacteria and aerobic bacteria (Guo et al., 2010). With acetate as the predominant pathway, for instance, a maximum of 4 mol H_2/mol glucose can be theoretically produced whereas a maximum of 2 mol H_2/mol glucose can be achieved when the fermentation pathway follows butyrate production (Fang & Liu, 2002).

$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2$; acetate pathway …….(5)

$C_6H_{12}O_6 \rightarrow 2CH_3CH_2CH_2COOH + 2CO_2 + 2H_2$; butyrate pathway …… (6)

Homoacetogenic bacteria, for instance, *Clostridium aceticum*, produce acetate from carbon dioxide and hydrogen and this reaction depletes hydrogen (Guo et al., 2010).

$2CO_2 + 4H_2 \rightarrow CH_3COOH + 2H_2O$ ……..(7)

As the propionate pathway leads to hydrogen consumption, a zero-hydrogen balance is brought about by both ethanol and lactic acid pathways (Guo et al., 2010).

$C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COOH + 2H_2$ ……..(8)

$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2$ ……(9)

$C_6H_{12}O_6 \rightarrow 2CH_3CHOHCOOH + 2CO_2$……(10)

$CH_3CH_2OH + H_2O \rightarrow CH_3COO^- + 2H_2 + H^+$ ……...(11)

$CH_3CH_2COO^- + 3H_2O \rightarrow CH_3COO^- + 3H_2 + H^+ + HCO_3^-$……(12)

$CH_3CH_2CH_2COO^- + 2H_2O \rightarrow 2CH_3COO^- + 2H_2 + H^+$……..(13)

The major challenge of biohydrogen research is to achieve higher yields of hydrogen and at the same time ensuring process stability. The nature of the inoculum, type of substrate, and the process conditions among others are the determining factors that control the formation of end products.

2.3 Factors Affecting Biohydrogen Production

The most common factors that influence biological hydrogen production among several other factors include temperature, nature of substrate, pH, hydrogen partial pressure, and inoculum.

2.3.1 Temperature

Temperature greatly affects hydrogen production potential and the metabolic activities of microorganisms (Karlsson et al., 2008). Mesophilic (37 ℃) and thermophilic (55 ℃) temperature ranges are mostly employed for biohydrogen production (Gadow et al., 2012). Most of the studies in the literature on biohydrogen have employed mesophilic conditions, nonetheless, the literature indicates that thermophilic temperatures enhance hydrolysis of complex organic matter, thereby increasing hydrogen yields (Guo et al., 2010). Thermophilic temperatures have also been observed to enhance the rates of substrate utilization and abate the dissolution of

hydrogen (Karlsson et al., 2008). Lee et al. (2008) observed a greater hydrogen yield of 250 $mLH_2/gCOD_{added}$ at mesophilic temperature than thermophilic (120 $mL/gCOD_{added}$) when hydrogen yields at both temperature conditions were investigated using starch and municipal sewage sludge as the seed. Kargi et al. (2012) observed a higher hydrogen yield of 180 $mLH_2/gCOD_{degraded}$ for using cheese whey as the substrate and anaerobically digested sludge (ADS) as seed at a temperature of 55 ℃ (thermophilic) than 115 $mLH_2/gCOD_{degraded}$ at mesophilic temperature (37 ℃). Yokoyama et al. (2007) used cow dung slurry to study the influence on hydrogen yields at different temperatures: 37 ℃, 56 ℃, 60 ℃, 68 ℃, 75 ℃, and 80 ℃ and observed higher hydrogen yields of 310 $mLH_2/gCOD_{added}$ and 350 $mLH_2/gCOD_{added}$ at 60 ℃ and 75 ℃, respectively. Gadow et al. (2012) examined cellulose utilization at mesophilic (36 ℃) to hyper-thermophilic temperatures (85 ℃) and the maximum yields of hydrogen occurred at the hyper-thermophilic temperatures (75 – 85 ℃) . A summary of hydrogen production at various temperatures is shown in Table 2.1 and from the results obtained by the different researchers suggest that the type of inoculum used for the hydrogen production process had an impact on the hydrogen yields.

Table 2.1: Review of the literature on biological hydrogen production from the various inoculum

Substrate/Feedstock	Seed/Inoculum	Reactor Configuration	Temperature [℃]	pH	H_2 Yield [mol H_2/mol.hexose$_{added}$]	Reference
Glucose	*Thermanaerobacterium thermsacharoliticum*	Batch	65	6.0	2.32	Ren et al., 2008
Potato starch residue	*Clostridium butyricum*	Batch	36	5.35	2.6	Yokoi et al 2002
Glucose	*Thermtoga efi*	Batch	60	7.4	3.30	Van Niel et al., 2002
Glucose	*Eschericha Coli B-2L*	CSTR	36	6.5	3.21	Chittibabu et al., 2006
Glucose	*Enterobacter Cloacae IT-BT07*	Batch	37	6.3	2.25	Kumar & Das, 2000
Glucose	*Rhodopseudomonas palustris*	Batch	36	6.5	2.67	Oh et al.,2002
Glucose	*Caldicellulosiruptor saccharolyticus*	Batch	68	7.2	3.5	Mars et al., 2010
Glucose	*Klebsiella Pneumonia ECU-15*	Batch	37	6.5	2.70	Niu et al., 2010
Glucose	*Enterobacter aerogenes HO-39*	Batch	37	7.0	1.02	Yokoi et al., 2002

Wheat	*Rhodobacter ssphaeroides-RV*	Hybrid bioreactor	35	7.5	0.65	Bharathiraja et al., 2016
Glucose	*Clostridium butyricum CWB11009*	Batch	35	5.3	1.8	Masset et al., 2010
Cellulose	*Clostridium termitidis CT1112*	Batch	37	7.3	0.65	Ramachandran et al., 2008

2.3.2 pH

The metabolic pathways of the microbial populations in mixed cultures are greatly dependent on pH and this affects hydrogen yields. Therefore, pH is an essential parameter that influences biological hydrogen production (Wang & Wan, 2009). The preferred pH range for food wastes is 5 – 6, while that for crop and animal remains is reported to be neutral (Guo et al., 2010). Lay et al. (2012) reported a pH range of 4.7 – 5.7 and a hydrogen yield of 280 $mLH_2/gCOD_{added}$ at 35 ℃ to be the optimum for hydrogen production using starch as the substrate. Yossan et al. (2012) used effluent from palm oil processing and reported an optimum pH of 6 and a maximum hydrogen yield of 1.05 mmol H_2/g $COD_{degraded}$. Masset et al. (2010) also examined hydrogen yields from glucose and starch, and reported optimum pH of 5.2 and 5.6 with respective hydrogen yields of 1.52 and 1.7 mol/mol hexose consumed. Alcohol production is favored over hydrogen for pH values below 4.1 or greater than 6.1 (Lay et al., 2012). Lee et al. (2008) reported a pH of 5.5 to 6.0 to be optimum for enhanced starch utilization at 37 ℃. Several different optimal pH values at both mesophilic and thermophilic temperatures have been documented in the literature, which could be associated with the diversity of the source of the inoculum, nature of the substrate, and the temperature of operation. The preferable end products are acetate and butyrate, but butyrate is selectively produced at low pH (Guo et al., 2010). The pH range of 4.5 – 6 favors acetate and butyrate metabolic pathways while ethanol is produced at neutral and alkaline conditions with accompanying propionate production, which is hydrogen-consuming (Guo et al., 2010; Fang & Liu, 2002). Fang & Liu (2002) examined the influence of pH on hydrogen yield by using glucose as the substrate and mixed culture inoculum and observed a pH of 5.5 and a

hydrogen yield of 2.1 mol H_2/mol.hexose to be the optimum, and the hydrogen content was 40% of the biogas produced. At neutral and alkaline pH, there was an observed decline of the hydrogen content in the biogas, the hydrogen yield, and the specific hydrogen production rates. Moreover, in biological hydrogen production systems using mixed culture at a pH above 6 stimulate methanogenic activity (Fang & Liu, 2002). Shin & Youn (2005) used food waste and anaerobic digester sludge (ADS) and observed a pH of 5.5 and a hydrogen yield of 2.2 mol H_2/mol.hexose to be the optimum and a substrate decomposition efficiency of 60.5% was achieved. Fang & Liu, (2002) also observed that an increase in pH beyond 6.0 correspondingly decreased hydrogen producers. With a sharp pH change active biomass growth is adversely affected (Lay et al., 2012).

2.3.3 Nature of Inoculum

Microbial populations are essentially responsible for the breakdown of organic matter to produce hydrogen and some other end products of digestion. The most common group of microorganisms that are notable for producing hydrogen are the strict anaerobes such as *Acitomytes* and *Propionibacterium*, either under mesophilic or thermophilic conditions. Some facultative bacteria such as *Escherichia coli* and *Listeria* have also been identified to produce high hydrogen yields (Chittibabu et al., 2006). There have been several studies in the literature that examined hydrogen production potential from various inocula. Nasr et al. (2011) used the mixed culture from ADS, mulch (Akutsu et al., 2008), connatural microflora (Puhakka et al., 2012) among others. Also, pure bacterial culture such as *Clostridium beijerinckii* has also been studied for hydrogen production (Gomez-Flores et al., 2015). An extensive literature review on various inoculums for biohydrogen production has been documented in Table 2.2.

Table 2.2: Summary of different substrates and their respective inoculum studied for biological hydrogen production

Substrate	**Seed/Inoculum**	**Hydrogen Yield**	**Reference**
Cellobiose	*Clostridium sp. RI*	80 mL H_2/$gCOD_{added}$	Ho et al., 2010
Starch	Soil inoculum	108 mL H_2/$gCOD_{added}$	Logan et al., 2007
Maltose	*Enterobacter aerogenes strain HO-37*	220 mL H_2/$gCOD_{added}$	Yokoi et al., 2002
Sucrose	Anaerobic digester sludge	180 mLH_2/$gCOD_{added}$	Hussy et al., 2005
Sucrose	*Clostridium pasteurianum*	170 mL H_2/$gCOD_{added}$	Zhang et al., 2005
Arabinose	*Escherichia coli* strain DJT135	152 mL/$gCOD_{added}$	Ghosh et al., 2009
Arabinose	Mixed culture sludge	215 mL H_2/$gCOD_{added}$	Danko et al.,2008
Galactose	Mixed Culture sludge	172 mL H_2/$gCOD_{added}$	Yokoi et al., 1995
Mannose	*Citrobacter sp. CMC-1*	192 mL H_2/$gCOD_{added}$	Mangayil et al., 2011
Xylose	Anaerobic mixed culture	214 mL H_2/$gCOD_{added}$	Lin et al., 2008

Xylose	*Enterobacter aerogenes IAM 1182*	110 $mLH_2/gCOD_{added}$	Ren et al., 2006
Glucose	Anaerobic digester sludge	260 mL $H_2/gCOD_{degraded}$	(Kim & Kim, 2012)
Glucose	Anaerobic digester sludge	175 $mLH_2/gCOD_{added}$	Hafez et al., 2010
Cellulose	*Clostridium cellulolyticum*	250 $mLH_2/gCOD_{added}$	Ren et al., 2006
Cellulose	*Clostridium termitidis*	85 mL $H_2/gCOD_{added}$	Ramachandran et al., 2008
Sugarcane bagasse hydrolysate	Elephant dung	108 mL $H_2/gCOD_{added}$	Fangkum & Reungsang, 2011
Thin stillage	Anaerobic digester sludge	213 mL $H_2/gCOD_{added}$	Nasr et al., 2003
Molasses	Mixed culture	240 $mLH_2/gCOD_{added}$	Ren et al., 2006
Cheese Whey	*Clostridium saccharobutylacetonicum*	128 $mLH_2/gCOD_{added}$	Ferchichi et al., 2005
Potato wastewater	Soil inoculum	85 mL $H_2/gCOD_{added}$	Van Ginkel et al., 2005
Food waste and sewage	Anaerobic digester sludge	102 $mLH_2/gCOD_{added}$	Kim et al., 2004

sludge			
Sugarcane bagasse	*Clostridium butyricum*	214 $mLH_2/gCOD_{added}$	Pattra et al., 2008

Mixed cultures are more useful and have an urge over pure cultures as they provide a broader range of catabolic and hydrolytic breakdown of complex organic molecules. Moreover, the action of pure cultures is specific with respect to the substrate, whereas mixed microbial cultures are able to degrade a wide range of feedstocks (Wang & Wan, 2009). Masset et al. (2010) used white starch powder and a pure bacterial strain of *Clostridium butyricum* as the inoculum and observed a maximum hydrogen yield of 2 mol/mol_{hexose} at 37 ℃. A relatively higher yield of 2.3 mol/mol_{hexose} was however observed by Akutsu et al. (2008) at 37 ℃ using activated sludge containing mixed bacterial cultures when starch was used as the substrate. Datar et al. (2007) obtained a maximum hydrogen yield of 3 mol/mol $_{hexose}$ with ADS and corn stover as the substrate at 55 ℃, whereas, Ren et al. (2010) obtained 2.2 mol/mol_{hexose} as the maximum hydrogen yield at the same operation temperature with a pure culture of *Thermoanaerobacterium thermosaccharolyticu.* Moreover, in adverse conditions, the odds of survival of hydrogen-producing bacteria are way much higher than hydrogen-consuming bacteria. Hydrogen-producing bacteria have the ability to form preservative spores in harsh environments such as elevated temperatures, extreme acidity, and alkalinity. Hydrogen-consuming bacteria, on the other hand, do not have the ability to withstand such adverse environments (Zhu & Béland, 2006). In essence, several pretreatment methods have been

adapted to repress the activity of hydrogen-consuming bacteria (Sinha & Pandey, 2011). Some of the most commonly adapted pretreatment methods include heat-pretreatment at 70 ℃, aeration, acidity, and alkalinity, and chemical inhibitors such as chloroform and iodopropane (Sinha & Pandey, 2011). Wang et al. (2011) pretreated inoculum at 80 ℃ for 30 mins and realized that the hydrogen yield increased by 35% in comparison with the control experiment that was not pretreated. From the aforementioned study, alkaline (pH of 11) and acidic (pH of 4) pretreatments were employed and the researchers observed that the hydrogen yield increased by 20% (Wang et al.,2011). Zhu & Béland (2006) studied the influence of iodopropane and BESA pretreatment techniques and noticed that relatively higher hydrogen yields of 2.7 and 2.4 mol/mol hexose than the seed without pretreatment (2.2 mol H_2/mol hexose added). Ren et al. (2008) maintained a low dissolved oxygen concentration of <0.5 mg/L and employed repeated aeration as a pretreatment method and observed the hydrogen yield from corn starch to have increased by 25%.

2.3.4 Feedstocks for Biological Hydrogen Production

Carbohydrates have been studied and determined to be the best source of carbon for biological hydrogen production (Hawkes et al., 2002). Several substrates (Table 2.2), mostly soluble sugars, have been examined for fermentative hydrogen production because of their ease of biodegradability, and their abundance in several industrial, agricultural and domestic effluents (Hallenbeck et al., 2009). Nonetheless, classic sources of carbohydrates are more costly for commercial scale production of fermentative hydrogen, consequently, waste feedstocks from agriculture, domestic and industrial

among others are more sustainable sources (Elsharnouby et al., 2013; Hawkes et al., 2002; Chong et al., 2009).

2.3.5 The Partial Pressure of Hydrogen

Several studies in the literature have reported the partial pressure of hydrogen to be a limiting factor in the biological hydrogen production process (Guo et al., 2010). In fermentative hydrogen production, ferredoxins are re-oxidized by bacteria and hydrogen bearing coenzymes, and these reactions become inimical at high hydrogen levels in the liquid phase, thus increasing the inhibition of end-products (Hawkes et al., 2002). Increasing hydrogen concentration decreases hydrogen synthesis and this shifts the metabolic activity towards the production of ethanol, butanol, lactate among other reduced substrates (Elbeshbishy et al., 2011). Lee et al. (2012) observed low concentrations of propionate (5 mg/L) at very low hydrogen partial pressure of 20 Pa. The production of volatile fatty acids (VFAs) as a result of the oxidative breakdown of long chain fatty acids is not favorable thermodynamically with associated positive Gibbs energy, therefore, extremely low hydrogen levels are needed to overcome this thermodynamic hindrance (Guo et al., 2010). Furthermore, supplementary production of hydrogen from acetate is another thermodynamically unfavorable reaction which is highly responsive to the concentrations of hydrogen.

Several methods have been employed to curtail hydrogen partial pressure in the liquid medium. Some of the techniques employed include ultrasonication, gas sparging, stripping by membrane absorption, and rapid mechanical mixing (Elbeshbishy et al., 2011). Elbeshbishy et al. (2011) have reported gas sparging to be the most common technique employed to reduce the concentrations of dissolved gases within hydrogen

bioreactors. Hussy et al. (2005) noticed a 47% rise in hydrogen yield from 1.27 to 1.88 mol H_2/mol hexose using nitrogen gas for sparging. The hydrogen yield from xylose was observed to be 2.8 times higher in a stirred culture than an unstirred one using *Clostridium thermocellum* as inoculum (Guo et al., 2010). Liang et al. (2002) studied the performance of silicone rubber membrane to isolate biogas from the liquid phase and noticed an enhancement in the hydrogen yield by 15%. Elbeshbishy et al. (2011) realized an improvement in the headspace hydrogen content by 31% when the ultrasonication method was employed to reduce the dissolved hydrogen and carbon dioxide from the liquid. Carbon dioxide sequestration has also been demonstrated to improve biohydrogen production (Lackner, 2003 ; Nasr et al., 2011).

2.4 Protein degradation

Proteins are made up of several amino acid units linked to one another by amide or peptide bonds. The hydrolysis of proteins into their constituent amino acids is mediated by extracellular enzymes called proteases (Randall et al., 2003). The degradation of proteins in anaerobic digesters seems to be quite different from in the rumen of animals. In the rumen, for example, proteins are fermented by carbohydrate-degrading bacteria and the fermentation of amino acids does not yield enough energy for growth (Uyar et al., 2011). Proteolytic bacteria in anaerobic digesters, however, mainly degrade proteins and the processes that are involved yield energy (Yokoi et al., 2009). The principal proteolytic bacteria as shown by most studies (Randall et al., 2003; Uyar et al., 2011; Yokoi et al., 2009) in anaerobic digesters are the gram-positive bacteria, mainly from the genus Clostridia and these have a significant role in amino acid fermentation (Wei et al., 2012).

2.4.1 Amino acid fermentation (Stickland reaction)

Amino acids are fermented in two principal ways: (1) Amino acid pairs can be degraded through the Stickland reaction (2) Single amino acids can be fermented in the process that requires hydrogen-utilizing bacteria (McInerney, 2005). The Stickland reaction is the most common for amino acid fermentation and it involves the coupled oxidation and reduction of amino acids to organic acids (Randall et al., 2003). The electron donating amino acid is oxidized to volatile carboxylic acid (one carbon atom shorter than the parent amino acid). For instance, alanine with three carbon atoms is converted to acetic acid with two carbon atoms. The electron accepting amino acid is reduced to a volatile carboxylic acid, as with the number of carbon atoms as the parent amino acid (Wei et al., 2012). An example is glycine with two carbon atoms converting to acetic acid. The general mechanism of the Stickland reaction is shown in Fig.2.1. Microorganisms which degrade amino acids avoid the usage of hydrogen ions as electron acceptors to generate hydrogen gas (Yokoi et al., 2009). Amino acids can either act as Stickland acceptors, donors or both (Moser-Engeler et al., 2010). It is only histidine that is oxidized and cannot be degraded by Stickland reaction. There is usually a 10% shortage of Stickland amino acid acceptors in a typical mixture of amino acids which can result in the production of hydrogen (Tanisho et al., 2005).

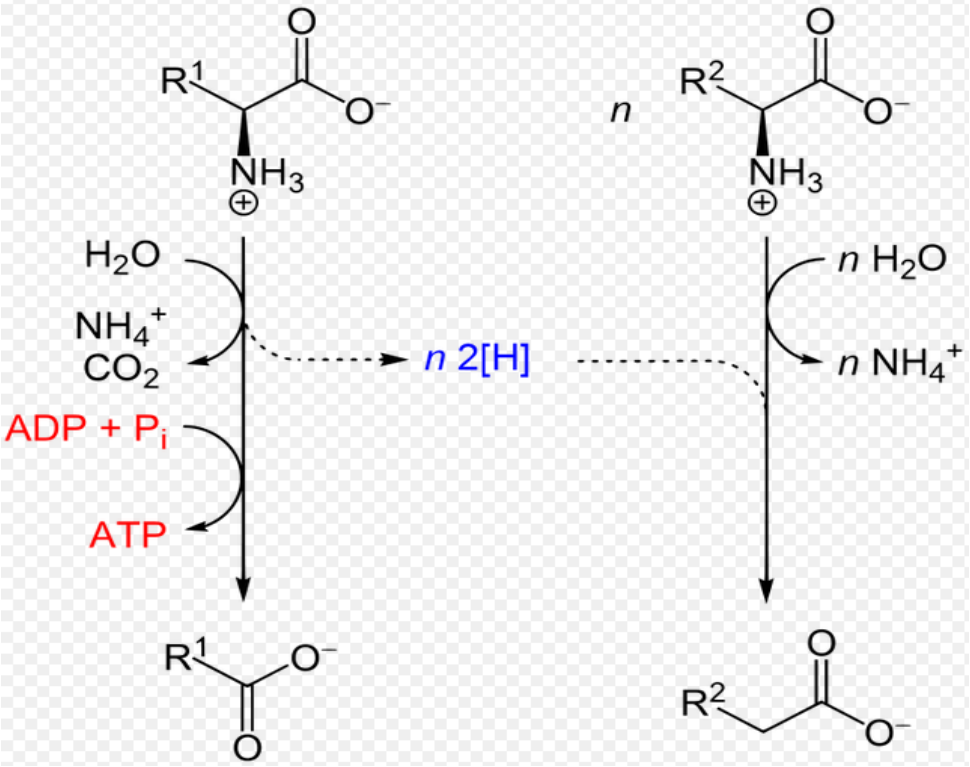


Fig. 2.1. The general mechanism of the Stickland reaction [McInerney, 2001]

2.4 References

Abreu, A. A., Karakashev, D., Angelidaki, I., Sousa, D. Z., & Alves, M. M. (2012). Biohydrogen production from arabinose and glucose using extreme thermophilic anaerobic mixed cultures, 1–12.

Akutsu, Y., Lee, D. Y., Li, Y. Y., & Noike, T. (2009). Hydrogen production potentials and fermentative characteristics of various substrates with different heat-pretreated natural microflora. *International Journal of Hydrogen Energy*, *34*(13), 5365–5372. https://doi.org/10.1016/j.ijhydene.2009.04.052

Akutsu, Y., Li, Y. Y., Tandukar, M., Kubota, K., & Harada, H. (2008). Effects of seed sludge on fermentative characteristics and microbial community structures in thermophilic hydrogen fermentation of starch. *International Journal of Hydrogen Energy*, *33*(22), 6541–6548. https://doi.org/10.1016/j.ijhydene.2008.08.038

APHA/AWWA/WEF. (2012). Standard Methods for the Examination of Water and Wastewater. *Standard Methods*, 541. https://doi.org/ISBN 9780875532356

Astrid E. Mars, Teun Veuskens , Miriam A.W. Budde, Patrick F.N.M. van Doeveren, Steef J. Lips, Robert R. Bakker, Truus de Vrije, P. A. M. C. (2010). Biohydrogen production from untreated and hydrolyzed potato steam peels by the extreme thermophiles Caldicellulosiruptor saccharolyticus and Thermotoga neapolitana. *International Journal of Hydrogen Energy*, *35*(15), 7730–7737. https://doi.org/10.1016/J.IJHYDENE.2010.05.063

Bahl, H., Andersch, W., Braun, K., & Gottschalk, G. (1982). Effect of pH and Butyrate Concentration on the Production of Acetone and Butanol by Clostridium acetobutylicum Grown in Continuous Culture A ~ ", *101*, 17–20.

Batstone, D. J., Keller, J., & Blackall, L. L. (2004). The influence of substrate kinetics on

the microbial community structure in granular anaerobic biomass. *Water Research*, *38*(6), 1390–1404. https://doi.org/10.1016/j.watres.2003.12.003

Benemann, J. R., Miyamoto, K., & Hallenbeck, P. C. (1980). Bioengineering aspects of biophotolysis. *Enzyme and Microbial Technology*, *2*(2), 103–111. https://doi.org/10.1016/0141-0229(80)90064-2

Bharathiraja, B., Sudharsanaa, T., Bharghavi, A., Jayamuthunagai, J., & Praveenkumar, R. (2016). Biohydrogen and Biogas – An overview on feedstocks and enhancement process. *Fuel*, *185*, 810–828. https://doi.org/10.1016/j.fuel.2016.08.030

Bhatia, S., Othman, Z., & Ahmad, A. L. (2007). Pretreatment of palm oil mill effluent (POME) using Moringa oleifera seeds as natural coagulant, *145*, 120–126. https://doi.org/10.1016/j.jhazmat.2006.11.003

Breure, A. M., Mooijman, K. A., & van Andel, J. G. (1986). Protein degradation in anaerobic digestion: influence of volatile fatty acids and carbohydrates on hydrolysis and acidogenic fermentation of gelatin. *Applied Microbiology and Biotechnology*, *24*(5), 426–431. https://doi.org/10.1007/BF00294602

Caccavo, F., Lonergan, D. J., Lovley, D. R., Davis, M., Stolz, J. F., & Mcinerneyl, M. J. (1994). Oxidizing dissimilatory metal-reducing microorganism. *Applied and Environmental Microbiology*, *60*(10), 3752–3759.

Chen, C. C., Lin, C. Y., & Lin, M. C. (2002). Acid-base enrichment enhances anaerobic hydrogen production process. *Applied Microbiology and Biotechnology*, *58*(2), 224–228. https://doi.org/10.1007/s002530100814

Chen, Y., Randall, A. A., & McCue, T. (2004). The efficiency of enhanced biological phosphorus removal from real wastewater affected by different ratios of acetic to propionic acid. *Water Research*, *38*(1), 27–36. https://doi.org/10.1016/j.watres.2003.08.025

Cheng, S. S., Chang, S. M., & Chen, S. T. (2002). Effects of volatile fatty acids on a thermophilic anaerobic hydrogen fermentation process degrading peptone. *Water*

Science and Technology, *46*(4–5), 209–214.

Chittibabu, G., Nath, K., & Das, D. (2006). Feasibility studies on the fermentative hydrogen production by recombinant Escherichia coli BL-21. *Process Biochemistry*, *41*(3), 682–688. https://doi.org/10.1016/j.procbio.2005.08.020

Choi, J. D. R., Chang, H. N., & Han, J. I. (2011). Performance of microbial fuel cell with volatile fatty acids from food wastes. *Biotechnology Letters*, *33*(4), 705–714. https://doi.org/10.1007/s10529-010-0507-2

Chong, M. L., Sabaratnam, V., Shirai, Y., & Hassan, M. A. (2009). Biohydrogen production from biomass and industrial wastes by dark fermentation. *International Journal of Hydrogen Energy*, *34*(8), 3277–3287. https://doi.org/10.1016/j.ijhydene.2009.02.010

Craven, S. E., & Russell, R. B. (1988). Increased Sporulation of Clostridium perfringens in a Medium Prepared with the Prereduced Anaerobically Sterilized Tech- nique or with Carbon Dioxide or Carbonate, *51*(9), 700–706.

Danko, A. S., Abreu, Â. A., & Alves, M. M. (2008). Effect of arabinose concentration on dark fermentation hydrogen production using different mixed cultures. *International Journal of Hydrogen Energy*, *33*(17), 4527–4532. https://doi.org/10.1016/j.ijhydene.2008.05.060

Das, D., & Veziroä, T. N. (2001). Hydrogen production by biological processes : a survey of literature, *26*, 13–28.

Das, D., & Veziroglu, T. N. (2008). Advances in biological hydrogen production processes. *International Journal of Hydrogen Energy*, *33*(21), 6046–6057. https://doi.org/10.1016/j.ijhydene.2008.07.098

Datar, R., Huang, J., Maness, P. C., Mohagheghi, A., Czernik, S., & Chornet, E. (2007). Hydrogen production from the fermentation of corn stover biomass pretreated with a steam-explosion process. *International Journal of Hydrogen Energy*, *32*(8), 932–939. https://doi.org/10.1016/j.ijhydene.2006.09.027

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric Method for Determination of Sugars and Related Substances. *Analytical Chemistry*, *28*(3), 350–356. https://doi.org/10.1021/ac60111a017

Elbeshbishy, E. E. (2011). *Enhancement of Biohydrogen and Biomethane Production from Wastes Using Ultrasonication*. *PhD Thesis*.

Elbeshbishy, E., Hafez, H., & Nakhla, G. (2011). Hydrogen production using sono-biohydrogenator. *International Journal of Hydrogen Energy*, *36*(2), 1456–1465. https://doi.org/10.1016/j.ijhydene.2010.07.092

Elefsiniotis, P., Wareham, D. G., & Smith, M. O. (2004). Use of volatile fatty acids from an acid-phase digester for denitrification. *Journal of Biotechnology*, *114*(3), 289–297. https://doi.org/10.1016/j.jbiotec.2004.02.016

Elsharnouby, O., Hafez, H., Nakhla, G., & El Naggar, M. H. (2013). A critical literature review on biohydrogen production by pure cultures. *International Journal of Hydrogen Energy*, *38*(12), 4945–4966. https://doi.org/10.1016/j.ijhydene.2013.02.032

Fang, H. H. P., & Liu, H. (2002). Effect of pH on hydrogen production from glucose by a mixed culture. *Bioresource Technology*, *82*(1), 87–93. https://doi.org/10.1016/S0960-8524(01)00110-9

Fangkum, A., & Reungsang, A. (2011). Biohydrogen production from sugarcane bagasse hydrolysate by elephant dung: Effects of initial pH and substrate concentration. *International Journal of Hydrogen Energy*, *36*(14), 8687–8696. https://doi.org/10.1016/j.ijhydene.2010.05.119

Ferchichi, M., Crabbe, E., Gil, G. H., Hintz, W., & Almadidy, A. (2005). Influence of initial pH on hydrogen production from cheese whey. *Journal of Biotechnology*, *120*(4), 402–409. https://doi.org/10.1016/j.jbiotec.2005.05.017

Freudenberg, W., Mayer, F., & Andreesen, J. R. (1989). Hicrnbinlngy of glycine reductase , all involved in anaerobic glycine metabolism, 182–188.

Gadow, S. I., Li, Y. Y., & Liu, Y. (2012). Effect of temperature on continuous hydrogen production of cellulose. *International Journal of Hydrogen Energy*, *37*(20), 15465–15472. https://doi.org/10.1016/j.ijhydene.2012.04.128

Gallert, C., Bauer, S., & Winter, J. (1998). Effect of ammonia on the anaerobic degradation of protein by a mesophilic and thermophilic biowaste population. *Applied Microbiology and Biotechnology*, *50*(4), 495–501. https://doi.org/10.1007/s002530051326

Gerber, A., Mostert, E. S., Winter, C. T., & De Villiers, R. H. (1986). The effect of acetate and other short-chain carbon compounds on the kinetics of biological nutrient removal. *Water SA*.

Ghosh, D., & Hallenbeck, P. C. (2009). Fermentative hydrogen yields from different sugars by batch cultures of metabolically engineered Escherichia coli DJT135. *International Journal of Hydrogen Energy*, *34*(19), 7979–7982. https://doi.org/10.1016/j.ijhydene.2009.08.004

Guo, L., Li, X. M., Bo, X., Yang, Q., Zeng, G. M., Liao, D. xiang, & Liu, J. J. (2008). Impacts of sterilization, microwave and ultrasonication pretreatment on hydrogen producing using waste sludge. *Bioresource Technology*, *99*(9), 3651–3658. https://doi.org/10.1016/j.biortech.2007.07.026

Guo, X. M., Trably, E., Latrille, E., Carrre, H., & Steyer, J. P. (2010). Hydrogen production from agricultural waste by dark fermentation: A review. *International Journal of Hydrogen Energy*, *35*(19), 10660–10673. https://doi.org/10.1016/j.ijhydene.2010.03.008

Hafez, H., Nakhla, G., El. Naggar, M. H., Elbeshbishy, E., & Baghchehsaraee, B. (2010). Effect of organic loading on a novel hydrogen bioreactor. *International Journal of Hydrogen Energy*, *35*(1), 81–92. https://doi.org/10.1016/j.ijhydene.2009.10.051

Hallenbeck, P. C., & Benemann, J. R. (2002). Biological hydrogen production; Fundamentals and limiting processes. *International Journal of Hydrogen Energy*, *27*(11–12), 1185–1193. https://doi.org/10.1016/S0360-3199(02)00131-3

Hallenbeck, P. C., Ghosh, D., Skonieczny, M. T., & Yargeau, V. (2009). Microbiological and engineering aspects of biohydrogen production. *Indian Journal of Microbiology*, *49*(1), 48–59. https://doi.org/10.1007/s12088-009-0010-4

Han, S. K., & Shin, H. S. (2004). Biohydrogen production by anaerobic fermentation of food waste. *International Journal of Hydrogen Energy*, *29*(6), 569–577. https://doi.org/10.1016/j.ijhydene.2003.09.001

Hawkes, F. R., Dinsdale, R., Hawkes, D. L., & Hussy, I. (2002). Sustainable fermentative hydrogen production: Challenges for process optimisation. *International Journal of Hydrogen Energy*, *27*(11–12), 1339–1347. https://doi.org/10.1016/S0360-3199(02)00090-3

Ho, K. L., Chen, Y. Y., & Lee, D. J. (2010). Biohydrogen production from cellobiose in phenol and cresol-containing medium using Clostridium sp. R1. *International Journal of Hydrogen Energy*, *35*(19), 10239–10244. https://doi.org/10.1016/j.ijhydene.2010.07.155

Hussy, I., Hawkes, F. R., Dinsdale, R., & Hawkes, D. L. (2005). Continuous fermentative hydrogen production from sucrose and sugarbeet. *International Journal of Hydrogen Energy*, *30*(5), 471–483. https://doi.org/10.1016/j.ijhydene.2004.04.003

Kargi, F., Eren, N. S., & Ozmihci, S. (2012). Bio-hydrogen production from cheese whey powder (CWP) solution: Comparison of thermophilic and mesophilic dark fermentations. *International Journal of Hydrogen Energy*, *37*(10), 8338–8342. https://doi.org/10.1016/j.ijhydene.2012.02.162

Karlsson, A., Vallin, L., & Ejlertsson, J. (2008). Effects of temperature, hydraulic retention time and hydrogen extraction rate on hydrogen production from the fermentation of food industry residues and manure. *International Journal of Hydrogen Energy*, *33*(3), 953–962. https://doi.org/10.1016/j.ijhydene.2007.10.055

Kim, D. H., & Kim, M. S. (2012). Thermophilic fermentative hydrogen production from various carbon sources by anaerobic mixed cultures. *International Journal of Hydrogen Energy*, *37*(2), 2021–2027. https://doi.org/10.1016/j.ijhydene.2011.07.043

Kim, S. H., Han, S. K., & Shin, H. S. (2004). Feasibility of biohydrogen production by anaerobic co-digestion of food waste and sewage sludge. *International Journal of Hydrogen Energy*, *29*(15), 1607–1616. https://doi.org/10.1016/j.ijhydene.2004.02.018

KINOSHITA, S., UDAKA, S., & SHIMONO, M. (2008). Studies on the Amino Acid Fermentation. *The Journal of General and Applied Microbiology*, *3*(3), 193–205. https://doi.org/10.2323/jgam.3.193

Kotzé, J. ., Thiel, P. ., & Hattingh, W. H. . (1969). Anaerobic digestion II. The characterization and control of anaerobic digestion. *Water Research*, *3*(7), 459–494. https://doi.org/10.1016/0043-1354(69)90014-1

Kumar, N., & Das, D. (2000). Enhancement of hydrogen production by Enterobacter cloacae IIT-BT 08. *Process Biochemistry*, *35*(6), 589–593. https://doi.org/10.1016/S0032-9592(99)00109-0

Kumar, R., Singh, R., Kumar, N., Bishnoi, K., & Bishnoi, N. R. (2009). Response surface methodology approach for optimization of biosorption process for removal of Cr (VI), Ni (II) and Zn (II) ions by immobilized bacterial biomass sp . Bacillus brevis, *146*, 401–407. https://doi.org/10.1016/j.cej.2008.06.020

Lane, C., & Nor, F. (2019). The Amino Acid-fermenting Clostridia, *2*, 47–56.

Lay, C. H., Kuo, S. Y., Sen, B., Chen, C. C., Chang, J. S., & Lin, C. Y. (2012). Fermentative biohydrogen production from starch-containing textile wastewater. *International Journal of Hydrogen Energy*, *37*(2), 2050–2057. https://doi.org/10.1016/j.ijhydene.2011.08.003

Lee, K. S., Hsu, Y. F., Lo, Y. C., Lin, P. J., Lin, C. Y., & Chang, J. S. (2008). Exploring optimal environmental factors for fermentative hydrogen production from starch using mixed anaerobic microflora. *International Journal of Hydrogen Energy*, *33*(5), 1565–1572. https://doi.org/10.1016/j.ijhydene.2007.10.019

Lee, K. S., Tseng, T. S., Liu, Y. W., & Hsiao, Y. D. (2012). Enhancing the performance

of dark fermentative hydrogen production using a reduced pressure fermentation strategy. *International Journal of Hydrogen Energy*, *37*(20), 15556–15562. https://doi.org/10.1016/j.ijhydene.2012.04.039

Levin, D. B., Pitt, L., & Love, M. (2004). Biohydrogen production: Prospects and limitations to practical application. *International Journal of Hydrogen Energy*, *29*(2), 173–185. https://doi.org/10.1016/S0360-3199(03)00094-6

Liang, T. M., Cheng, S. S., & Wu, K. L. (2002). Behavioral study on hydrogen fermentation reactor installed with silicone rubber membrane. *International Journal of Hydrogen Energy*, *27*(11–12), 1157–1165. https://doi.org/10.1016/S0360-3199(02)00099-X

Lin, C. Y., Chang, C. C., & Hung, C. H. (2008). Fermentative hydrogen production from starch using natural mixed cultures. *International Journal of Hydrogen Energy*, *33*(10), 2445–2453. https://doi.org/10.1016/j.ijhydene.2008.02.069

Liu, H., Wang, J., Liu, X., Fu, B., Chen, J., & Yu, H. Q. (2012). Acidogenic fermentation of proteinaceous sewage sludge: Effect of pH. *Water Research*, *46*(3), 799–807. https://doi.org/10.1016/j.watres.2011.11.047

Lowry Lewis. (1951). The folin by oliver. *Readings*, *193*(1), 265–275. https://doi.org/10.1016/0304-3894(92)87011-4

Mangayil, R., Santala, V., & Karp, M. (2011). Fermentative hydrogen production from different sugars by Citrobacter sp. CMC-1 in batch culture. *International Journal of Hydrogen Energy*, *36*(23), 15187–15194. https://doi.org/10.1016/j.ijhydene.2011.08.076

Masset, J., Hiligsmann, S., Hamilton, C., Beckers, L., Franck, F., & Thonart, P. (2010). Effect of pH on glucose and starch fermentation in batch and sequenced-batch mode with a recently isolated strain of hydrogen-producing Clostridium butyricum CWBI1009. *International Journal of Hydrogen Energy*, *35*(8), 3371–3378. https://doi.org/10.1016/j.ijhydene.2010.01.061

Massey, L. K., & Sokatch, J. R. (1976). Branched-chain amino acid catabolism in bacteria. *... and Molecular Biology ...*, *40*(1), 42–54. Retrieved from http://mmbr.asm.org/cgi/reprint/40/1/42.pdf%5Cnpapers2://publication/uuid/53E3C8D0-6756-4B75-BE8A-C4F88B59D967

Mata-Alvarez, J., Macé, S., & Llabrés, P. (2000). Anaerobic digestion of organic solid wastes. An overview of research achievements and perspectives. *Bioresource Technology*, *74*(1), 3–16. https://doi.org/10.1016/S0960-8524(00)00023-7

Mengmeng, C., Hong, C., Qingliang, Z., Shirley, S. N., & Jie, R. (2009). Optimal production of polyhydroxyalkanoates (PHA) in activated sludge fed by volatile fatty acids (VFAs) generated from alkaline excess sludge fermentation. *Bioresource Technology*, *100*(3), 1399–1405. https://doi.org/10.1016/j.biortech.2008.09.014

Moser-Engeler, K.M Udert, Wild, S. (1998). 1998_-_R_Moser_Engeler_-_Productsfromprimarysludgefermentationandtheirsuita[retrieved_2018-10-22].pdf.

Nasr, N., Elbeshbishy, E., Hafez, H., Nakhla, G., & El Naggar, M. H. (2011). Bio-hydrogen production from thin stillage using conventional and acclimatized anaerobic digester sludge. *International Journal of Hydrogen Energy*, *36*(20), 12761–12769. https://doi.org/10.1016/j.ijhydene.2011.07.032

Niu, K., Zhang, X., Tan, W. S., & Zhu, M. L. (2010). Characteristics of fermentative hydrogen production with Klebsiella pneumoniae ECU-15 isolated from anaerobic sewage sludge. *International Journal of Hydrogen Energy*, *35*(1), 71–80. https://doi.org/10.1016/j.ijhydene.2009.10.071

Oh, Y. K., Seol, E. H., Lee, E. Y., & Park, S. (2002). Fermentative hydrogen production by a new chemoheterotrophic bacterium Rhodopseudomonas palustris P4. *International Journal of Hydrogen Energy*, *27*(11–12), 1373–1379. https://doi.org/10.1016/S0360-3199(02)00100-3

Owen, W. F., Stuckey, D. C., Healy, J. B., Young, L. Y., & McCarty, P. L. (1979). Bioassay for monitoring biochemical methane potential and anaerobic toxicity. *Water Research*, *13*(6), 485–492. https://doi.org/10.1016/0043-1354(79)90043-5

Peng, L., Xu, X., Mu, H., Høy, C., & Adler-nissen, J. (2002). Production of structured phospholipids by lipase-catalyzed acidolysis : optimization using response surface methodology, *31*, 523–532.

Prakasham, R. S., Brahmaiah, P., Sathish, T., & Sambasiva Rao, K. R. S. (2009). Fermentative biohydrogen production by mixed anaerobic consortia: Impact of glucose to xylose ratio. *International Journal of Hydrogen Energy*, *34*(23), 9354–9361. https://doi.org/10.1016/j.ijhydene.2009.09.104

Puhakka, J. A., Karadag, D., & Nissilä, M. E. (2012). Comparison of mesophilic and thermophilic anaerobic hydrogen production by hot spring enrichment culture. *International Journal of Hydrogen Energy*, *37*(21), 16453–16459. https://doi.org/10.1016/j.ijhydene.2012.02.121

Ramachandran, U., Wrana, N., Cicek, N., Sparling, R., & Levin, D. B. (2008). Hydrogen production and end-product synthesis patterns by Clostridium termitidis strain CT1112 in batch fermentation cultures with cellobiose or α-cellulose. *International Journal of Hydrogen Energy*, *33*(23), 7006–7012. https://doi.org/10.1016/j.ijhydene.2008.09.022

Ramsay, I. R., & Pullammanappallil, P. C. (2001). Protein degradation during anaerobic wastewater treatment. *Biodegradation*, *12*, 247–257.

Randall, A. A., Chen, Y., Liu, Y. H., & McCue, T. (2003). Polyhydroxyalkanoate form and polyphosphate regulation: Keys to biological phosphorus and glycogen transformations? *Water Science and Technology*, *47*(11), 227–233.

Ren, N., Cao, G., Wang, A., Lee, D. J., Guo, W., & Zhu, Y. (2008). Dark fermentation of xylose and glucose mix using isolated Thermoanaerobacterium ihermosaccharolyticum W16. *International Journal of Hydrogen Energy*, *33*(21), 6124–6132. https://doi.org/10.1016/j.ijhydene.2008.07.107

Ren, N., Li, J., Li, B., Wang, Y., & Liu, S. (2006). Biohydrogen production from molasses by anaerobic fermentation with a pilot-scale bioreactor system. *International Journal of Hydrogen Energy*, *31*(15), 2147–2157.

https://doi.org/10.1016/j.ijhydene.2006.02.011

Ren, N. Q., Cao, G. L., Guo, W. Q., Wang, A. J., Zhu, Y. H., Liu, B. feng, & Xu, J. F. (2010). Biological hydrogen production from corn stover by moderately thermophile Thermoanaerobacterium thermosaccharolyticum W16. *International Journal of Hydrogen Energy*, *35*(7), 2708–2712. https://doi.org/10.1016/j.ijhydene.2009.04.044

Ren, N. Q., Guo, W. Q., Wang, X. J., Xiang, W. S., Liu, B. F., Wang, X. Z., … Chen, Z. B. (2008). Effects of different pretreatment methods on fermentation types and dominant bacteria for hydrogen production. *International Journal of Hydrogen Energy*, *33*(16), 4318–4324. https://doi.org/10.1016/j.ijhydene.2008.06.003

Ren, Y., Wang, J., Liu, Z., Ren, Y., & Li, G. (2009). Hydrogen production from the monomeric sugars hydrolyzed from hemicellulose by Enterobacter aerogenes. *Renewable Energy*, *34*(12), 2774–2779. https://doi.org/10.1016/j.renene.2009.04.011

Ren, Z., Ward, T. E., Logan, B. E., & Regan, J. M. (2007). Characterization of the cellulolytic and hydrogen-producing activities of six mesophilic Clostridium species. *Journal of Applied Microbiology*, *103*(6), 2258–2266. https://doi.org/10.1111/j.1365-2672.2007.03477.x

Riaño, B., Molinuevo, B., & García-González, M. C. (2011). Potential for methane production from anaerobic co-digestion of swine manure with winery wastewater. *Bioresource Technology*, *102*(5), 4131–4136. https://doi.org/10.1016/j.biortech.2010.12.077

Ries, E. F., & Macedo, G. A. (2011). Improvement of Phytase Activity by a New Saccharomyces cerevisiae Strain Using Statistical Optimization, *2011*. https://doi.org/10.4061/2011/796394

Romão, B. B., Batista, F. R. X., Ferreira, J. S., Costa, H. C. B., Resende, M. M., & Cardoso, V. L. (2014). Biohydrogen production through dark fermentation by a microbial consortium using whey permeate as substrate. *Applied Biochemistry and Biotechnology*, *172*(7), 3670–3685. https://doi.org/10.1007/s12010-014-0778-5

Shin, H. S., & Youn, J. H. (2005). Conversion of food waste into hydrogen by thermophilic acidogenesis. *Biodegradation*, *16*(1), 33–44. https://doi.org/10.1007/s10531-004-0377-9

Silvestre, G., Fernández, B., & Bonmatí, A. (2015). Significance of anaerobic digestion as a source of clean energy in wastewater treatment plants. *Energy Conversion and Management*, *101*, 255–262. https://doi.org/10.1016/j.enconman.2015.05.033

Sinha, P., & Pandey, A. (2011). An evaluative report and challenges for fermentative biohydrogen production. *International Journal of Hydrogen Energy*, *36*(13), 7460–7478. https://doi.org/10.1016/j.ijhydene.2011.03.077

Stein, H. (n.d.). Amino Acid Composition of Lactoglobulin and Bovine Serum Albumin, (3).

Tanisho, S., & Ishiwata, Y. (1995). Continuous hydrogen production from molasses by fermentation using urethane foam as a support of flocks. *International Journal of Hydrogen Energy*, *20*(7), 541–545. https://doi.org/10.1016/0360-3199(94)00101-5

Tommaso, G., Ribeiro, R., Varesche, M. B. A., Zaiat, M., & Foresti, E. (2000). Influence of multiple substrates on anaerobic protein degradation in a packed-bed bioreactor Gas line Affluent Effluent, 23–31.

Ueno, Y., Otsuka, S., & Morimoto, M. (1996). Hydrogen production from industrial wastewater by anaerobic microflora in chemostat culture. *Journal of Fermentation and Bioengineering*, *82*(2), 194–197. https://doi.org/10.1016/0922-338X(96)85050-1

Uyar, B., Eroglu, I., Yücel, M., & Gündüz, U. (2009). Photofermentative hydrogen production from volatile fatty acids present in dark fermentation effluents. *International Journal of Hydrogen Energy*, *34*(10), 4517–4523. https://doi.org/10.1016/j.ijhydene.2008.07.057

Van Ginkel, S. W., Oh, S. E., & Logan, B. E. (2005). Biohydrogen gas production from food processing and domestic wastewaters. *International Journal of Hydrogen*

Energy, *30*(15), 1535–1542. https://doi.org/10.1016/j.ijhydene.2004.09.017

Van Niel, E. W. J., Budde, M. A. W., De Haas, G., Van der Wal, F. J., Claassen, P. A. M., & Stams, A. J. M. (2002). Distinctive properties of high hydrogen producing extreme thermophiles, Caldicellulosiruptor saccharolyticus and Thermotoga elfii. *International Journal of Hydrogen Energy*, *27*(11–12), 1391–1398. https://doi.org/10.1016/S0360-3199(02)00115-5

Venkata Mohan, S., Lalit Babu, V., & Sarma, P. N. (2008). Effect of various pretreatment methods on anaerobic mixed microflora to enhance biohydrogen production utilizing dairy wastewater as substrate. *Bioresource Technology*, *99*(1), 59–67. https://doi.org/10.1016/j.biortech.2006.12.004

Wang, J., & Wan, W. (2009). Factors influencing fermentative hydrogen production: A review. *International Journal of Hydrogen Energy*, *34*(2), 799–811. https://doi.org/10.1016/j.ijhydene.2008.11.015

Wang, Y. Y., Ai, P., Hu, C. X., & Zhang, Y. L. (2011). Effects of various pretreatment methods of anaerobic mixed microflora on biohydrogen production and the fermentation pathway of glucose. *International Journal of Hydrogen Energy*, *36*(1), 390–396. https://doi.org/10.1016/j.ijhydene.2010.09.092

Wei, Y. (2004). Enhanced Biohydrogen Production from Sewage Sludge with Alkaline, *38*(11), 3195–3202.

Xia, Y., Cai, L., Zhang, T., & Fang, H. H. P. (2012). Effects of substrate loading and co-substrates on thermophilic anaerobic conversion of microcrystalline cellulose and microbial communities revealed using high-throughput sequencing. *International Journal of Hydrogen Energy*, *37*(18), 13652–13659. https://doi.org/10.1016/j.ijhydene.2012.02.079

Xiao, B., Han, Y., & Liu, J. (2010). Evaluation of biohydrogen production from glucose and protein at neutral initial pH. *Renewable Energy*, *35*(12), 6152–6160. https://doi.org/10.1016/j.ijhydene.2010.03.084

Yokoi, H., Maki, R., Hirose, J., & Hayashi, S. (2002). Microbial production of hydrogen from starch-manufacturing wastes. *Biomass and Bioenergy*, *22*(5), 389–395. https://doi.org/10.1016/S0961-9534(02)00014-4

Yokoi, Haruhiko, Saitsu, A., Uchida, H., Hirose, J., Hayashi, S., & Takasaki, Y. (2001). Microbial hydrogen production from sweet potato starch residue. *Journal of Bioscience and Bioengineering*, *91*(1), 58–63. https://doi.org/10.1016/S1389-1723(01)80112-2

Yokoyama, H., Waki, M., Moriya, N., Yasuda, T., Tanaka, Y., & Haga, K. (2007). Effect of fermentation temperature on hydrogen production from cow waste slurry by using anaerobic microflora within the slurry. *Applied Microbiology and Biotechnology*, *74*(2), 474–483. https://doi.org/10.1007/s00253-006-0647-4

Zhang, Y., Liu, G., & Shen, J. (2005). Hydrogen production in batch culture of mixed bacteria with sucrose under different iron concentrations. *International Journal of Hydrogen Energy*, *30*(8), 855–860. https://doi.org/10.1016/j.ijhydene.2004.05.009

Zheng, X., Chen, Y., & Liu, C. (2010). Waste activated sludge alkaline fermentation liquid as carbon source for biological nutrients removal in anaerobic followed by alternating aerobic-anoxic sequencing batch reactors. *Chinese Journal of Chemical Engineering*, *18*(3), 478–485. https://doi.org/10.1016/S1004-9541(10)60246-7

Zhu, H., & Béland, M. (2006). Evaluation of alternative methods of preparing hydrogen producing seeds from digested wastewater sludge. *International Journal of Hydrogen Energy*, *31*(14), 1980–1988. https://doi.org/10.1016/j.ijhydene.2006.01.019

Zhu, H., Suzuki, T., Tsygankov, A. A., Asada, Y., & Miyake, J. (1999). Hydrogen production from tofu wastewater by Rhodobacter sphaeroides immobilized in agar gels. *International Journal of Hydrogen Energy*, *24*(4), 305–310. https://doi.org/10.1016/S0360-3199(98)00081-0

Chapter 3

Effect of pH on the Production of Volatile Fatty Acids from a Proteinaceous Substrate[1]

3.1 Introduction

Several valuable products are generated through the development of renewable means utilizing biomass. Acidogenic fermentation is one of the processes that use acidogens to convert organic matter to volatile fatty acids (VFAs). VFAs are very essential substrates for diverse applications, including, the biological removal of nutrients (nitrogen and phosphorus) from wastewater (Zheng et al., 2010), biofuels (Uyar *et al.*, 2009; Choi et al., 2011), and the manufacturing of biodegradable plastics (Mengmeng et al., 2009). The commercial production of VFAs is generally through chemical processes that usually require high amounts of raw materials as non-renewable petrochemicals. Acidogenic fermentation can relatively enhance the recycling of organics and at the same time produce VFAs. Among the VFAs, acetate, and propionate have been observed to be the most essential substrates that buttress enhanced biological phosphorus removal (EBPR)(Randall *et al.*, 2003; Gerber *et al.*, 1986; Chen et al., 2004). Mengmeng *et al.*, (2009) reported that 6 – 9 mg of VFAs is required to biologically remove 1 mg of phosphorus. However, in wastewater especially when the influent chemical oxygen demand (COD) is very low, these levels of VFAs are not always available. Moreover, the removal of phosphorus is determined by the available VFAs supply as they are being

[1] A version of this chapter is currently under review in Biohemical Engineering Journal

consumed by other microorganisms (Mengmeng et al., 2009). Thus, VFAs supplementation becomes necessary to ensure adequate phosphorus removal efficiency.

To elevate the VFA concentration in the influent wastewater, two approaches have been implemented in order to enhance biological nutrient removal. The first strategy is by adding chemically produced VFAs such as acetic and propionic acids to wastewater in order to enhance biological nutrient removal (Chen et al, 2004). To minimize the cost of the supplementary carbon dosing and also achieve effluent requirements, another approach is the fermentation of sludge produced in wastewater treatment facilities (Moser-Engeler et al., 1998; Elefsiniotis et al., 2004).

One of the most influential parameters affecting the anaerobic digestion process is pH, and its effect has been extensively studied in the literature for carbohydrate-rich substrates and wastewaters (Fang & Liu, 2002). Alkaline pHs have been reported to enhance the solubilization of solid organic matter in sludge, thereby increasing their bioavailability to acidogenic microorganisms in anaerobic bioreactors (Zheng et al., 2010). As proteins also form a major component of organic matter in wastewaters and its fractional composition usually comes next to that of carbohydrates, there is, however, limited knowledge on the acidification of proteins in the literature, thus necessitating the need for an extensive examination of proteins acidification under both acidic and alkaline conditions.

For instance, the protein content of dairy wastewater contributes to over forty percent of the entire chemical oxygen demand (Wei, 2004). Processing industries including cheese, whey, fish, abattoir, and some other vegetable processing generally produce significant

volumes of protein-containing wastewater effluents. Anaerobic degradation models for the fermentation of sewage sludge and the treatment of wastewater have been extensively examined. Nonetheless, most of these studies centered on the degradation of complex carbohydrates (Yokoi *et al.*, 2001; Ueno et al., 1996; Tanisho et al., 1995; Zhu *et al.*, 1999), as they constitute the major organic matter. Carbohydrate hydrolysis, digestion of sugars, acetogenesis, and methanation were used to obtain the yields of biomass, substrate consumption, and product formation in the models. With the knowledge of these, coupled with the reaction kinetics, the mass balance equations were derived. In the same vein, mathematical models that describe protein degradation under anaerobic conditions can also be generated by the same aforementioned procedure. Nonetheless, the feasibility of this would require protein degradation stoichiometry.

Relatively few studies reported the anaerobic degradation of proteins (Liu et al., 2012; Ramsay & Pullammanappallil, 2001; Cheng et al., 2002) for the purposes of modeling. Typically, the overall protein catabolic reaction resulting in the yields of acetate, propionate, butyrate, ammonia, and carbon dioxide was obtained from an average chemical formula for proteins. This kind of stoichiometric equation would obviously have narrow applicability. The stoichiometry developed from known mechanisms and reaction pathways is an alternative approach and could have a generic and wider scope of application.

Carbohydrate degradation studies present the stoichiometry for the fermentation of intermediary products like propionate and butyrate to acetate and that of methane production from acetate and hydrogen. However, the stoichiometric reactions of the remaining organics which predominantly include proteins hydrolysis and the subsequent

fermentation of amino acids need to be examined, and the relevance of this knowledge to the anaerobic mixed-culture condition has not been discussed in the literature.

In this paper, a methodology was explored to develop the stoichiometric reaction for the degradation of proteins to organic acids based on microbial degradation studies documented in the literature. For the purposes of illustration, the method was applied to BSA as the model protein to evaluate stoichiometric coefficients which were then compared to actual values obtained from laboratory anaerobic batch studies.

3.2 Materials and Methods

3.2.1 Batch Expeimental Setup

Batch anaerobic experiments were conducted to examine the influence of pH (5 – 9) on the acidification of BSA as the model protein. The batches were run at a working volume of 200 mL in a series of 250 mL serum reactors. The seed for the fermentation tests was an anaerobic digester sludge obtained from the Stratford municipal treatment facility (Stratford, Ontario, Canada). In an effort to get rid of the soluble organics that may interfere with the fermentation process, 500 mL of sludge sample diluted in 1 L of distilled water was centrifuged at 3000 rpm for 20 min. After decanting the supernatant, the pellets were re-suspended in 1000 mL of distilled water and was followed by heating at 70 ℃ for 30 min in order to suppress methanogenic bacteria. To each serum reactor, 30 mL of the inoculum, 40 mL of BSA stock solution of 25 g/L (i.e BSA in the bottle is 5 g/L), 10 mL nutrient solution (composition shown in Table 3.1) and 120 mL of distilled water was added. The initial pHs were adjusted accordingly to 5.03 ± 0.01, 6.03 ± 0.02, 7.02 ± 0.01, 8.04 ± 0.01, and 9.02 ± 0.02 with 0.5 N HCl and NaOH solutions. A total of 21 bottles were prepared for each initial pH and at each pH value, three controls

without BSA were used and the remaining were sacrificial bottles for liquid analyses. Three bottles were sacrificed each time for liquid analyses and all results reported were the average values and standard deviations of the triplicates analyzed. An oxygen-free nitrogen gas (99.9%) was used to sparge the headspace of each serum bottle for 2 min to ensure anaerobic conditions. The reactors were finally placed in a swirling action shaker (Max 4000, Incubated and Refrigerated Shaker, Thermo Scientific, Ca) that operated at 160 – 180 rpm and at a temperature of 37 $\pm$ 1 ℃. The pH was not controlled throughout the fermentation period.

Table 3.1. Composition of the nutrient solution

Component	Conc., g/L
K_2HPO_4	250
$MgSO_4.7H_2O$	100
$CaCl_2.2H_2O$	10
$FeCl_2.4H_2O$	2
H_3BO_3	0.05
$ZnCl_2$	0.05
$CuCl_2$	0.03
$MnCl_2.4H_2O$	0.5
$(NH_4)_6Mo_7O_{24}$	0.05
$AlCl_3$	0.05
$CoCl_2.6H_2O$	0.05
$NiCl_2$	0.05

3.2.2 Analytical Methods

The biogas generated in the anaerobic reactors was periodically released using 5 – 100 mL glass sized syringes. The liquid samples were filtered through a 0.45 μm paper prior to using the gas chromatograph (Varian, 8500, Varian Inc. Toronto, Canada) with a flame ionization detector equipped with a fused silica column to measure VFA concentrations,

inclusive of acetic, propionic, butyric, iso-butyric, valeric and iso-valeric acids. Methanol, ethanol, propanol, and butanol were the alcohols analyzed. Helium was the carrier gas and it flowed at 5 mL/min and the detector and column temperatures were 250 ℃ and 110 ℃, respectively. The carbohydrate and protein concentrations were determined by the phenol-sulphuric acid method (Dubois et al.,1956) using glucose as the standard solution, and the Lowry-Folin method (Lowry et al., 1951) using BSA as the protein standard solution, respectively. The procedures of Standard Methods (APHA, 2005) were followed to determine COD, NH_3, TSS and VSS concentrations. The pH measurement was by a digital pH meter (VWR, B10P, SympHony).

3.3 Results and Discussion

3.3.1 Substrate degradation

Table 3.2 shows the characteristics of the diluted seed pellets in 1 L of distilled water after centrifuging and decanting the seed supernatant. The initial characterization of the acidogenic reactors fermented under the different pH conditions is depicted in Table 3.3. It is noteworthy that BSA is completely soluble and therefore does not undergo hydrolysis. Moreover, the only source of carbohydrate and particulate proteins in the reactors was from the seed biomass. From Table 3.3, the initial soluble proteins concentration in all the reactors was 5100 ± 20 mg/L and that of total carbohydrates was 395 ± 15 mg/L. Obviously, soluble proteins accounted for almost all the organic matter in the anaerobic reactors whereas carbohydrates constituted a negligible fraction. Thus, the liquid organic by-products such as VFAs and alcohols that resulted from the acidification of carbohydrates had an insignificant effect and were therefore not taken into consideration.

Fig. 3.1 shows the degradation profiles of the soluble proteins for the five different initial pH values. The soluble proteins are bioavailable to acidogenic microorganisms and their degradation efficiency increased with increasing pH. Fermentation under alkaline conditions resulted in higher consumption of soluble proteins than acidic or neutral pH. This high consumption at pHs 8 and 9 indicated that alkaline conditions provided a favorable environment for substrate degradation by acidogenic microorganisms.

Table 3.2. Characteristics of the diluted inoculum used for the fermentation test[a]

Parameter	Average	Standard Deviation
pH	6.8	0.1
TSS	7404	162
VSS	4400	60
TCOD	7800	170
Particulate carbohydrate	2100	80
Soluble carbohydrate	530	30
Particulate proteins	1700	100
Soluble proteins	603	23
Ethanol	105	15
Propanol	84	12
Butanol	65	5
Nitrogen	323	13
Ammonia	25	8
TVFA	200	5

[a]All values are expressed in mg/L excluding pH

Table 3.3. Initial reactor conditions of the fermentation tests

pH	VSS (mg/L)	Particulate proteins (mg/L)	Soluble proteins (mg/L)	Particulate carbs (mg/L)	Soluble carbs (mg/L)	Ammonia (mg/L)	Ethanol (mg/L)	Propanol (mg/L)	Butanol (mg/L)	TVFA (mg/L)
5	658 ± 10	250 ± 15	5100 ± 30	310 ± 2	78 ± 2	3.8 ± 0.3	13.5 ± 0.5	11.5 ± 1.6	8.2 ± 1.2	30 ± 2
6	660 ± 20	265 ± 10	5050 ± 10	308 ± 4	84 ± 6	4.2 ± 0.6	14.6 ± 1.2	11.8 ± 1.2	7.8 ± 1.4	28 ± 2
7	650 ± 10	260 ± 20	5110 ± 25	312 ± 8	82 ± 4	4.4 ± 0.1	13.8 ± 0.2	11.4 ± 0.6	9.2 ± 2.1	29 ± 1
8	662 ± 12	248 ± 12	5120 ± 20	314 ± 2	86 ± 8	4.6 ± 0.4	14.2 ± 0.1	11.6 ± 0.8	8.8 ± 1.8	30 ± 2
9	668 ± 8	255 ± 5	5160 ± 40	316 ± 6	80 ± 2	4.3 ± 0.2	13.6 ± 0.3	12.2 ± 0.4	8.4 ± 0.4	29 ± 1

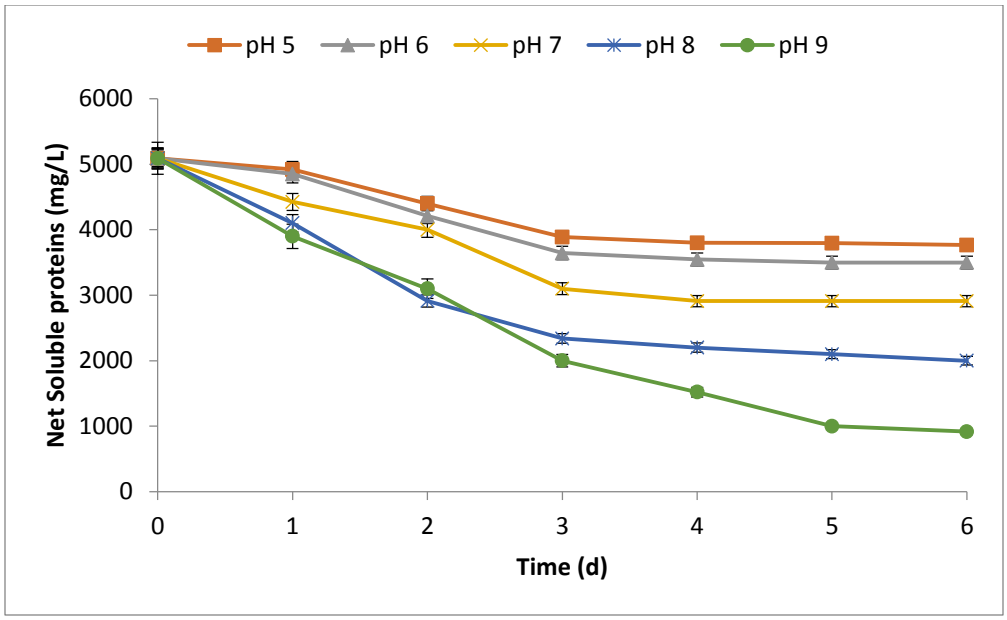


Fig. 3.1 Substrate degradation at the different initial pH conditions at a temperature of 37 ℃.

3.3.2 Effect of pH on VFA production

The volatile fatty acid production trend of BSA as influenced by the different initial pH values is shown in Fig.3.2. The initial TVFAs for the acidogenic reactors on the average was 30 mgCOD/L. During the first 2 days of fermentation, the order of TVFAs was: pH 8 (934 mgCOD/L) > pH 9 (500 mgCOD/L) > pH 7 (455 mgCOD/L) > pH 6 (400 mgCOD/L) > pH 5 (314 mgCOD/L). With the increase in fermentation time to 4 days, there was a marked increase in the TVFAs production with the exception at pH 5, and a similar trend of pH was noticed as that on day 2, that is, pH 8 (127 mgCOD/L) > pH 9 (1000 mgCOD/L) > pH 7 (930 mgCOD/L) > pH 6 (682 mgCOD/L) > pH 5 (325 mgCOD/L). Further examination showed that the TVFAs production on day 4 increased

linearly ($y_{TVFA} = 309pH - 1204, R^2 = 0.99$) with pH from 5 to 8. A further increase in the fermentation time, however, except at pH 9, did not reflect in the increase in TVFA production. As depicted in Fig. 3.2a, the TVFAs at pH 9 continued to rise with time reaching 1120 and 1285 mgCOD/L on days 5 and 6, respectively.

The foregoing analysis and results suggest that a significant amount of VFA can be produced and stabilized at an initial fermentation pH of 8. Granted, high VFA could also be produced at pH 9, but a much longer time (6 days) was required to produce a similar amount to that at pH 8 on day 3. It can be inferred that the desired conditions for VFA production from BSA were an initial pH of 8 and incubation time of 3 days. The relatively lower TVFAs (80 mgCOD/L) produced at pH 9 on day 1 could be associated with the extreme environment presented by the stronger alkaline condition to the acidogens responsible for the acidogenic fermentation process.

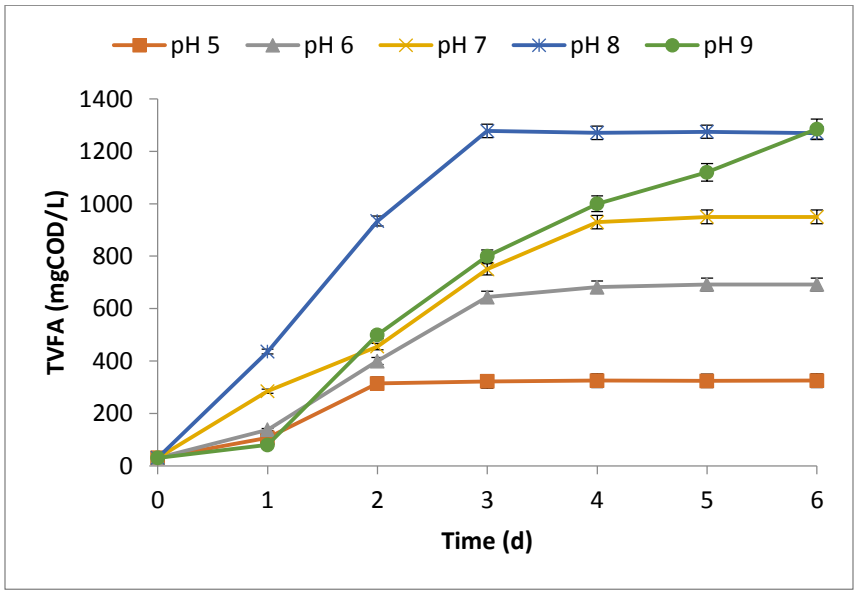
The anaerobic digestion process can be holistically considered in three principal stages; hydrolysis, acidogenesis, and finally, methanogenesis. VFA production depends on the provision of substrates for the acidogens and subsequent production of methane by methanogenic activity. In this study, the preheating of the seed sludge at 70 ℃ for 30 min prior to inoculation inhibited methane-producing bacteria responsible for VFA utilization (Silvestre et al., 2015; Riaño et al., 2011; Owen et al., 1999) as confirmed by no methane production by the biogas analysis.

Cheng et al. (2002) reported that alkaline pH enhanced acetate production in a thermophilic anaerobic degradation of peptone and the proportion in the total VFA increased significantly. In our study, six single VFAs; acetate, propionate, butyrate, iso-

valeric, iso-butyrate and valeric were produced. As depicted in Fig. 3.2b, at pH 5, there were only three acids produced; acetate (37.4%), propionate (13.7%) and butyrate (48.9%) in which butyrate was the most prevalent VFA. Over the range of pH examined, all the six volatile acids were observed and the percentage of acetate increased from 24.1% at pH 7.0 to 48.2% at pH 9.0. The proportions of acetate were higher under alkaline condition relative to acidic pH ($P < 0.05$). Contrarily, the fraction of valeric acid declined steadily when the pH was increased. Valeric and iso-butyric acids declined from 14.8%, and 19.9% at pH 6 to 6.0% and 8.1% at pH 9, respectively. In addition, the proportions of propionic acid were relatively stable at pH 6 - 9 whereas iso-valeric acid was essentially the same for pHs 8 and 9.

The soluble protein degradation rates, ($r_{sol.\ p}$) were impacted by the initial pH values. The higher the initial pH, the higher the rate of soluble proteins consumption. The maximum absolute soluble proteins consumption rates for pH 5 - 9 obtained from the slope of the linear fits of Fig. 3.1 are in the order: pH 9 (711 mg/L.d) > pH 8 (499 mg/L.d) > pH 7 (381 mg/L.d) > pH 6 (291 mg/L.d) > pH 5 (244 mg/L.d). Furthermore, there was a corresponding increase in the maximum TVFA (obtained from the slope of the linear fit of Fig. 3.2a) production rate (r_{TVFA}) with increasing pH, which were in the order: pH 5 (48 mg/L.d) < pH 6 (121 mg/L.d) < pH 7 (163 mg/L.d) < pH 8 (205 mg/L.d) < pH 9 (227 mg/L.d). The degree of volatile fatty acid production ($r_{TVFA}/r_{sol.\ p)}$ for the pH range studied was within the the range of 20% – 43% with pH 7 achieving the highest degree of VFA production and pH 5 the least.

(a)



(b)

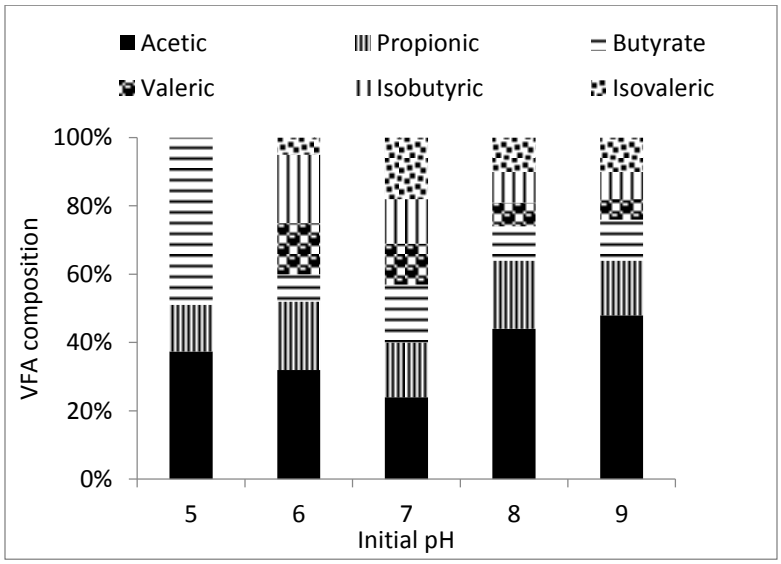


Fig.3.2. Production of VFA from BSA at 37 ℃ **(a)** Effect of pH values and fermentation time on TVFA production **(b)** Single VFAs distribution at the different initial pH values

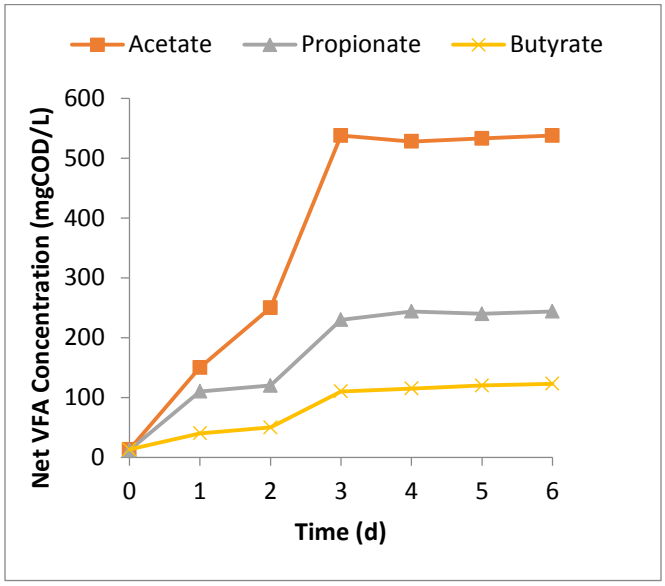
3.3.3 Changes in reactor conditions during acidification at the optimum pH of 8

Fig.3.3 shows the production of individual VFAs and alcohols. There was a rapid increase in the acetic, propionic, and butyric acid concentrations, achieving 150, 110, and 40 mgCOD/L on day 1, respectively. Their concentrations continued to increase thereafter and acetic acid plateaued at 538 mgCOD/L by day 3, propionic acid at 244 mgCOD/L on day 4 and butyric acid at 123 mgCOD/L by day 5.

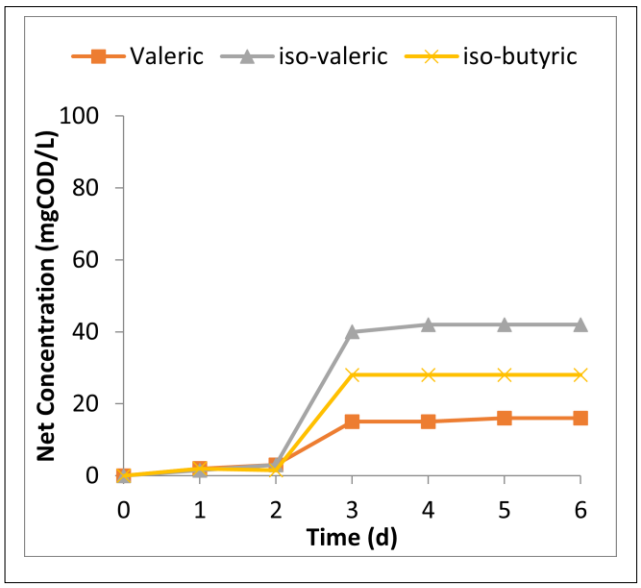
Contrarily, Fig.3.3b depicts that the production of the higher molecular weight volatile acids, that is, valeric, iso-valeric and iso-butyrate were largely time-dependent and not directly in response to proteins degradation. In the first 2 days, their concentrations were negligible even though proteins were significantly degraded. Thereafter, their concentrations increased reaching 15, 25, and 40 mgCOD/L, respectively, by day 3 and remained stable over the fermentation period. These higher molecular weight acids could be produced either by the Stickland reaction or through the individual amino acid reductive de-amination process (Gallert et al., 1998). Nonetheless, the production of these three higher molecular weight acids was not statistically significant ($P>0.05$) compared with acetic, propionic and butyric acids.

As shown in Fig. 3.3c, alcohol production was relatively lower than VFAs. Ethanol dominated the three alcohols produced, peaking at 32 mgCOD/L on day 6. The production of propanol and butanol was similar, each reaching a maximum concentration of 15 mgCOD/L by day 6. Throughout the 6-day acidification test, methanol was not detected.

(a)


Acetate
Propionate
Butyrate
Net VFA Concentration (mgCOD/L)
600
500
400
300
200
100
0
0
1
2
3
4
5
6
Time (d)

(b)



(c)

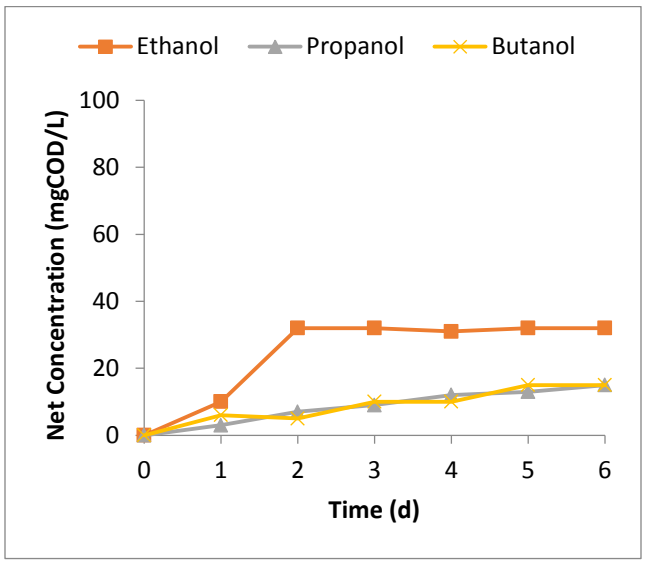


Fig. 3.3. Changes in reactor conditions during a 6-day acidogenic fermentation test at a pH of 8 and at 37 ℃ **(a)** dominant VFA concentrations **(b)** higher molecular weight VFA concentrations **(c)** concentrations of alcohols

3.4 Development of stoichiometry for protein degradation

Microbial degradation studies of amino acid fermentation in both pure and mixed culture environments and their mediated reactions are presented in this section. Based on this information, the stoichiometry for the fermentation of proteins was derived.

Proteins consist of amino acid units linked to one another by peptide or amide bonds and are being hydrolyzed by enzymes called proteases into peptides and amino acids(Caccavo et al., 1994). There are significant variations in the size and structure of amino acids. Amino acids degrade via diverse pathways by virtue of nature and concentration involved(Massey & Sokatch, 1976). Organic acids, ammonia, carbon dioxide and small amounts of hydrogen and sulfur-containing compounds are the most common products of amino acid fermentation. (Randall et al., 2003). Several researchers have examined the fermentation of amino acids including (Massey et al. 1996; Kotzé et al. 1999; Kinoshita et al. 2008). A brief summary of the biochemical reactions of amino acid fermentation by the aforementioned papers is provided below.

Amino acids are notably degraded in two ways: (1) A pair of amino acids may follow the Stickland reaction to be degraded; (2) A single amino acid may be degraded in the presence of hydrogen-utilizing bacteria. Amino acid biodegradation typically follows the Stickland reaction. This is an oxidation-reduction reaction process in which one amino acid acts as an electron donor and the other an electron acceptor. Some amino acids such as Leucine can serve as both electron donor and acceptor. The Stickland reaction provides the cell with closely 0.5 mole ATP per mole amino acid converted (Freudenberg et al., 1989). Amino acid decomposition through the Stickland reaction occurs swiftly relative to uncoupled amino acid fermentation (Kinoshita et al., 2008).

Table 3.4 shows a list of anaerobic bacteria commonly known to ferment amino acids. On the premise of the work by Mead (1977) and McInerney (1988), five bacteria groups (I-IV) have been identified based on their involvement in Stickland reactions and the typical amino acid utilized. Table 4 also presents the nature of the enzyme produced by each bacteria established on the information from Hippe et al. (1992).

Group I bacteria carry out Stickland reactions. These bacteria all degrade proline in the fermentation process to produce intermediates as α-aminobutyrate, δ-aminovalerate and γ-aminobutyrate. Clostridial species have only been identified with this type of reaction. The commonly known amino acids involved in Stickland reactions are proline, arginine, ornithine, glycine, leucine, isoleucine, valine, serine, lysine, alanine, cysteine, methionine, aspartate, threonine, phenylalanine, tyrosine, and tryptophan.

Microorganisms that are not involved in Stickland reactions but degrade amino acids are listed in Groups II, III, IV, and V. These are predominantly obligate spore-formers and some non-sporing obligate anaerobes. Glycine is being utilized by all of Group II bacteria and some species also degrade arginine, histidine, and lysine. All of Group III bacteria utilize histidine, serine, and glutamate and other species decompose arginine, aspartate, threonine, trypsin, and tryptophan. Group IV bacteria which is only *C. putrefaciens* utilize serine and threonine. *C. propionicum* which is only in Group V use alanine, serine, threonine, and Cysteine. δ-aminovalerate production which is a characteristic of Stickland reactions is not being produced by any of these bacteria.

With a mixture of amino acids under a mixed-culture condition, uncoupled fermentation of amino acids occurs only when there is a deficit in the amino acids that are electron

acceptors (Lane & Nor, 1994). For some proteins such as casein, albumin, and gelatin, this would only account for not more than 10% of the entire amino acids degraded (Lane & Nor, 1994). This thus suggests that amino acids are preferably fermented through Stickland reactions during anaerobic conditions.

Table 3.5 provides a summary of the stoichiometric equations for the fermentation of various amino acids. These equations entail the most common pathways described in the literature but not inclusive of the pathways of some specific bacterial species such as *C. propionicum.* In most cases, a single amino acid degrades via more than one pathway. Therefore, all the reactions have been denoted either as Stickland or non-Stickland reactions.

Table 3.4 Anaerobic bacteria groups which degrade amino acids

Group	Species	Enzyme produced	Amino acids involved	Characteristics
I	C. bifermentans	proteo, saccharolytic	proline,serine,	all species utilize
	C. sordellii	proteo, saccharolytic	argine, glycine	proline
	C. botulinum	proteo/saccharolytic	leucine,	δ-aminovalerate
	C. caloritolerans	-	isoleucine, valine	α-aminobutyrate
	C. sporogenes	proteo, saccharolytic	ornithine,lysine,	γ-aminobutyrate
	C. cochlearium	specialist	alanine	
	C. difficile	saccharolytic	cysteine,	produced
	C. putrificum	proteo, saccharolytic	methionine,	
	C. sticklandii	specialist	aspartate	
	C. ghoni	proteolytic	threonine,	
	C. mangenotii	proteo, saccharolytic	phenylalanine	
	C. scatologenes	saccharolytic	tyrosine,	
	C. lituseburense	proteo, saccharolytic	tryptophan	
	C. aerofoetidum	-	glutamate	
	C. butyricum	saccharolytic		
	C. caproicum	-		
	C. carnofoetidum	-		
	C. indolicum	-		
	C. mitelmani	-		
	C. saprotoxicum	-		

II	C. botulinum C. histolyticum C. cochelearium C. histolyticum C. cochlearium C. subterminale C. botulinum P. anaerobius P. variabilis P. micros	proteo, saccharolytic proteolytic specialist proteolytic specialist proteolytic - - 	glycine, arginine, histidine lysine	all species use glycine δ-aminovalerate absent
III	C. cochlearium C. tetani C. tetanomorphum C. lentoputrescens C. limosum C. malenomenatum C. microsporum C. perfringens C. butyricum P. asaccharolyticus P. prevotii P. activus	specialist proteolytic saccharolytic - proteolytic specialist - proteo/saccharolytic saccharolytic - - -	glutamate, serine, histidine arginine, aspartate, threonine tyrosine, tryptophan, cysteine	δ-aminovalerate absent histidine, serine and glutamate utilized all species
IV	C. putrefaciens	proteolytic	Serine, threonine	δ-aminovalerate absent
V	C. propionicum	specialist	Alanine, serine, threonine, cysteine and methionine	δ-aminovalerate absent

Sources: Mead (1977); Kinoshita et al. (2008); Elsden & Hilton (1978, 1978 and 1979); Nisman (1954). C- Clostridium; P- Peptostreptococcus; Specialist – specialized species that utilize only one or few amino acids

Table 3.5. Catabolic reactions for amino acid fermentation (stoichiometry)

No.	Catabolic reaction	Type	Reference
1	$C_6H_{13}O_2N(Leu) + 2H_2O \rightarrow C_5H_{10}O_2(3-methylbutyrate) + NH_3 + CO_2 + 2H_2 + ATP$	Stickland	Elsden & Hilton (1978)
2	$C_6H_{13}O_2N(Leu) + H_2 \rightarrow C_6H_{12}O_2(4-methylvalerate) + NH_3$	Stickland	Elsden & Hilton (1978)
3	$C_6H_{13}O_2N(Ile) + 2H_2O \rightarrow C_5H_{10}O_2(2-$methylbutyrate) + $NH_3 + CO_2 + 2H_2 + ATP$	Stickland	Elsden & Hilton (1978)
4	$C_5H_{11}O_2N(val) + 2H_2O \rightarrow C_4H_8O_2\,(2-methylpropionate) + NH_3 + CO_2 + 2H_2 + ATP$	Stickland	Elsden & Hilton (1978), Mead (1977)
5	$C_9H_{11}O_2N(Phe) + 2H_2O \rightarrow C_8H_8O_2(phenylactate) + NH_3 + CO_2 + 2H_2 + ATP$	Stickland	Elsden & Hilton (1978)
6	$C_9H_{11}O_2N(Phe) + H_2 \rightarrow C_9H_{10}O_2(phenylpropionate) + NH_3$	Stickland	Elsden & Hilton (1978)
7	$C_9H_{11}O_2N(Phe) + 2H_2O \rightarrow C_6H_6(phenol) + C_2H_4O_2(acetate) + NH_3 + CO_2 + H_2 + ATP$	Non-Stickland	Elsden & Hilton (1978)
8	$C_9H_{11}O_3N(Tyr) + 2H_2O \rightarrow C_8H_8O_3(hydroxyphenol\ acetate) + NH_3 + CO_2 + 2H_2 + ATP$	Stickland	Elsden & Hilton (1978)
9	$C_9H_{11}O_3N(Tyr) + H_2 \rightarrow C_9H_{10}O_3(hydroxyphenyl\ propionate) + NH_3$	Stickland	Elsden & Hilton (1978)
10	$C_9H_{11}O_3N(Tyr) + 2H_2O \rightarrow C_6H_6O\ (cresol) + C_2H_4O_2(acetate) + NH_3 + CO_2 + H_2 + ATP$	Stickland	Elsden & Hilton (1976)
11	$C_{11}H_{12}O_3N_2(Trp) + 2H_2O \rightarrow C_{10}H_9O_2N(indole\ acetate) + NH_3 + CO_2 + 2H_2 + ATP$	Stickland	Elsden & Hilton (1976)
12	$C_{11}H_{12}O_3N_2(Tyr) + H_2 \rightarrow C_{11}H_{11}O_2(indole\ propionate) + NH_3$	Stickland	Elsden & Hilton (1976)
13	$C_{11}H_{12}O_3N_2(Trp) + 2H_2O \rightarrow C_8H_7N(indole) + C_2H_4O_2(acetic\ acid) + NH_3 + CO_2 + H_2$	Non-stickland	Elsden & Hilton (1976)

14	$C_2H_5O_2N(Gly) + H_2 \rightarrow C_2H_4O_2(acetate) + NH_3$	Stickland	Seto (1980)
15	$C_2H_5O_2N(Gly) + 1/2H_2O \rightarrow 3/4C_2H_4O_2(acetate)$ +NH_3 +1/2CO_2 + 1/4ATP	Non-Stickland	Lebertz et al (1988)
16	$C_3H_7O_2N(Ala) + 2H_2O \rightarrow C_2H_4O_2(acetate) + NH_3 + CO_2 + 2H_2 + ATP$	Stickland	Bader (1982)
17	$C_3H_6O_2N(Cys) + 2H_2O \rightarrow C_2H_4O_2(acetate) + NH_3 + CO_2 + H_2S + 1/2H_2 + ATP$	Stickland	Mead (1977)
18	$C_5H_{11}O_2NS(Met) + 2H_2O \rightarrow C_3H_6O_2(propionate) + CO_2 + NH_3 + CH_4S + H_2 + ATP$	Stickland	Elsden & Hilton (1978)
19	$C_3H_7O_3N(Ser) + H_2O \rightarrow C_2H_4O_2(acetate) + NH_3$ +CO_2 + H_2 + ATP	Either	Ely (1954)
20	$C_4H_9O_3N(Thr) + H_2O \rightarrow C_3H_6O_2(propionate) + NH_3 + CO_2 + H_2 + ATP$	Non-Stickland	Seto (1980)
21	$C_4H_9O_3N(Thr) + H_2 \rightarrow C_2H_4O_2(acetate) + 1/2C_4H_8O_2(butyrate)$+$NH_3$ + ATP	Stickland	Thressa (1959)
22	$C_4H_7O_4N(Asp) + 2H_2O \rightarrow C_2H_4O_2(acetate) + NH_3 + 2CO_2 + 2H_2 + 2ATP$	Either	Elsden & Hilton (1978)
23	$C_5H_9O_4N(Glu) + H_2O \rightarrow C_2H_4CO_2(acetate) + 1/2C_4H_8O_2(butyrate) + NH_3$ + CO_2 + 2ATP	Stickland	Mead (1977)
24	$C_5H_9O_4N(Glu) + 2H_2O \rightarrow C_2H_4O_4(acetate) + NH_3$+ CO_2 + H_2 + 2ATP	Non-Stickland	Bader (1982)
25	$C_6H_9O_2N_3(His) + 4H_2O \rightarrow CH_3ON(formamide) + C_2H_4O_2(acetate) +$ $1/2C_4H_8O_2$(butyrate) + 2NH_3 + CO_2 + 2ATP	Stickland	Barker (1961)
26	$C_6H_9O_2N_3(His) + 5H_2O \rightarrow CH_3ON(formamide) + 2C_2H_4O_2(acetate) + 2NH_3$ + CO_2 + H_2 + 2ATP	Non-Stickland	Barker (1961)
27	$C_6H_{14}O_2N_4(Arg) + 6H_2O \rightarrow C_2H_4O_2(acetate) + 4NH_3 + 2CO_2 + 3H_2 + 2ATP$	Stickland	Barker (1961)
28	$C_6H_{14}O_2N_4(Arg) + 3H_2O \rightarrow 1/2C_2H_4O_2(acetate) +$ 1/2$C_3H_6O_2$(propionate)+H_2 + ½ $C_5H_{10}O_2$ (valerate) + 4NH_3 + CO_2 + ATP	Stickland	Elsden & Hilton (1978)
29	$C_5H_9O_2N(Pro) + H_2O + H_2 \rightarrow 1/2C_2H_4O_2(acetate)$ +1/2 $C_3H_6O_2$ (propionate) + 1/2$C_5H_{10}O_2$ (valerate) +NH_3	Stickland	Mead (1977)

30	$C_6H_{14}O_2N_2(Lys) + 2H_2O \rightarrow C_2H_4O_2(acetate) + C_2H_4O_2(acetate) + C_4H_8O_2(butyrate)$+ $2NH_3$ +ATP	Either	Mead (1977)

3.4.1 Degradation of BSA

A methodology based on the application of the above information is presented in this section to develop the stoichiometry for the anaerobic degradation of BSA substrate.

Step 1 – Assumptions

In the derivation of the stoichiometry, a number of assumptions were made. First, as BSA is completely soluble, there was no hydrolysis step and this made the fermentation of the amino acids rapid. Second, the fermentation pathways followed by the amino acids were assumed to essentially remain constant and were dominated by a single pathway irrespective of substrate concentration and the incubation time. If the amino acids are being degraded and are not accumulating in the reactive system, and in the case that the initial assumption holds, then the odds that the fermentation pathways will change are not very likely. On the premise of these assumptions, the degradation of BSA to organic acids can be represented by an overall single catabolic reaction necessitated by a single bacterial group. The coefficients of this stoichiometric reaction in question would be determined.

Step 2 – Determination of amino acid content of BSA

The dominant amino acids that constitute BSA and their respective compositions were obtained from the literature (Stein, 1948), and are shown respectively in columns 1 and 2 of Table 3.6. This information was used to calculate the molecular formula for BSA and is represented as $C_{0.93}H_{1.90}O_{0.51}N_{0.23}$

Step 3 – The selection of dominant amino acids and catabolic reactions

Some amino acids are known to degrade in only one pathway, and in this case, were selected as the dominant fermentation pathways. These amino acids include; alanine,

serine, leucine, aspartate, lysine, cysteine, proline, valine, and methionine. With the other amino acids, several fermentation pathways were known to exist, and those that follow the Stickland reaction to being degraded were chosen as the dominant pathway. For instance, glycine, histidine, threonine, and glutamate were in this case (Moser-Engeler et al, 1998). It was observed that these Stickland reactions consumed or yielded little hydrogen with accompanying similar ATP yields relative to the other oxidation reactions and under anaerobic conditions are favorable energetically.

In a situation where a choice between Stickland reactions existed, preference was given to proteolytic bacteria mediated pathways. For instance, arginine reaction known for a range of proteolytic bacteria was chosen over one involved with the specialist bacteria, *C. Sticklandii*. Elsden & Hilton (1976) conducted batch studies and reported that the Stickland reaction, be it oxidation or reduction, is determined by the growth media and microbial species involved. Nevertheless, oxidation reactions that yielded ATP were assumed favorable in an anaerobic condition as a result of the general presence of hydrogen-consuming methane bacteria that has the potential to reduce the requirement for electron acceptors (Lane & Nor, 1991). Therefore, the oxidation reactions were chosen as the dominant for tryptophan, leucine, tyrosine, and phenylalanine.

Step – 4 Determination of the overall stoichiometry for BSA degradation to organic acids

The overall stoichiometry for BSA degradation to organic acids was determined based on the foregoing steps 1 – 3 and is shown in Table 3.6. Each column of the table denotes a product of amino acid degradation such as acetic, propionic, butyric, valeric, ammonia, carbon dioxide and hydrogen. For each product column, the

stoichiometric coefficient (α) for the product in the reaction equations (from Table 3.5) was placed. Thereafter, the amino acid content (on the basis of one carbon mole of BSA) was then multiplied by the corresponding stoichiometric coefficient of the product. The total summation gives the overall stoichiometric coefficient (α) for that product.

The overall degradation of BSA to organic acids represented by a single stoichiometric reaction is shown below, and with each overall α provided in Table 3.6 To maintain a correct carbon balance, all aromatic acids produced as a result of amino acid fermentation were lumped together as a single compound. This is necessary because aromatic amino acids can account for almost 20% of the protein carbon (Lane & Nor, 2001; Bau et al., 2013).

$C_{0.93}H_{1.90}O_{0.51}N_{0.23} + {}_{\alpha\,\text{water}}H_2O \rightarrow {}_{\alpha\,\text{acet}}CH_3COOH + {}_{\alpha\,\text{prop}}CH_3CH_2COOH + {}_{\alpha\,\text{buty}}CH_3CH_2CH_2COOH + {}_{\alpha\,\text{vale}}CH_3CH_2CH_2CH_2COOH + {}_{\alpha}NH_3 + {}_{\alpha\ CO2}CO_2 + {}_{\alpha\ H2}H_2 + \alpha_{aromatic\ acid}C_wH_xO_yN_z + \beta$ ATP

From table 3.6, the total stoichiometric coefficients of CO_2 and each organic acid product were multiplied by the total number of carbon atoms (in parenthesis) in each product and were added together in order to estimate the value of $\alpha_{aromatic\ acid}$ in the above equation. It was also assumed that the aromatic acid is composed of the minimum number of carbon atoms of 6, and therefore w = 6.

BSA	Acetic	Propionic	Butyric	Valeric	CO_2	$C_wH_xO_yN_z$
1(0.93) =	0.124(2) +	0.018(3) +	0.05(4) +	0.041(5) +	0.157(1) +	$6\alpha_{aromatic}$

From the above, $\alpha_{aromatic\ acid}$ is estimated to be 0.011

Table 3.6 Stoichiometric coefficient determination for BSA degradation

Amino Acid (AA)	**(mol content/mol c-BSA)**	**Acetic acid (mol/mol AA)***	**Propionic acid (mol/mol AA)**	**Butyric acid (mol/mol AA)**	**Valeric acid (mol/mol AA)**	**Ammonia (mol /mol AA)**	**Carbon dioxide (mol /mol AA)**	**Hydrogen (mol /mol AA)**	**ATP (mol/mol AA)**	**Eqn Used (Table 3.5)**
Arginine	0.005	0.5	0.5		0.5	4	1	1	1	28
Histidine	0.005	1		0.5		2	1		2	25
Lysine	0.0122	1		1		2			1	30
Tyrosine	0.0082	1				1	1	1	1	10
Tryptophan	0.0016					1	1	2	1	11
Phenylalanine	0.0078					1	1	2	1	5
Cysteine	0.0003	1				1	1	0.5	1	17
Methionine	0.0049		1			1	1	1	1	18
Threonine	0.0075	1		0.5		1		-1	1	21
Serine	0.0147	1				1	1	1	1	19
Leucine	0.0167				1	1	1	2	1	1
Isoleucine	0.0106				1	1	1	2	1	3

Valine	0.0128			1		1	1	2	1	4
Glutamate	0.0369	1		0.5		1	1		2	23
Aspartate	0.0124	1				1	2	2	2	22
Glycine	0.0058	1				1		-1		14
Alanine	0.0076	1				1	1	2	1	16
Proline	0.0217	0.5	0.5		0.5	1		-1		29
Total (α)		**0.124**	**0.018**	**0.050**	**0.041**	**0.225**	**0.157**	**0.134**	**0.219**	

*AA – Amino Acid

3.5 Experimental Validation

Anaerobic batch experiments were conducted using BSA as the principal substrate and anaerobic sludge as the seed in 250 mL serum reactors and at a working volume of 200 mL. In this case, the fermentation test was carried out at the optimum pH of 8, and the detailed experimental design and setup is as described above in section 3.2.1 of this paper. Three batch bottles were prepared in triplicate and 15 mL of samples were taken at each sampling event for liquid analyses. Two additional bottles were prepared with the seed only (no substrate addition) to discount the effect of the seed in the reactive system.

The measurements for this experiment included ammonia, hydrogen and organic acid concentrations. However, hydrogen production was negligible.

Two different batches of experimental runs were conducted. For run 1, the substrate concentration was 2 g/L and an incubation time of 3 days, whereas run 2 was operated under a 100% increase in substrate concentration (4 g/L) and at the same fermentation time. Nine sampling events were taken for the 3-day fermentation test for the measurement of ammonia and organic acids.

From the concentrations of ammonia and organic acid measured, a molar ratio of organic acid to ammonia was evaluated at each sampling event. By multiplying each ratio by 0.225 mole of NH_3 (stoichiometric yield of NH_3/mol amino acid degraded from Table 3.6), the stoichiometric coefficients were calculated and then averaged over the nine sampling events. The total stoichiometric values (in Table 3.6) for BSA fermentation to organic acids are tabulated in Table 3.7 and are compared with the experimental values

obtained for the two batch runs. The measured stoichiometric values shown are the mean and standard deviations. The comparative analysis was made using ammonia as the basis.

3.5.1 Analysis

The experimental stoichiometric values were compared with the theoretical and this comparison indicated that each coefficient was within the range of variation of the experiment, except propionic acid with a lower recorded ratio for the experiments. On examining Table 3.6, the fermentation of δ-aminovalerate which is an intermediary product of arginine and proline fermentation constitutes closely to 70% of the theoretical propionic acid produced (i.e. 0.013 of 0.018 in Table 3.6). This suggests that there was an alternative fermentation pathway for δ-aminovalerate. Nonetheless, the data in Table 3.7 does not clearly show the alternative product to propionic acid. However, this inconsistency only relatively represents a small portion of the entire acids within the reactive system.

The stoichiometric coefficients obtained for each organic acid from the two batch experimental runs were found to be similar. To provide a wider range for comparison, the individual butyric and valeric acid isomers were provided separately. From the two batch experimental runs, the butyric, valeric and propionic acids coefficients compared very well.

Over the course of the two batch experimental runs, there were changes in the coefficients for each organic acid product, and this reflected the influence of the BSA concentration on amino acid degradation pathways. With the first batch run, on the average, the stoichiometric coefficients varied by $\pm$ 15%, while those of the second batch

run varied by ±12%. The difference in substrate concentration imposed on the two reactive systems was extreme (100% increment for the batch run 2). With this wide concentration difference and the values of ±15% and ±12% for stoichiometric coefficients reinforce the assumption that amino acid fermentation pathways remained the same and that the fermentation of proteins can be described by a single stoichiometry.

Table 3.7 – Theoretical and measured stoichiometric coefficients for batch anaerobic fermentation of BSA

Amino acid fermentation product	**Measured stoichiometric coefficient (Run 1)**	**Measured stoichiometric coefficient (Run 2)**	**Theoretical stoichiometric coefficient**
Acetic acid	0.142(±0.011, 7.7%)	0.114(±0.012, 11%)	0.124
Propionic acid	0.006(±0.001, 17%)	0.007(±0.001, 14%)	0.018
Butyric acid	0.048(±0.006, 13%)	0.045(±0.007, 11%)	0.050
• Iso	0.015(±0.002, 13%)	0.015(±0.002, 13%)	0.013
Valeric acid	0.045(±0.005, 11%)	0.046(±0.005, 11%)	0.041
• Iso	0.030(±0.008, 27%)	0.031(±0.004, 13%)	0.027

The percentage values in parenthesis were calculated as Standard Deviation/Average

3.6 Conclusions

From the results of this study, the following conclusions can be drawn:

- Alkaline pH conditions favored protein degradation over neutral or acidic pH; however, the degree of acidification indicated by the ratio of the maximum TVFA production to the maximum protein degradation rate was highest at neutral pH (40%)
- The optimum conditions for the production of VFA from the model protein, BSA, were a pH of 8 and fermentation time of 3 days. At these conditions, maximum VFA is produced and maintained stable over the fermentation time. Higher pH values than 8 could achieve the same level of VFA production but would require the doubling of the optimum fermentation time.

- The production of acetic, propionic, and butyric acids was in direct response to protein degradation whereas those of higher molecular weight VFAs (iso-butyrate, valerate, and iso-valerate) depended on the length of the incubation time.

- The theoretically derived stoichiometric coefficients generally compared very well to those obtained experimentally. Therefore, the representation by a single stoichiometry for the overall catabolic reaction of anaerobic protein fermentation to organic acids was validated.
- The variation in the prediction of stoichiometric coefficient for propionic acid seems to occur from an alternative pathway for proline and arginine fermentation,

conceivably the degradation of δ-aminovalerate devoid of propionic acid production.

- Under two extremely differently feed concentrations, that is, when BSA concentration was doubled, amino acid fermentation predominantly occurred by a single pathway.

3.7 References

APHA/AWWA/WEF. (2012). Standard Methods for the Examination of Water and Wastewater. *Standard Methods*, 541. https://doi.org/ISBN 9780875532356

Bader, D., Masaki, T., & Fischman, D. A. (1982). immunochemical Analysis of Myosin Heavy Chain during Avian Myogenesis In Vivo and In Vitro, 763–770.

Caccavo, F., Lonergan, D. J., Lovley, D. R., Davis, M., Stolz, J. F., & Mcinerneyl, M. J. (1994). Oxidizing dissimilatory metal-reducing microorganism. *Applied and Environmental Microbiology*, *60*(10), 3752–3759.

Chen, Y., Randall, A. A., & McCue, T. (2004). The efficiency of enhanced biological phosphorus removal from real wastewater affected by different ratios of acetic to propionic acid. *Water Research*, *38*(1), 27–36. https://doi.org/10.1016/j.watres.2003.08.025

Cheng, S. S., Chang, S. M., & Chen, S. T. (2002). Effects of volatile fatty acids on a

thermophilic anaerobic hydrogen fermentation process degrading peptone. *Water Science and Technology*, *46*(4–5), 209–214.

Choi, J. D. R., Chang, H. N., & Han, J. I. (2011). Performance of microbial fuel cell with volatile fatty acids from food wastes. *Biotechnology Letters*, *33*(4), 705–714. https://doi.org/10.1007/s10529-010-0507-2

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric Method for Determination of Sugars and Related Substances. *Analytical Chemistry*, *28*(3), 350–356. https://doi.org/10.1021/ac60111a017

Elefsiniotis, P., Wareham, D. G., & Smith, M. O. (2004). Use of volatile fatty acids from an acid-phase digester for denitrification. *Journal of Biotechnology*, *114*(3), 289–297. https://doi.org/10.1016/j.jbiotec.2004.02.016

Elsden, S. R., Hilton, M. G., & Waller, J. M. (1976). The end products of the metabolism of aromatic amino acids by clostridia. *Archives of Microbiology*, *107*(3), 283–288. https://doi.org/10.1007/BF00425340

Ely, R. E. (1954). Dairy H~lsbandry Research Branch, 6–9. https://doi.org/10.3168/jds.S0022-0302(55)95022-0

Fang, H. H. P., & Liu, H. (2002). Effect of pH on hydrogen production from glucose by a mixed culture. *Bioresource Technology*, *82*(1), 87–93. https://doi.org/10.1016/S0960-8524(01)00110-9

Freudenberg, W., Mayer, F., & Andreesen, J. R. (1989). Hicrnbinlngy of glycine reductase , all involved in anaerobic glycine metabolism, 182–188.

Gallert, C., Bauer, S., & Winter, J. (1998). Effect of ammonia on the anaerobic degradation of protein by a mesophilic and thermophilic biowaste population. *Applied Microbiology and Biotechnology*, *50*(4), 495–501. https://doi.org/10.1007/s002530051326

Gerber, A., Mostert, E. S., Winter, C. T., & De Villiers, R. H. (1986). The effect of acetate and other short-chain carbon compounds on the kinetics of biological

nutrient removal. *Water SA*.

KINOSHITA, S., UDAKA, S., & SHIMONO, M. (2008). Studies on the Amino Acid Fermentation. *The Journal of General and Applied Microbiology*, *3*(3), 193–205. https://doi.org/10.2323/jgam.3.193

Kotzé, J. ., Thiel, P. ., & Hattingh, W. H. . (1969). Anaerobic digestion II. The characterization and control of anaerobic digestion. *Water Research*, *3*(7), 459–494. https://doi.org/10.1016/0043-1354(69)90014-1

Lane, C., & Nor, F. (2019). The Amino Acid-fermenting Clostridia, *2*, 47–56.

Lebertz, H., & Andreesen, J. R. (1988). Hicrebielngy, 11–14.

Liu, H., Wang, J., Liu, X., Fu, B., Chen, J., & Yu, H. Q. (2012). Acidogenic fermentation of proteinaceous sewage sludge: Effect of pH. *Water Research*, *46*(3), 799–807. https://doi.org/10.1016/j.watres.2011.11.047

Lowry Lewis. (1951). The folin by oliver. *Readings*, *193*(1), 265–275. https://doi.org/10.1016/0304-3894(92)87011-4

Massey, L. K., & Sokatch, J. R. (1976). Branched-chain amino acid catabolism in bacteria. *... and Molecular Biology ...*, *40*(1), 42–54. Retrieved from http://mmbr.asm.org/cgi/reprint/40/1/42.pdf%5Cnpapers2://publication/uuid/53E3C8D0-6756-4B75-BE8A-C4F88B59D967

Mengmeng, C., Hong, C., Qingliang, Z., Shirley, S. N., & Jie, R. (2009). Optimal production of polyhydroxyalkanoates (PHA) in activated sludge fed by volatile fatty acids (VFAs) generated from alkaline excess sludge fermentation. *Bioresource Technology*, *100*(3), 1399–1405. https://doi.org/10.1016/j.biortech.2008.09.014

Moser-Engeler, K.M Udert, Wild, S. (1998). 1998_-_R_Moser_Engeler_-_Productsfromprimarysludgefermentationandtheirsuita[retrieved_2018-10-22].pdf.

Nisman, B. (n.d.). The Stickland Reaction, (10), 16–42.

Owen, W. F., Stuckey, D. C., Healy, J. B., Young, L. Y., & McCarty, P. L. (1979).

Bioassay for monitoring biochemical methane potential and anaerobic toxicity. *Water Research*, *13*(6), 485–492. https://doi.org/10.1016/0043-1354(79)90043-5

Ramsay, I. R., & Pullammanappallil, P. C. (2001). Protein degradation during anaerobic wastewater treatment. *Biodegradation*, *12*, 247–257.

Randall, A. A., Chen, Y., Liu, Y. H., & McCue, T. (2003). Polyhydroxyalkanoate form and polyphosphate regulation: Keys to biological phosphorus and glycogen transformations? *Water Science and Technology*, *47*(11), 227–233.

Riaño, B., Molinuevo, B., & García-González, M. C. (2011). Potential for methane production from anaerobic co-digestion of swine manure with winery wastewater. *Bioresource Technology*, *102*(5), 4131–4136. https://doi.org/10.1016/j.biortech.2010.12.077

Silvestre, G., Fernández, B., & Bonmatí, A. (2015). Significance of anaerobic digestion as a source of clean energy in wastewater treatment plants. *Energy Conversion and Management*, *101*, 255–262. https://doi.org/10.1016/j.enconman.2015.05.033

Stein, H. (n.d.). Amino Acid Composition of Lactoglobulin and Bovine Serum Albumin, (3).

Tanisho, S., & Ishiwata, Y. (1995). Continuous hydrogen production from molasses by fermentation using urethane foam as a support of flocks. *International Journal of Hydrogen Energy*, *20*(7), 541–545. https://doi.org/10.1016/0360-3199(94)00101-5

Thressa, C. (1959). Metabolism of Amino Acids, 544–548.

Ueno, Y., Otsuka, S., & Morimoto, M. (1996). Hydrogen production from industrial wastewater by anaerobic microflora in chemostat culture. *Journal of Fermentation and Bioengineering*, *82*(2), 194–197. https://doi.org/10.1016/0922-338X(96)85050-1

Uyar, B., Eroglu, I., Yücel, M., & Gündüz, U. (2009). Photofermentative hydrogen production from volatile fatty acids present in dark fermentation effluents. *International Journal of Hydrogen Energy*, *34*(10), 4517–4523.

https://doi.org/10.1016/j.ijhydene.2008.07.057

Wei, Y. (2004). Enhanced Biohydrogen Production from Sewage Sludge with Alkaline, *38*(11), 3195–3202.

Yokoi, H., Saitsu, A., Uchida, H., Hirose, J., Hayashi, S., & Takasaki, Y. (2001). Microbial hydrogen production from sweet potato starch residue. *Journal of Bioscience and Bioengineering*, *91*(1), 58–63. https://doi.org/10.1016/S1389-1723(01)80112-2

Zheng, X., Chen, Y., & Liu, C. (2010). Waste activated sludge alkaline fermentation liquid as carbon source for biological nutrients removal in anaerobic followed by alternating aerobic-anoxic sequencing batch reactors. *Chinese Journal of Chemical Engineering*, *18*(3), 478–485. https://doi.org/10.1016/S1004-9541(10)60246-7

Zhu, H., Suzuki, T., Tsygankov, A. A., Asada, Y., & Miyake, J. (1999). Hydrogen production from tofu wastewater by Rhodobacter sphaeroides immobilized in agar gels. *International Journal of Hydrogen Energy*, *24*(4), 305–310. https://doi.org/10.1016/S0360-3199(98)00081-0

Chapter 4

Co-fermentation of Carbohydrates and Proteins for Biohydrogen Production[2]

4.1 Introduction

The anaerobic digestion process (AD) has shown promise in full-scale operation to be an economical technology (Silvestre et al., 2015). Biological hydrogen production as an AD process has recently become the subject of accelerated research and has attracted the attention of many researchers worldwide. Dark fermentation among the other biological hydrogen production methods is of considerable importance to generate hydrogen from a wide range of organic wastes (Chong et al., 2009). Fermentative hydrogen production has been assessed for diverse organic wastes ranging from waste molasses (L. Guo et al., 2008), dairy wastewater (Venkata et al., 2008), sewage sludge (Mata-Alvarez et al., 2000) among others.

Fermentative hydrogen production from monosaccharide carbohydrates has been extensively examined and reported in the literature (Fang & Liu, 2002; Elsharnouby et al., 2013). The majority of studies on co-digestion only explored the different classes of carbohydrates and there are no documented reports on hydrogen production from co-fermentation of carbohydrates and proteins, despite the fact that proteins and

[2] A version of this chapter is currently under review in International Journal of Hydrogen Energy

carbohydrates in most organic wastes account for over two-thirds of the total organic matter. Prakasham et al (2009) examined the influence of the combination of glucose and xylose on hydrogen yield with enriched hydrogen producing mixed culture from compost dung as inoculum. It was evident that, on a weight basis, a glucose-to-xylose ratio of 2:3 at a temperature of 37 ℃ enhanced the hydrogen yield as compared to the fermentation of the pure individual sugars. The co-substrates resulted in an increase in hydrogen yield by 23% when compared to the fermentation of glucose only, and a 9% increase relative to using xylose only as the substrate. Xia et al. (2012) examined the co-digestion of glucose, xylose, and starch in batch tests using anaerobically digested sludge as inoculum for the thermophilic (55 ℃) degradation of microcrystalline cellulose for hydrogen production and it was observed that xylose tripled the conversion efficiency of cellulose relative to the control without any co-substrate addition. Abreu et al. (2012) investigated thermophilic (65 ℃) biohydrogen production in a batch system by co-digesting xylose and glucose at a ratio of 2:3 on mass basis using a pure bacterial strain of *Thermoanaerobacterium thermosaccharolyticum W16* and observed that the fraction of glucose in the substrate mixture impacted negatively on the degradation of xylose. The glucose degradation rate, however, remained essentially the same and was not affected by the xylose content in the substrate mixture. Furthermore, at a co-substrate mixture ratio of 4:1 on mass basis, the maximum hydrogen yield of 2.4 mol /mol $glucose_{consumed}$ was achieved, and this was not significantly different from those obtained from singly fermented substrates. Batch studies using pure culture strains of *Clostridium perfringes* for mesophilic (37 ℃) biohydrogen production by co-digesting various monosaccharides with cellulose achieved hydrogen yields that ranged from 0.6 to 1.7 mol H_2/mol hexose

(Craven & Russell, 1998). With a corn starch feed of 2 g/L in a batch system operating at 36 ℃, Ramachandran et al. (2008) achieved a hydrogen yield of 0.6 mol H_2/mol $hexose_{degraded}$. Mangayil et al. (2011) achieved a maximum hydrogen yield of 1.7 mol H_2/mol $hexose_{consumed}$ using *Clostridium Cellulolyticum* as inoculum and starch as the substrate.

The anaerobic co-digestion studies documented in the literature focused primarily on the biodegradability of these organic materials and their impact on the fermentative hydrogen production process was not, however, discussed. Breure et al. (1996) studied the effect of varying carbohydrate concentrations on the acidification of gelatin. A relatively higher glucose concentration as a second substrate was used to supplement the gelatin-containing culture upon achieving steady-state conditions. The results revealed that by increasing the carbohydrate concentrations in the feed, protein degradation progressively decreased. In a continuous flow system, Tomaso et al. (2003) examined the impact of lipids on the anaerobic fermentation of carbohydrates. It was reported that the rate of degradation of the carbohydrate was adversely impacted by the presence of the lipid. The rate of degradation of carbohydrate only was 72.8 mg carbohydrate/L.h whereas that of the co-substrate mixture of carbohydrate and lipid was 51.3 mg carbohydrate/L.h. Nonetheless, the latter showed greater process stability.

Relatively few studies in the literature reported fermentative hydrogen production from pure protein substrates. Cheng et al., (2002) studied the anaerobic degradation of peptone in a batch system using a thermophilic (55 ℃) anaerobic organic nitrogen-fed wastewater as seed at neutral pH and achieved a maximum hydrogen yield of 0.16 mmol/$gCOD_{added}$. Abrupt pH drops during the fermentation tests were avoided as a result of the production

of ammonia from the anaerobic degradation of the peptone. Xiao et al. (2010) evaluated biohydrogen production from 5 gCOD/L of peptone in a batch test at neutral pH and observed a maximum hydrogen yield of 0.11 mmol $H_2/gCOD_{added}$. Akutsu et al. (2009) investigated the effect of heat treatment of inocula on the hydrogen yield of different kinds of substrates. Eight different inocula were used on various substrates (starch, glycerol, oil, and peptone). Considerable hydrogen yields were observed for starch (20.4 – 175.5 mL $H_2/g\text{-}COD_{starch}$) and glycerol (11.6 – 38.2 mL $H_2/g\text{-}COD_{glycerol}$); for peptone and oil, there was almost no production of hydrogen.

The pH of the fermentation medium is one of the essential parameters that influence hydrogen production, metabolic pathways and also microbial community structures in mixed cultures (Wang & Wan, 2009). During anaerobic digestion, proteins are hydrolyzed to peptides and amino acids whereas carbohydrates first undergo enzymatic hydrolysis to produce sugars which are further degraded to produce VFAs by acidogenic microorganisms (Fangkum & Reungsang, 2011). The subsequent acidification of the amino acids and sugars produce volatile fatty acids, hydrogen, ammonia, and reduced sulfur. The VFAs produced during acidogenesis accumulate and further decrease the fermentation pH and may reach a level detrimental to hydrogen-producing microorganisms, and eventually, a system failure may occur (Batstone et al., 2004). Ward et al. (2007) reported 4.5 – 5.7 to be an optimum pH range for biohydrogen production from starch. For glucose and starch, an optimal pH of 5.3 and 5.6 with their respective yields of hydrogen of 1.5 and 1.7 mol H_2/mol_{hexose} was reported (Mangayil et al., 2011). Ward et al. (2007) reported that the substrate fermentation pathway favors the production of alcohols over hydrogen at pH <4.0 and pH>6. Enhanced substrate

utilization efficiency with hydrogen yields of 1.7 and 1.6 mol H_2/mol_{hexose} have been reported at a pH of 5.5 and 6.0 respectively (Ren et al., 2008). Several different pH values have been documented in the literature to be the optimum and these discrepancies could be associated with the diversity of substrate, the temperature of operation, and the source of inoculum. The favored liquid organic by-products are acetate and butyrate but at low pH (3.9 – 4.1), butyrate is selectively produced(Carrre & Steyer, 2010). At pH ≥ 7.0, propionate which is a hydrogen-consuming pathway is favored (Fang & Liu, 2002). Fang and Liu (2002) reported an optimum pH of 5.5 and a maximum yield of 2.1 mol H_2/mol_{hexose} for hydrogen production from glucose using mixed culture as inoculum. Also, a reduction in the hydrogen yield, as well as the specific hydrogen production rate, was observed at pH values higher than 6.0. Moreover, Fang and Liu (2002) also reported that the hydrogen production mechanism using mixed culture shifts towards methanogenesis at a pH higher than 6.

From the foregoing literature review, notwithstanding the significantly low yields of hydrogen from proteins, the anaerobic degradation of proteins produces ammonia which has the potential to counterbalance the effects of the accumulated VFAs in reactive systems, and the feasibility of this was worthy of exploration. While the aforementioned studies discussed the impact of co-digestion of different types of carbohydrates on the hydrogen production process, in this study, the optimum co-digestion ratio of carbohydrates and proteins that achieved the maximum hydrogen yield, production rate, and process stability was established. Response surface methodology (RSM) is a group of statistical tools for designing experiments, analyzing the relationship between process variables and predicting optimal conditions for desired responses. The application of

RSM has achieved notoriety in the areas of food processing, adsorption and biochemical processes where several process variable responses feature (Kumar et al., 2009). Presently, there is no documented literature on the optimization of co-fermentation of carbohydrates and proteins for the hydrogen production process. This study employed the central composite design (CCD) in RSM to determine the optimum VFA and ammonia concentrations and the hydrogen produced as the responses to the co-fermentation process at which pH control was not necessary.

4.2 Materials and Methods

4.2.1 Seed preparation and substrate

The seed for the fermentation tests was an anaerobically digested sludge obtained from the Stratford municipal wastewater treatment facility (Stratford, Ontario, Canada). In an effort to get rid of the soluble organics that may interfere with the fermentation process, the seed was centrifuged at 3000 rpm for 20 min and the supernatant containing dissolved organics was discarded. The characteristics of the seed sludge after suspension of the seed pellets in 1 L of distilled water is shown in Table 4.1. To inhibit methane production and enriching hydrogen producing microorganisms, the seed sludge was heated at 70 ℃ for 30 min. BSA and starch, both substrates obtained from Sigma Aldrich in Ontario, Canada, were the respective model protein and carbohydrate used for the batch acidogenic co-fermentation hydrogen production process.

4.2.2 Anaerobic co-fermentation for hydrogen production

Batch anaerobic co-fermentation studies were conducted using five different mixture ratios (C1 – C5) of BSA and starch for hydrogen production. Table 4.2 depicts the percentage substrate mixture compositions on the basis of COD (COD of BSA is 1.2 gCOD/g BSA and that of starch is 1.07 gCOD/g starch) and the amount in terms of mass. The experiments were carried out at a working volume of 200 mL in a series of 250 mL serum reactors. 40 mL of inoculum, 10 mL of nutrient solution and the required mass of starch and BSA dissolved in 150 mL of distilled water was added to each bottle. The total substrate mass COD added to each bottle was 800 mg COD. With 0.5 N NaOH and HCl, the pH of each reactor bottle was adjusted to 5.5 ± 0.1 prior to the fermentation test. An oxygen-free nitrogen gas (99.9%) was used to sparge the headspace of each serum bottle for two minutes to ensure the anaerobic condition. The reactor bottles were finally placed in a swirling action shaker (Max 4000, Thermo Scientific, CA) with an operating temperature of 37 ± 1℃ and a swirling speed at 160 – 180 rpm. Three bottles containing only the seed, the nutrient solution, and distilled water (without substrates) were prepared to serve as the controls. Thirty bottles were prepared for each mixture ratio and for each liquid sampling event, three bottles were sacrificed for analyses.

Table 4.1 - Characteristics of the seed sludge after suspension in distilled water

Parameter	Seed Sludge	Number of Samples Analyzed
TSS (mg/L)	12404 ± 262	3
VSS (mg/L)	9400 ± 67	3
Particulate proteins (mg/L)	2750 ± 114	3
Soluble proteins (mg/L)	1071 ± 43	3
Particulate carbs(mg/L)	4473 ± 89	3
Soluble carbs(mg/L)	980 ± 40	3
Nitrogen (mg/L)	963 ± 84	3
Ammonia (mg/L)	35 ± 2	3
TCOD (mg/L)	12800 ± 174	3
SCOD (mg/L)	2271 ± 34	3
TVFA (mg/L)	450 ± 10	3
Ethanol (mg/L)	150 $\pm$ 10	3

Table 4.2 – Substrate composition in each bottle at the various mixtures expressed in terms of mass and percentage on a COD basis

Substrate mixture	BSA (%)	Starch (%)	BSA (mg)	Starch (mg)	Total substrate mass COD_{added} (mg COD)
C1	100	0	670	0	800
C2	80	20	536	150	800
C3	50	50	335	375	800
C4	20	80	134	600	800
C5	0	100	0	750	800

1.2 g COD/ g BSA ; 1.07 gCOD/ g Starch

4.3 Analytical Methods

The biogas generated in the anaerobic reactors was periodically measured several times using 5 – 100 mL sized glass syringes. The gas drawn from the headspace of the reactors was released to equilibrate to the ambient pressure (Owen et al., 1979). A gas chromatograph with an equipped thermal conductivity detector (Model 310, SRI Instruments, Torrence, CA) attached to a molecular sieve column was used to characterize methane and hydrogen from the produced gas. Argon was the carrier gas and it flowed at 20 mL/min. The detector temperature was 105 ℃ and that of the column was 90 ℃. Free ammonia (NH_3) and COD were determined by HACH methods and test kits

(HACH Odyssey DR/2500) and pH measurements were by a digital pH meter (VWR, B10P, SympHony). Protein concentrations were determined by Lowry's method (Lowry and Lewis, 1951) using a protein standard solution of BSA. Carbohydrates were determined by the phenol-sulphuric acid method using glucose as the standard solution (Dubois et al.,1956). To measure VFA and ethanol concentrations, the samples were filtered through a 0.45 μm paper prior to using the gas chromatograph, (Varian 8500, Varian Inc. Toronto, Canada). The respective detector and column temperatures of the gas chromatograph were 250 ℃ and 110 ℃ and the carrier gas was helium and it flowed at 5 mL/min. Volatile and total suspended solids were determined by standard methods (APHA, 2005).

4.4 Results and Discussion

4.4.1 Substrate degradation

The initial and final batch characteristics of the various substrate mixtures are summarized in Table 4.3. Fig. 4.1 depicts the degradation of proteins for C1 – C5. The only source of particulate proteins in the acidogenic reactors was the seed biomass and its initial concentration in C1 – C5 was 540±20 mg/L, as the BSA is soluble and does not undergo hydrolysis. As illustrated in Fig.4.1a, there was a steady decline in particulate proteins for all the substrate mixtures and the final concentrations ranged from 35 – 145 mg/L for C1 – C5, respectively, indicating an average degradation of 80% particulate proteins which was similarly observed for the seed controls. More degradation was observed for the mixtures with low starch content (Table 4.3). This phenomenon could be attributed to rapid hydrolysis of more carbohydrates to produce glucose as the main

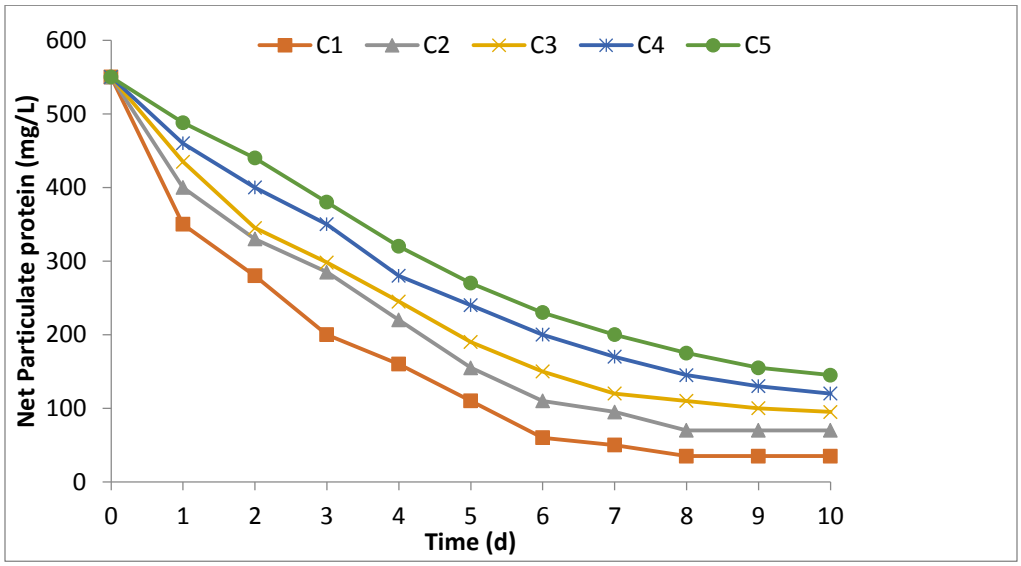
hydrolysate which can suppress protease that is responsible for the decomposition of proteins (Breure et al, 1996). The initial VSS concentrations for C1 – C5 on the average was from 1862 to 4385 mg/L, respectively, with the corresponding initial particulate proteins to biomass ratio range of 0.14 – 0.29. The final particulate proteins-to-biomass ratio for C1 – C5 ranged between 0.04 – 0.08, thus indicating the complete degradation of the particulate proteins in C1 – C5.

As shown in Fig. 4.1b, there was an increase in the soluble proteins concentration for the first 2 days and was immediately followed by a quick decline until leveling off after 5 days. The reduction in particulate proteins for all mixtures in the first 2 days was not equal to the increase in soluble proteins for all samples, indicating that the BSA was degraded over the fermentation time. The soluble proteins concentrations were influenced by both the solubilization rate of particulate proteins and the degradation rate of soluble proteins to VFAs and ammonia. The rate of solubilization exceeded that of the degradation at the beginning of the batches and this was reflected in the increase in soluble proteins for the first 2 days of the fermentation time. However, from day 2 to day 6, the converse was observed as more soluble proteins were degraded which resulted in the sharp decline until leveling off after day 5. The respective maximum soluble proteins concentrations for C1 – C4 plateaued at 3580, 3074, 2059 and 1019 mg/L. The ratios between the peak soluble proteins and that of the initial particulate proteins concentations for the various mixtures were in the range of 1.9 – 6.6. This underscored the relationship existing between the particulate proteins concentrations prior to the fermentation test and that of the soluble proteins within the different ratio mixtures.

Table 4.3. Initial and final reactor conditions for the acidogenic fermentation test

Mixture	VSS (mg/L)		Particulate proteins (mg/L)		Soluble Proteins (mg/L)		Particulate Carbs (mg/L)[a]		Soluble Carbs (mg/L)		Ammonia (mg/L)		TCOD (mg/L)		Ethanol (mg/L)		TVFA (mg/L)	
	Initial	Final	Initial	Final	Initial	Final	Initial	Final	initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final
C1	1862 ± 95	945 ± 10	540 ± 20	35 ± 2	3565 ± 40	600 ± 50	894 ± 22	250 ± 10	210 ± 12	20 ± 2	6 ± 1	456 ± 8	6470 ± 80	4912 ± 102	30 ± 5	485 ± 60	90 ± 5	725 ± 15
C2	2162 ± 80	1020 ± 50	540 ± 20	70 ± 5	2894 ± 70	700 ± 20	1456 ± 102	150 ± 5	384 ± 24	50 ± 5	6 ± 1	351 ± 12	6610 ± 30	4968 ± 150	30 ± 5	848 ± 50	90 ± 5	930 ± 50
C3	3050 ± 50	1310 ± 20	540 ± 20	95 ± 5	1889 ± 170	520 ± 10	2300 ± 50	160 ± 10	665 ± 35	80 ± 4	6 ± 1	230 ± 5	6510 ± 70	4821 ± 110	30 ± 5	1050 ± 80	90 ± 5	1020 ± 70
C4	3780 ± 110	1650 ± 50	540 ± 20	120 ± 10	888 ± 30	470 ± 15	3144 ± 112	230 ± 15	946 ± 22	150 ± 15	6 ± 1	80 ± 2	6495 ± 40	4567 ± 120	30 ± 5	1325 ± 115	90 ± 5	875 ± 20
C5	4385 ± 25	1870 ± 40	540 ± 20	145 ± 5	214 ± 12	95 ± 10	3707 ± 153	240 ± 12	1134 ± 86	250 ± 10	6 ± 1	35 ± 2	6650 ± 50	4839 ± 170	30 ± 5	1624 ± 124	90 ± 5	1150 ± 50
Seed Only	1888 ± 120	978 ± 18	560 ± 40	28 ± 4	220 ± 10	37 ± 5	880 ± 14	60 ± 5	200 ± 10	38 ± 4	6 ± 1	24 ± 5	2580 ± 40	1917 ± 70	30 ± 5	220 ± 10	90 ± 5	210 ± 10

(a)



(b)

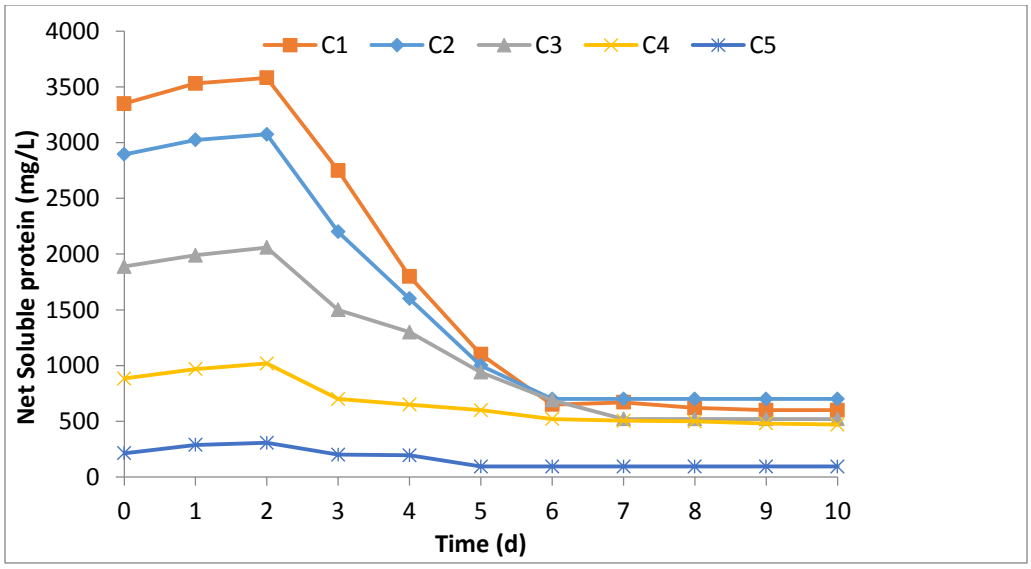
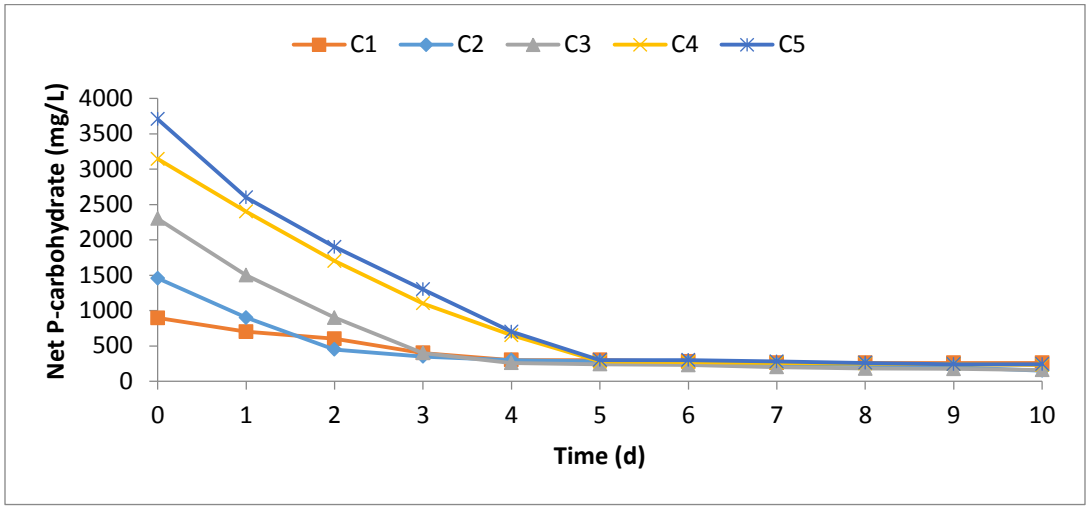


Fig.4.1. Changes in the concentration of proteins with time at initial pH of 5.5 and at 37 ℃ (a) particulate protein (b) soluble protein

The degradation profile of particulate and soluble carbohydrates for C1 – C4 is shown in Fig.4.2. From Fig. 2a, there was a similar trend of particulate carbohydrate degradation as observed in the case of particulate proteins, but with a rather sharp decline from the beginning until leveling off on the 5^{th} day of the incubation time. Almost 50% of the particulate carbohydrates were degraded for C4 and C5 and over 60% for C2 and C3 in the first 2 days of the batch test. The soluble carbohydrate concentrations peaked at 674, 955, 1396, and 1554 mg/L for C2 – C5, and that accounted for 46%, 42%, 44%, and 42% of the initial particulate carbohydrates, respectively.

The average particulate proteins concentration in all the five substrate mixtures was 540 $\pm$ 20 mg/L. The rates of degradation of particulate proteins in the mixtures were determined by the slopes of the linear fits of Fig. 4.1a for C1 – C5. It is apparent from Fig. 4.1a that the rates of particulate proteins degradation were in an inverse relationship with the starch content in the mixture. That is, the lower the starch content, the higher the rate of degradation of particulate proteins. With no supplementation of starch at C1 (100% BSA), the highest absolute particulate proteins degradation rate of 55 mg/Ld was achieved whereas for C5 (100% starch) the least degradation rate of 38 mg/Ld was obtained. With the co-substrate mixtures (C2 – C4) the rates of degradation were in the order: C2 (80% BSA + 20% starch) (48 mg/Ld) > C3 (50% BSA + 50% starch) (45 mg/Ld) > C4 (20% BSA + 80% starch) (40 mg/Ld), suggesting that the starch concentration impacted negatively the particulate proteins degradation rates. This is plausible since starch is more readily biodegradable than proteins.

(a)


C1
C2
C3
C4
C5
Net P-carbohydrate (mg/L)
4000
3500
3000
2500
2000
1500
1000
500
0
0
1
2
3
4
5
6
7
8
9
10
Time (d)

(b)

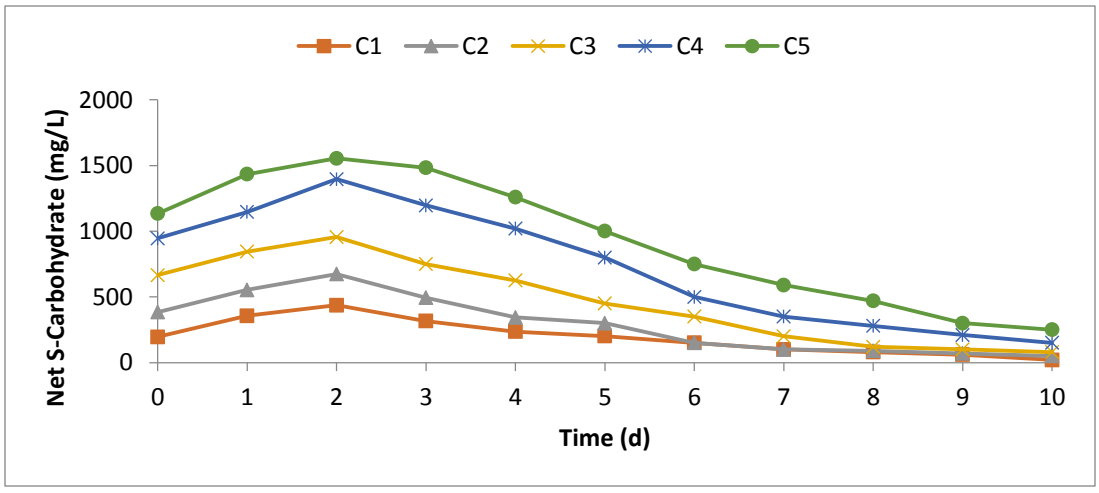

C1
C2
C3
C4
C5
Net S-Carbohydrate (mg/L)
2000
1500
1000
500
0
0
1
2
3
4
5
6
7
8
9
10
Time (d)

Fig. 4.2. Variation of carbohydrate concentrations with time at initial pH of 5.5 and at 37 ℃ (a) particulate carbohydrate (b) soluble carbohydrate

4.4.2 Hydrogen production

To examine the effect of carbohydrate-to-protein ratio on hydrogen production potential, BSA and starch were fermented individually (C1 and C5 respectively) and also in ratio combinations (C2 – C4) inoculated with the preheated anaerobically digested sludge at 70 ℃. The net cumulative hydrogen production after discounting the effect of the hydrogen produced from the seed control is shown in Fig.4.3 for the five substrate mixtures (C1 – C5). The highest hydrogen of 280 mL was obtained for C4 (20% BSA + 80% starch) and the lowest of 10 mL for C1 (proteins only). The carbohydrate only (C5) produced 251 mL of hydrogen. Xiao et al. (2010) observed a lower hydrogen yield of 0.04 mL/mg peptone than 0.16 mL/mg glucose. Ordinarily, carbohydrates are the most preferred carbon source for fermentative hydrogen production (Prakasham et al., 2009), which was a reflection on the relative volumes produced from the BSA only (C1) and the starch only (C5) as mono-substrates. The synergistic impact of the co-substrates in C2 – C4 was evaluated by using the masses of the fermented proteins and carbohydrates in C1 – C5, coupled with the hydrogen produced from C1 (10 mL) and C5 (251 mL). The expected hydrogen from C2, C3, and C4 and their respective experimental cumulative hydrogen are shown in Table 4.4. The highest experimental cumulative hydrogen production of 280 mL for C4 (20% BSA + 80% starch) was 38% higher than the calculated hydrogen of 203 mL. It is imperative to emphasize that the co-fermentation of carbohydrates and proteins resulted in a relatively higher hydrogen production than the fermentation of proteins only (C1) or carbohydrates only (C5). Thus, the hydrogen produced from the co-substrates of

carbohydrates and proteins (C2 –C4) was relatively higher than that from the sum of each fraction in the different mixtures.

Table 4.5 shows the COD balance and the fraction of $TCOD_{added}$ that was converted to hydrogen-COD. The $TCOD_{added}$ for each mixture was 800 mgCOD. The $TCOD_{effluent}$ included organic acids, alcohols and residuals. As 1 mol (25400 mL) of hydrogen at 37 ℃ has a COD equivalent of 16000 mgCOD, the COD of hydrogen produced from each mixture was calculated. The fraction of $TCOD_{added}$ converted to hydrogen-COD ranged from 0.81% to 35%, with C1 (100% BSA) and C4 (20% BSA+80% starch) achieving the least and highest, respectively.

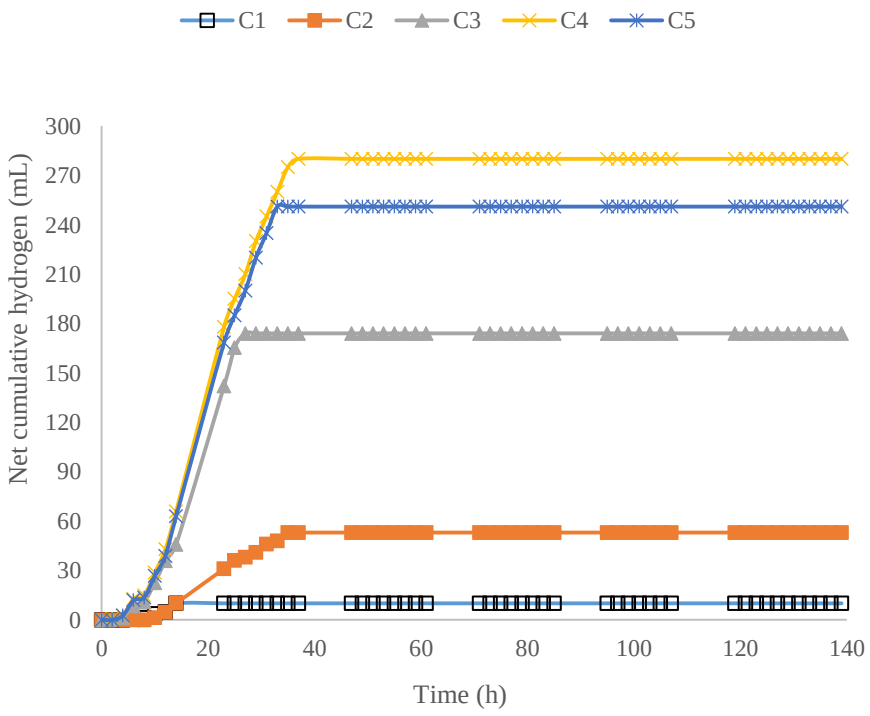


Fig.4.3. Cumulative hydrogen production from the five substrate mixtures at initial pH of 5.5 and at 37 ℃

Table 4.4. Synergistic effect of co-fermentation of carbohydrates and proteins at different ratios

Mixture	Expected hydrogen (mL)	Measured hydrogen (mL)	(%) Difference
C2	58	63	9
C3	131	164	25
C4	203	280	38

Table 4.5. COD balance and the fraction of feed COD converted to hydrogen-COD

Mixture	COD_{added} (mgCOD)	$COD_{effluent}$ (mgCOD)	Vol. of H_2 produced (mL)	COD of H_2 (mgCOD)	COD of H_2/COD_{added} (%)
C1	800	791	10	6.5	0.81
C2	800	754	63	41.1	5.1
C3	800	686	164	107.1	13.4
C4	800	505	280	182.9	35
C5	800	536	251	163.9	31

4.4.3 Kinetic analysis and hydrogen yields

As shown in Table 4.6 and on the premise of the Gompertz model (equation 1) below, the lag phases for C1 – C5 lasted between 4 to 10 h. The lag phase time for BSA only (C1) was longer than that of starch only (5), 10 h versus 4 h.

$$P = P_{max}.exp\left(-exp\left[\frac{R_m}{p}(L-t)+1\right]\right) \quad \ldots\ldots\ldots\ldots\ldots (1)$$

P denotes the total cumulative hydrogen produced, P_{max} represents maximum cumulative hydrogen and R_m stands for the hydrogen production rate. L and t are the lag phase and the fermentation times respectively.

The hydrogen produced was normalized by the mass of substrate COD added (mL $H_2/gCOD_{added}$). From Table 4.5, the maximum hydrogen yield of 350 mL $H_2/gCOD_{added}$ occurred at C4 (20% BSA + 80% starch). The lowest hydrogen yield of 13 mL $H_2/gCOD_{added}$ was obtained from C1 (100% BSA). As depicted in Table 4.4, the supplementation of carbohydrates with proteins only had a positive impact on the hydrogen yield at C4 (20% BSA + 80% starch). Thereafter, a negative response occurred and manifested in the decrease in hydrogen yields by 35% for C3 and 75% for C2 with respect to the yield of hydrogen obtained from C5.

The hydrogen production rate obtained from the slope of the Gompertz cumulative hydrogen production curve (exponential phase) is depicted in Table 4.5. The highest production rate of 11.2 mL/h occurred at C4 and was 918% higher than that of proteins only, C1 (1.1 mL/h) and 33% higher than that of starch only, C5 (8.4 mL/h). The hydrogen production rate of 8.4 mL/h for C5 (starch only) was 950% greater than 1.1 mL/h for C1 (proteins only). Thus, the hydrogen yield and the production rate obtained

for C4 reinforced that the optimum carbohydrate-to-protein ratio for enhanced fermentative hydrogen production is 4:1.

Table 4.6 – Gompertz kinetic model and hydrogen yields for the five different mixtures

Substrate mixture	Gompertz model				H_2 yield (mL H_2/gCOD_{added})	H_2 yield (mol H_2/mol glucose)
	P (mL)	R_m (mL/h)	L (h)	R^2		
C1	10	1.1	10	0.99	13	0.08
C2	63	2.1	8.2	0.99	79	0.4
C3	164	6.3	7.3	0.99	205	1.6
C4	280	11.2	3.8	0.99	350	2.5
C5	251	8.4	4	0.98	314	1.8

4.4.4 pH Change

Carbohydrate and protein degradation produce various products such as hydrogen, carbon dioxide, water, volatile fatty acids, amino acids, and ammonia among others (Das & Veziroä, 2001; Hawkes et al., 2002). The generation of VFAs, amino acids and NH_4^+-N has the capacity to effect pH changes during the fermentation process. The changes in pH and the ammonia production for C1 – C5 are shown in Fig. 4.4, C1 (100% BSA) produced the highest concentration of ammonia of 456 mg/L. The reactors containing only starch (C5) produced the least concentration of ammonia of 35 mg/L. The ammonia

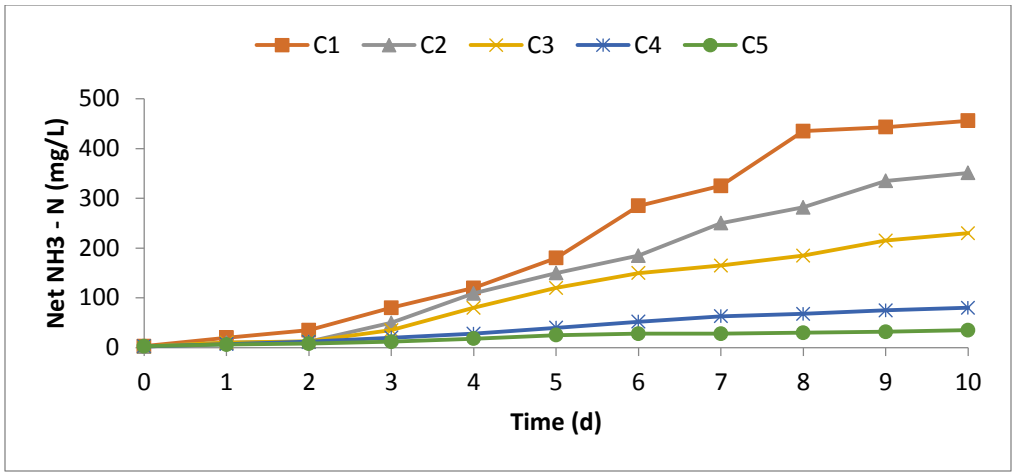
produced in C1 is 13 folds of that of C5. For the substrate mixtures, the ammonia production in C2 – C4 were 351, 230 and 80 mg/L, respectively. There was more ammonia produced with respect to high protein content in the substrate mixtures.

For all samples (C1 – C5), the initial pH at the start of the experiment was adjusted to 5.5 as reported by (Fang & Liu, 2002) to be the optimum initial pH value for fermentative hydrogen production from carbohydrates. There was a considerable pH drop from the initial pH to 4.1 for C5 (starch only) for the first two days. This occurrence could be associated with the rapid acidification of starch during the early stage of fermentation (Craven & Russell, 1998), and at no supplementation of proteins for its subsequent degradation to produce ammonia to counteract the effect of the accumulated VFAs resulted in the observed significant pH drop. Bahl et al (1992) reported that there was an inhibition of *Clostridium sp.* for fermentative hydrogen production at a pH range of 4.0 – 5.0. This, therefore, suggested that the hydrogen yield by C5 (starch only) was impacted negatively by the extreme pH condition presented by the rapid acidification process, despite the available fact that carbohydrates are the most preferred carbon source for hydrogen production (Yokoi et al., 2002; Mangayil et al., 2011; Ren et al., 2009). For C1 (BSA only), there was a sudden increase in pH to 6.2 (beyond optimum pH for hydrogen production) as opposed to that of C5 (starch only) for the first two days of the experiment. The rapid increase in pH beyond the optimum initial pH is attributed to the acidification of proteins to produce ammonia, which in aqueous solution produce ammonium and hydroxide (Ramsay & Pullammanappallil, 2001). No statistical correlation existed between the change in pH and the change in ammonia production. Significantly low hydrogen yields from proteins have been reported by (Xiao et al., 2010;

Cheng et al., 2002; Gallert et al., 1998) which are in accordance with the hydrogen yield from C1(BSA only) in our study.

As depicted in Fig.4.4b, the abrupt pH drop observed for C5 (starch only) was prevented for the mixture ratios C2(80% BSA + 20% starch), C3(50% BSA + 50% starch) and C4(20% BSA + 80% starch). It has been established that carbohydrate materials yield significant amounts of volatile fatty acids whereas those of proteins provide great buffering capacity as a result of the degradation of proteins to produce ammonia(Gallert et al., 1998). For C2 and C3, their respective ratio combinations of carbohydrates and proteins only successfully avoided the abrupt pH drops but failed to ensure a buffer system as there was an increase in pH from the initial of 5.5 to 6.2 and 6.0, respectively, for the first two days of fermentation and some level of pH control was required. The pH changes over the fermentation time for C4 (20% BSA + 80% starch), however, invariably remained nearly stable at the optimum initial pH of 5.5 and this resulted in the highest hydrogen yield and rate of 2.5 mol H_2/mol glucose equivalent and 11.2 mL/h respectively. It is imperative to emphasize here that adequate buffering capacity was only achieved at this carbohydrate-to-protein ratio, as the required amounts of products necessary to counterbalance the effects of VFAs were produced at this co-substrate mixture ratio and therefore pH control is not necessary at this ratio.

(a)



(b)

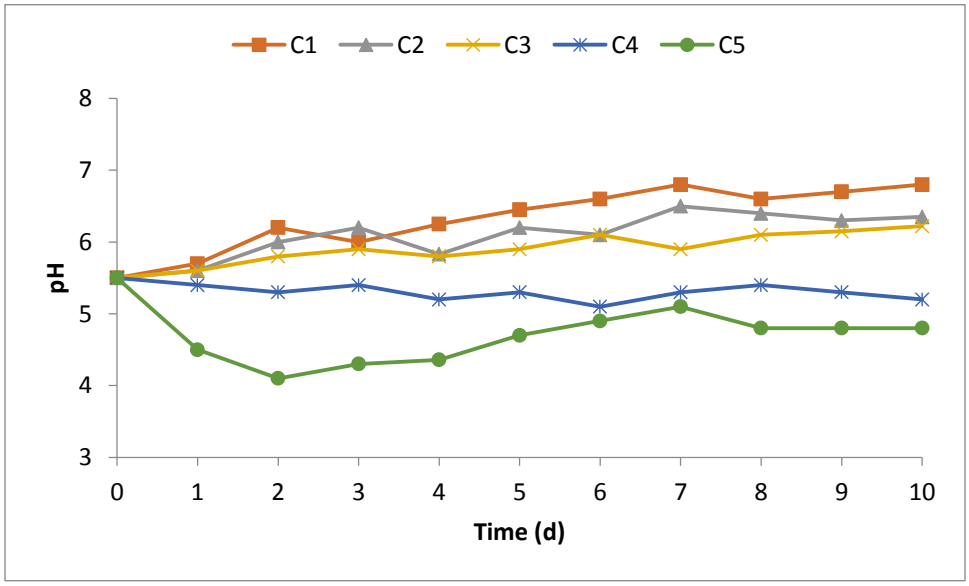


Fig. 4.4. Ammonia production and pH changes from initial pH of 5.5 and at 37 ℃ (a) ammonia concentration (b) pH changes

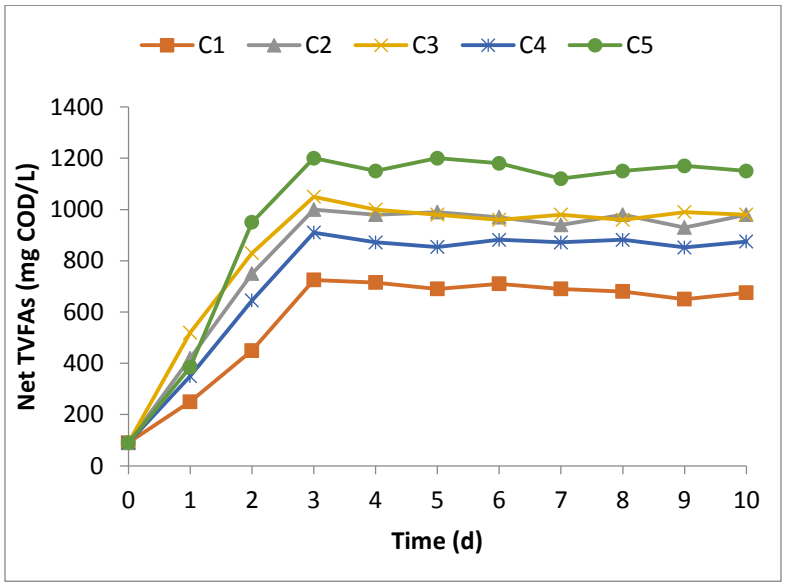
4.4.5 Volatile fatty acids (VFAs)

The TVFAs yields and the liquid organic by-products (single VFAs and ethanol) examined at the peak production of TVFAs are shown in Fig.4.5 for the five substrate

mixtures. There was an increase (almost linear) in the TVFAs concentrations for all the substrate mixtures and reached peak concentrations of 725, 1000, 1050, 910, and 1200 mgCOD/L (Fig.4.5a) for C1 – C5, respectively, on day 3 and invariably remained stable over the fermentation period. The liquid organic by-products differed in composition for C1 (BSA only) and C5 (starch only). Acetate was the main single VFA in the seed control reactors. The dominant single VFAs in C1 (BSA only) were acetate (198 mgCOD/L), butyrate (300 mgCOD/L), propionate (150 mgCOD/L), and the ethanol produced was the lowest (80 mgCOD/L) whereas those for C5 (starch only) were acetate (550 mgCOD/L), butyrate (430 mgCOD/L), ethanol (840 mgCOD/L), and the propionic acid (95 mgCOD/L) was the least produced. The varying compositions observed for the liquid organic by-products for C1 (BSA only) and C5 (starch only) indicated that there were different pathways for hydrogen production: the pathway of C1 (BSA only) was butyrate-type fermentation and that of C5 (starch only) followed ethanol-type. *Ethanoligenens sp.* have been observed to produce acetate and ethanol from carbohydrate-rich substrates and the fermentation pathway follows that of ethanol-type as the presence of this strain yield more ethanol as end-product in the pH range of 5.2 – 5.6 (Azbar and Levin, 2012).

By examining the three substrate mixtures containing co-substrates of starch and BSA (C2 – C4), there was consumption of both starch and BSA as evidenced by the soluble substrate degradation data (Fig.4.1b and Fig.4.2b). Thus, the production of hydrogen and liquid organic by-products was synchronic with butyrate-ethanol type pathways and this resulted in the synergistic effect in the hydrogen production in C2 – C4 (Table 4.4) than the fermentation of mono-substrate of starch and BSA.

(a)



(b)

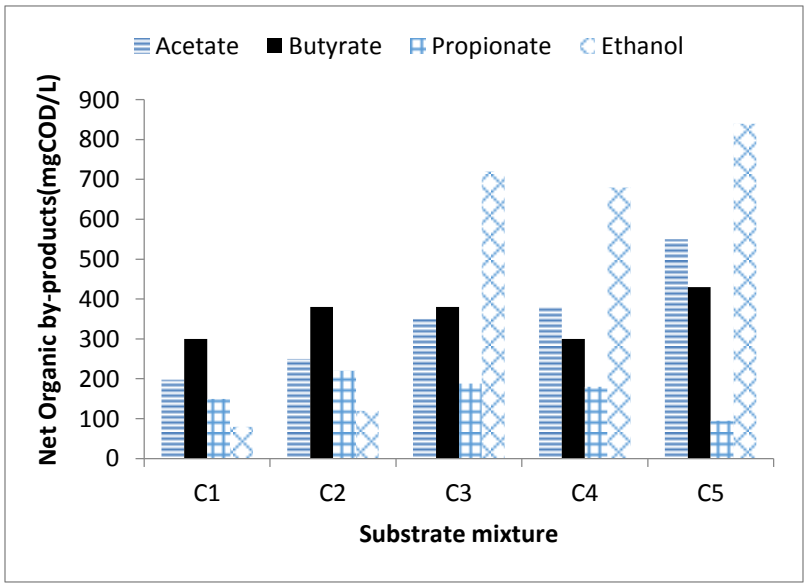


Fig. 4.5 – Liquid organic by-products produced (a) TVFAs production over the fermentation time (b) Single VFAs and ethanol produced at the peak (day 3) of TVFAs production

4.5 Statistical Analysis

4.5.1 The Box-Behnken Design

The Box-Behnken 3-point design was used to develop mathematical models that correlate the variable factors in the experiment and to optimize them for the co-fermentation process for the optimum ratio at C4(20%BSA + 80% Starch) using MINITAB-16. The experimental variable factors, carbohydrate concentration (A), protein concentration (B), and the pH (C), and with their low (-α) and high (+α) levels within the tested range were considered. The production of VFA, ammonia, and hydrogen was the response to the fermentation process. The Box-Behnken 3-point design model equations generated with highly significant coded terms are given below:

VFA (Y) = +520.6*A – 38.1*B + 201*C – 43.9*A^2 – 20.7B^2 – 119*C^2 – 428*A*B – 513*A*C – 14.5* B*C…….(3)

Ammonia (Y) = +130.10*A – 40.2*B + 170*C – 20.4*A^2 – 14.2*B^2 – 100.3*C^2 + 108*A*B – 70*A*C – 41.9*B*C…… (4)

Hydrogen (Y) = +20*A + 8.6*B + 88.2*C + 145.8*A^2 – 25.6*B^2 – 124*C^2 + 15.5 *A*B – 33.4*A*C + 2.5*B*C……..(5)

The above model equations (equations 3 – 5) illustrate how the single variables (quadratic) or double interactions influenced the production of VFA, ammonia, and hydrogen. The negative coefficient values depict that the single or double interactions of the independent variables negatively affected the responses (VFA, ammonia, and hydrogen), whereas the positive coefficient values indicated an increase in response within the tested range. The suitability of the generated models was validated by the

analysis of variance (ANOVA) provided in Appendix B. The high coefficient of determination (R^2) values of the polynomial models makes them desirable and also enhance the model terms (Kumar et al., 2009).

4.5.2 ANOVA tables

The extent of significance and suitability of the generated models were examined by considering the p-values of the analysis of variance (ANOVA). Ordinarily, model terms are significant when the p-value is <0.05 and insignificant when the p-value is >0.05. The ANOVA table for the response of VFA shows a highly significant model with model F and Prob > F values of 734.4 and <0.0001 respectively, suggesting that there is close to 0.01% chance that the model F value could occur due to noise. Also, from the ANOVA tables for ammonia and hydrogen responses, the model F values and p-values for ammonia and hydrogen responses are respectively 48.3, 1274 and <0.0001, suggesting that both models are highly significant.

The significance of the individual coefficients as well as the interactions between the factors was also tested by considering the p-values. For VFA the response shows that only two linear coefficients, carbohydrate concentration (A) and pH (C), one interaction term AC (carbohydrate concentration and pH) together with only one quadratic coefficient (C^2) had a significant effect on the production of VFA ($p < 0.05$). For all three responses, the pH impacted significantly ($p<0.05$). The polynomial coefficients (A^2 and C^2) were both significant ($p<0.05$) for hydrogen response. The foregoing analysis suggests that the carbohydrate and protein concentrations, as well as the pH, were the limiting operating factors of the process such that any changes in their magnitude could affect the process significantly.

4.5.3 Regression analysis

A test of significance of regression was employed to re-establish the significance of the models by evaluating the coefficients of determination (R^2). Reduced variability in the regression variables is determined by the actual R^2 value. A good model does not necessarily mean large R^2 value but the more comparable the adjusted R^2 value is to the actual R^2 value, the highly significant the model terms. The actual and adjusted R^2 values for the responses (VFA, ammonia, and hydrogen) have been reported in Table 4.7.With R^2 values closer to 1.0, the stronger the correlation between the observed and the predicted values. With higher R^2 values of 0.8451, 0.8745 and 0.8812 and adjusted R^2 values of 0.8977, 0.9024 and 0.8993 for VFA, ammonia, and hydrogen responses, respectively, implied the adequacy of the models, suggesting that 84.5%, 87.5%, and 88.1% variability can be estimated for by the model equations, respectively. Moreover, with a significantly high R^2 value closer to 1.0, the more fitted the model.

Adequate precision compares the predicted values with the average predicted errors within the design space. It also determines the signal-to-noise ratio and values higher than 4 are deemed desirable for suitable models (Peng et al., 2002). The Adequate precision values of 62.3, 24.5 and 85.3 (Table 4.7) for VFA, ammonia, and hydrogen, respectively, for the co-fermentation process, imply that the generated quadratic models have good signals and are adequate enough to navigate the design space for the optimum conditions for the co-fermentation process. Furthermore, the degree of precision is determined by the coefficient of variation (CV), and the lower the CV values (20 – 38)(Peng et al., 2002; Kumar et al., 2009) the more reliable the experiment. In our study, significantly low CV values of 5.3, 7.4, and 3.5 (Table 4.7) were achieved for VFA, ammonia, and

hydrogen, respectively, indicating a satisfactory precision and reliability of the experiment.

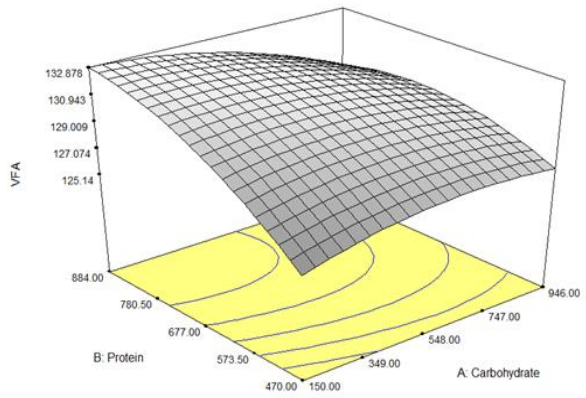
Table 4.7 – Measure of statistical significance and adequate precision

Statistical analysis	VFA	Ammonia	Hydrogen
R-Squared	0.8451	0.8745	0.8812
Adjusted R-Squared	0.8977	0.9024	0.8993
Adq. Precision	62.3	24.5	85.3
Coefficient of Variation (CV)	5.3	7.4	3.5

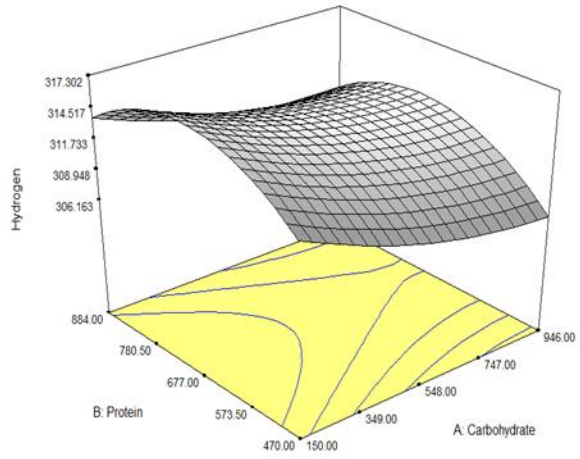
4.5.4 3D Contour plots

The regression equations used to investigate the interactions among the variable factors are represented graphically by the three dimensional contour plots (Fig. 4.6) and were used to determine the optimum conditions for the co-fermentation process. The significance of the interaction between the variables is indicated by the saddle nature of the 3D contour plot (Raus et al., 2011; Jamal et al., 2013; Bau et al., 2012). Results from Fig.4.6 indicate that the optimum regions for VFA and ammonia production at a fixed pH of 5.5 are respectively 125 – 133 mg/L and 41 – 47 mg/L, and at these concentrations of VFA and ammonia, the control of pH is not necessary.

(a)


132.878
130.943
129.009
127.074
125.14
VFA
884.00
780.50
677.00
573.50
470.00
B: Protein
150.00
349.00
548.00
747.00
946.00
A: Carbohydrate

(b)


317.302
314.517
311.733
308.948
306.163
Hydrogen
884.00
780.50
677.00
573.50
470.00
B: Protein
150.00
349.00
548.00
747.00
946.00
A: Carbohydrate

(c)

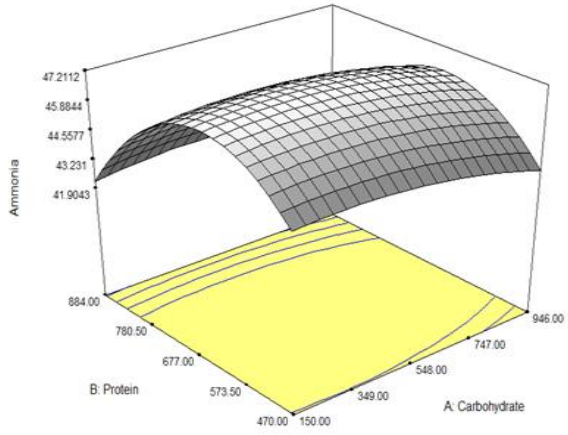

47.2112
45.8844
44.5577
43.231
41.9043
Ammonia
884.00
780.50
677.00
573.50
470.00
B: Protein
150.00
349.00
548.00
747.00
946.00
A: Carbohydrate

Fig. 4.6. 3D Contour plots of the co-fermentation of carbohydrates and proteins at a COD ratio of 4:1. Optimum fermentation time was 3 days for all the plots (a) VFA (b) ammonia (c) hydrogen

4.5.5 Empirical model validation

The optimized results and the quadratic models were validated by three supplementary experimental runs at an initial pH of 5.5 and each conducted in triplicate and averaged over for each point prediction by MINITAB-16. The actual experimental values obtained for the responses of VFA and ammonia were comparable with the predicted response values within an absolute error of 10% and these are shown in Table 4.8. These errors could be attributed to the experimental conditions in the laboratory.

Table 4.8- Comparison of point predicted response values with actual experimental values

Exp. run	Carbs (mg/L)	Proteins (mg/)	time (d)	VFA (mg/L)		Ammonia (mg/L)	
				Predicted	Actual	Predicted	Actual
1	230	185	3	163	154	45	48
2	460	368	3	275	288	58	63
3	920	736	3	855	825	76	82

4.7 Conclusions

The following conclusions can be drawn based on the outcome of this study:

- Co-fermentation of carbohydrates and proteins resulted in the synergistic impact on hydrogen production and the optimum hydrogen potential was 38% higher than the expected.

- The dominant hydrogen production pathways for carbohydrate and proteins were distinctive: carbohydrate degradation followed ethanol-type fermentation whereas butyrate pathway was observed for proteins. The synchronic effect of the two different pathways in the co-substrate mixture resulted in the synergistic impact on hydrogen production.

- A second-order polynomial adequately correlated the responses to the co-fermentation process. The predicted optimum concentration range of VFA (125 – 133mg/L) and ammonia (41 - 47 mg/L) that ensured an essentially constant pH of the fermentation medium were validated within an average absolute error of 10%.

- The fermentative hydrogen production process would be feasible without any pH control at a carbohydrate-to-protein COD ratio of 4:1.

4.8 References

Abreu, A. A., Karakashev, D., Angelidaki, I., Sousa, D. Z., & Alves, M. M. (2012). Biohydrogen production from arabinose and glucose using extreme thermophilic anaerobic mixed cultures, 1–12.

Akutsu, Y., Lee, D. Y., Li, Y. Y., & Noike, T. (2009). Hydrogen production potentials and fermentative characteristics of various substrates with different heat-pretreated natural microflora. *International Journal of Hydrogen Energy*, *34*(13), 5365–5372. https://doi.org/10.1016/j.ijhydene.2009.04.052

APHA/AWWA/WEF. (2012). Standard Methods for the Examination of Water and Wastewater. *Standard Methods*, 541. https://doi.org/ISBN 9780875532356

Bahl, H., Andersch, W., Braun, K., & Gottschalk, G. (1982). Effect of pH and Butyrate Concentration on the Production of Acetone and Butanol by Clostridium acetobutylicum Grown in Continuous Culture A ~ ", *101*, 17–20.

Batstone, D. J., Keller, J., & Blackall, L. L. (2004). The influence of substrate kinetics on the microbial community structure in granular anaerobic biomass. *Water Research*, *38*(6), 1390–1404. https://doi.org/10.1016/j.watres.2003.12.003

Bhatia, S., Othman, Z., & Ahmad, A. L. (2007). Pretreatment of palm oil mill effluent (POME) using Moringa oleifera seeds as natural coagulant, *145*, 120–126. https://doi.org/10.1016/j.jhazmat.2006.11.003

Breure, A. M., Mooijman, K. A., & van Andel, J. G. (1986). Protein degradation in anaerobic digestion: influence of volatile fatty acids and carbohydrates on hydrolysis and acidogenic fermentation of gelatin. *Applied Microbiology and Biotechnology*, *24*(5), 426–431. https://doi.org/10.1007/BF00294602

Cheng, S. S., Chang, S. M., & Chen, S. T. (2002). Effects of volatile fatty acids on a thermophilic anaerobic hydrogen fermentation process degrading peptone. *Water Science and Technology*, *46*(4–5), 209–214.

Chong, M. L., Sabaratnam, V., Shirai, Y., & Hassan, M. A. (2009). Biohydrogen production from biomass and industrial wastes by dark fermentation. *International Journal of Hydrogen Energy*, *34*(8), 3277–3287. https://doi.org/10.1016/j.ijhydene.2009.02.010

Craven, S. E., & Russell, R. B. (1988). Increased Sporulation of Clostridium perfringens in a Medium Prepared with the Prereduced Anaerobically Sterilized Tech- nique or with Carbon Dioxide or Carbonate, *51*(9), 700–706.

Das, D., & Veziroä, T. N. (2001). Hydrogen production by biological processes : a survey of literature, *26*, 13–28.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric Method for Determination of Sugars and Related Substances. *Analytical Chemistry*, *28*(3), 350–356. https://doi.org/10.1021/ac60111a017

Elsharnouby, O., Hafez, H., Nakhla, G., & El Naggar, M. H. (2013). A critical literature review on biohydrogen production by pure cultures. *International Journal of*

Hydrogen Energy, *38*(12), 4945–4966. https://doi.org/10.1016/j.ijhydene.2013.02.032

Fang, H. H. P., & Liu, H. (2002). Effect of pH on hydrogen production from glucose by a mixed culture. *Bioresource Technology*, *82*(1), 87–93. https://doi.org/10.1016/S0960-8524(01)00110-9

Fangkum, A., & Reungsang, A. (2011). Biohydrogen production from sugarcane bagasse hydrolysate by elephant dung: Effects of initial pH and substrate concentration. *International Journal of Hydrogen Energy*, *36*(14), 8687–8696. https://doi.org/10.1016/j.ijhydene.2010.05.119

Gallert, C., Bauer, S., & Winter, J. (1998). Effect of ammonia on the anaerobic degradation of protein by a mesophilic and thermophilic biowaste population. *Applied Microbiology and Biotechnology*, *50*(4), 495–501. https://doi.org/10.1007/s002530051326

Guo, L., Li, X. M., Bo, X., Yang, Q., Zeng, G. M., Liao, D. xiang, & Liu, J. J. (2008). Impacts of sterilization, microwave and ultrasonication pretreatment on hydrogen-producing using waste sludge. *Bioresource Technology*, *99*(9), 3651–3658. https://doi.org/10.1016/j.biortech.2007.07.026

Guo, X. M., Trably, E., Latrille, E., Carrre, H., & Steyer, J. P. (2010). Hydrogen production from agricultural waste by dark fermentation: A review. *International Journal of Hydrogen Energy*, *35*(19), 10660–10673. https://doi.org/10.1016/j.ijhydene.2010.03.008

Hawkes, F. R., Dinsdale, R., Hawkes, D. L., & Hussy, I. (2002). Sustainable fermentative hydrogen production: Challenges for process optimisation. *International Journal of Hydrogen Energy*, *27*(11–12), 1339–1347. https://doi.org/10.1016/S0360-3199(02)00090-3

Kumar, R., Singh, R., Kumar, N., Bishnoi, K., & Bishnoi, N. R. (2009). Response surface methodology approach for optimization of biosorption process for removal of Cr (VI), Ni (II) and Zn (II) ions by immobilized bacterial biomass sp . Bacillus brevis, *146*, 401–407. https://doi.org/10.1016/j.cej.2008.06.020

Lowry Lewis. (1951). The folin by oliver. *Readings*, *193*(1), 265–275. https://doi.org/10.1016/0304-3894(92)87011-4

Mangayil, R., Santala, V., & Karp, M. (2011). Fermentative hydrogen production from different sugars by Citrobacter sp. CMC-1 in batch culture. *International Journal of Hydrogen Energy*, *36*(23), 15187–15194. https://doi.org/10.1016/j.ijhydene.2011.08.076

Mata-Alvarez, J., Macé, S., & Llabrés, P. (2000). Anaerobic digestion of organic solid wastes. An overview of research achievements and perspectives. *Bioresource Technology*, *74*(1), 3–16. https://doi.org/10.1016/S0960-8524(00)00023-7

Owen, W. F., Stuckey, D. C., Healy, J. B., Young, L. Y., & McCarty, P. L. (1979). Bioassay for monitoring biochemical methane potential and anaerobic toxicity. *Water Research*, *13*(6), 485–492. https://doi.org/10.1016/0043-1354(79)90043-5

Peng, L., Xu, X., Mu, H., Høy, C., & Adler-nissen, J. (2002). Production of structured

phospholipids by lipase-catalyzed acidolysis : optimization using response surface methodology, *31*, 523–532.

Prakasham, R. S., Brahmaiah, P., Sathish, T., & Sambasiva Rao, K. R. S. (2009). Fermentative biohydrogen production by mixed anaerobic consortia: Impact of glucose to xylose ratio. *International Journal of Hydrogen Energy*, *34*(23), 9354–9361. https://doi.org/10.1016/j.ijhydene.2009.09.104

Ramachandran, U., Wrana, N., Cicek, N., Sparling, R., & Levin, D. B. (2008). Hydrogen production and end-product synthesis patterns by Clostridium termitidis strain CT1112 in batch fermentation cultures with cellobiose or α-cellulose. *International Journal of Hydrogen Energy*, *33*(23), 7006–7012. https://doi.org/10.1016/j.ijhydene.2008.09.022

Ramsay, I. R., & Pullammanappallil, P. C. (2001). Protein degradation during anaerobic wastewater treatment. *Biodegradation*, *12*, 247–257.

Ren, N., Cao, G., Wang, A., Lee, D. J., Guo, W., & Zhu, Y. (2008). Dark fermentation of xylose and glucose mix using isolated Thermoanaerobacterium ihermosaccharolyticum W16. *International Journal of Hydrogen Energy*, *33*(21), 6124–6132. https://doi.org/10.1016/j.ijhydene.2008.07.107

Ren, Y., Wang, J., Liu, Z., Ren, Y., & Li, G. (2009). Hydrogen production from the monomeric sugars hydrolyzed from hemicellulose by Enterobacter aerogenes. *Renewable Energy*, *34*(12), 2774–2779. https://doi.org/10.1016/j.renene.2009.04.011

Ren, Z., Ward, T. E., Logan, B. E., & Regan, J. M. (2007). Characterization of the cellulolytic and hydrogen-producing activities of six mesophilic Clostridium species. *Journal of Applied Microbiology*, *103*(6), 2258–2266. https://doi.org/10.1111/j.1365-2672.2007.03477.x

Ries, E. F., & Macedo, G. A. (2011). Improvement of Phytase Activity by a New Saccharomyces cerevisiae Strain Using Statistical Optimization, *2011*. https://doi.org/10.4061/2011/796394

Silvestre, G., Fernández, B., & Bonmatí, A. (2015). Significance of anaerobic digestion as a source of clean energy in wastewater treatment plants. *Energy Conversion and Management*, *101*, 255–262. https://doi.org/10.1016/j.enconman.2015.05.033

Tommaso, G., Ribeiro, R., Varesche, M. B. A., Zaiat, M., & Foresti, E. (2000). Influence of multiple substrates on anaerobic protein degradation in a packed-bed bioreactor Gas line Affluent Effluent, 23–31.

Venkata Mohan, S., Lalit Babu, V., & Sarma, P. N. (2008). Effect of various pretreatment methods on anaerobic mixed microflora to enhance biohydrogen production utilizing dairy wastewater as substrate. *Bioresource Technology*, *99*(1), 59–67. https://doi.org/10.1016/j.biortech.2006.12.004

Wang, J., & Wan, W. (2009). Factors influencing fermentative hydrogen production: A review. *International Journal of Hydrogen Energy*, *34*(2), 799–811. https://doi.org/10.1016/j.ijhydene.2008.11.015

Xia, Y., Cai, L., Zhang, T., & Fang, H. H. P. (2012). Effects of substrate loading and co-

substrates on thermophilic anaerobic conversion of microcrystalline cellulose and microbial communities revealed using high-throughput sequencing. *International Journal of Hydrogen Energy, 37*(18), 13652–13659. https://doi.org/10.1016/j.ijhydene.2012.02.079

Xiao, B., Han, Y., & Liu, J. (2010). Evaluation of biohydrogen production from glucose and protein at neutral initial pH. *Renewable Energy, 35*(12), 6152–6160. https://doi.org/10.1016/j.ijhydene.2010.03.084

Yokoi, H., Maki, R., Hirose, J., & Hayashi, S. (2002). Microbial production of hydrogen from starch-manufacturing wastes. *Biomass and Bioenergy, 22*(5), 389–395. https://doi.org/10.1016/S0961-9534(02)00014-4

Chapter 5
Conclusions and Recommendations

5.1 Conclusions

The following major findings resulted from the outcome of this research:

5.1.1 Effect of pH on the Acidification of a Proteinaceous Substrate

- Alkaline pH conditions favored proteins degradation over neutral or acidic pH; however the degree of acidification indicated by the ratio of the maximum TVFA production to the maximum protein degradation rate was highest at neutral pH (43%).
- The optimum conditions for the production of VFA from the model protein, BSA, were a pH of 8 and fermentation time of 3 days. At these conditions, maximum VFA is produced and maintained stable over the fermentation time. Higher pH values than 8 could achieve the same level of VFA production but would require the doubling of the optimum fermentation time.
- The production of acetic, propionic, and butyric acids was in direct response to protein degradation whereas those of higher molecular weight VFAs (iso-butyric, valeric, and iso-valeric) depended on the length of the incubation time.
- The theoretically derived stoichiometric coefficients generally compared very well to those obtained experimentally. Therefore, the representation by a single stoichiometry for the overall catabolic reaction of anaerobic protein fermentation to organic acids was validated.

- The variation in the predicted stoichiometric coefficient for propionic acid seems to occur from an alternative pathway for proline and arginine fermentation, conceivably the degradation of δ-aminovalerate devoid of propionic acid production.
- Under two extremely differently feed concentrations, that is, when BSA concentration was doubled, amino acid fermentation predominantly occurred by a single pathway.

5.1.2 Co-fermentation of Carbohydrates and Proteins for Biohydrogen Production

- Co-fermentation of carbohydrates and proteins resulted in the synergistic impact on hydrogen production and the optimum hydrogen potential was 38% higher than the expected.
- The dominant hydrogen production pathways for carbohydrates and proteins were distinctive: carbohydrates degradation followed ethanol-type fermentation whereas butyrate synthesis was observed for proteins. The synchronic effect of the two different pathways in the co-substrate mixtures resulted in the synergistic impact on hydrogen production.
- A second-order polynomial adequately correlated the responses to the co-fermentation process. The predicted optimum concentration range of VFA (125 – 133mg/L) and ammonia (41 - 47 mg/L) that ensured an essentially constant pH of the fermentation medium were validated within an average absolute error of 10%.

- The fermentative hydrogen production process would be feasible without any pH control at a carbohydrate-to-protein COD ratio of 4:1.

5.2 Recommendations

The following are recommended for further research:

- The kinetics of anaerobic degradation of proteins

- A comparison between the co-fermentation of carbohydrates and proteins at different ratios in continuous flow and batch systems.

Appendix A

Response equations in terms of actual factors:

VFA = -81.45 + 0.9209*A + 0.7682*B + 100.36*C – 96.58E-006*A^2 – 547.4969E-006B^2 - 0.7840*C^2 – 2.11607E-005*A*B – 10.7E-003*A*C – 0.1463E-003*B*C

NH_3 = -100.56 + 120.54749E-003*A + 50.5074*B + 21.0309*C – 23.290E-006*A^2 – 10.9435E-005*B^2- 2.9347*C^2 + 9.2664E-002*A*B – 23.5841E-005*A*C – 24.1858E-004*B*C

H_2 = -58.42 – 15.7188*A + 45.4686*B + 98.4473*C + 89.3042E-003*A^2 – 98.0573E-004*B^2 – 180.3499*C^2 + 26.3040E-009*A*B – 14.2629E-005*A*C + 26.1594E-003*B*C

A – Carbohydrate B – Protein C – pH

Appendix B

Analysis of variance for the responses of VFA, ammonia, and hydrogen

VFA

ANOVA for the test of significance for VFA production from co-fermentation of carbohydrates and proteins

Source		Sum of Squares	DF	Mean Square	F Value	Prob >F	
Model		3.708E+006	9	3.026E+005	734.40	<0.0001	*significant*
	A	5304.10	1	6073.10	18.56	0.0018	
	B	0.95	1	0.84	3.554E-004	0.9940	
	C	3.030E+006	1	2.018E+005	4064.55	<0.0001	
	A^2	369.60	1	369.78	0.07	0.4021	
	B^2	45.14	1	56.44	0.080	0.4354	
	C^2	9.018E+004	1	6.246E+005	1291.50	<0.0001	
	AB	89.44	1	35.25	0.010	0.6301	
	AC	2516.25	1	4943.89	25.84	0.0070	
	BC	45.56	1	45.56	0.081	0.8551	
Residual		5568.73	11	311.63			
Lack of Fit		2314.73	6	300.75	0.91	0.9040	*not significant*
Pure Error		5610.22	7	400.00			
Cor Total		3.6458E+007	21				

NH_3

ANOVA for the test of significance for ammonia production from co-fermentation of carbohydrates and proteins

Source		Sum of Squares	DF	Mean Square	F Value	Prob >F	
Model		1544.30	9	2160.11	48.30	<0.0001	*significant*
	A	106.55	1	98.25	8.44	0.2024	
	B	75.56	1	26.87	0.88	<0.0001	
	C	2544.44	1	24786.10	347.01	<0.0001	
	A^2	11.36	1	12.45	0.20	0.8435	
	B^2	19.56	1	19.16	23.13	0.4011	
	C^2	452.20	1	2254.36	12.40	0.0004	
	AB	5.01	1	5.33	0.084	0.9436	
	AC	369.00	1	369.00	4.84	0.0733	
	BC	84.00	1	83.11	0.47	<0.0001	
Residual		466.65	10	53.54			
Lack of Fit		230.25	5	42.22	0.44	0.8210	*not significant*
Pure Error		669.83	5	430.01			
Cor Total		16625.95	24				

H_2

ANOVA for the test of significance for hydrogen production from co-fermentation of carbohydrates and proteins

Source		Sum of Squares	DF	Mean Square	F Value	Prob >F	
Model		3.269E+004	9	22234.24	3441.02	<0.0001	*significant*
	A	24.10	1	36.10	2.61	<0.0001	
	B	36.11	1	32.78	4.63	0.1410	
	C	2.713E+005	1	2.959E+005	33547.23	<0.0001	
	A^2	88.23	1	87.05	7.86	0.0286	
	B^2	251.14	1	261.24	56.54	0.0373	
	C^2	42964.23	1	40860.21	8956.24	<0.0001	
	AB	7.21	1	7.33	0.56	0.5201	
	AC	8.63	1	5.89	0.41	<0.0001	
	BC	8.00	1	8.00	0.69	0.5268	
Residual		211.25	10	48.56			
Lack of Fit		68.10	5	12.45	0.88	0.7235	*not significant*
Pure Error		89.45	4	24.86			
Cor Total		3.719E+006	21				

Appendix C

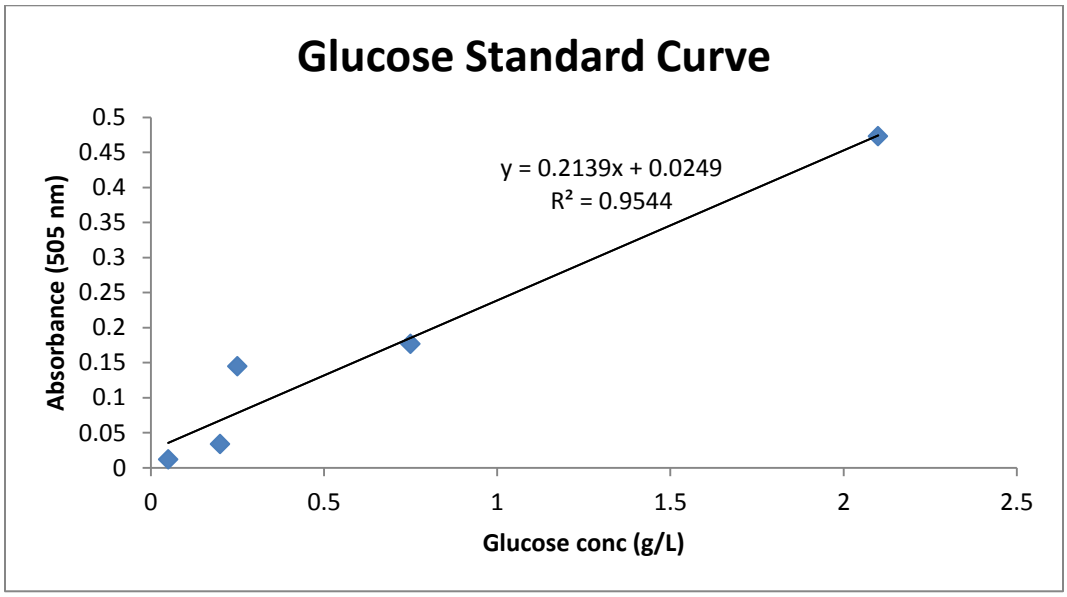
Data for Statistical Analysis

Std	Run	Block	Factor 1 A:Carbohydrat mg/L	Factor 2 B:Protein mg/L	Factor 3 C:Time d	Response 1 VFA mg/L	Response 2 Ammonia mg/L	Response 3 Hydrogen mL	Response 4 pH
12	1	Block 1	548.00	884.00	5.00	842	57	274	5.6
19	2	Block 1	548.00	677.00	5.00	840	58	274	5.4
1	3	Block 1	150.00	470.00	0.00	0	0	0	5.5
5	4	Block 1	150.00	470.00	10.00	875	80	280	5.2
8	5	Block 1	1396.00	884.00	10.00	785	54	281	5.5
15	6	Block 1	548.00	677.00	5.00	835	60	275	5.3
9	7	Block 1	150.00	677.00	5.00	854	55	285	5.4
10	8	Block 1	1396.00	677.00	5.00	800	60	278	5.1
6	9	Block 1	1396.00	470.00	10.00	800	60	273	5.4
11	10	Block 1	548.00	328.87	5.00	825	53	265	5.2
20	11	Block 1	548.00	677.00	5.00	845	52	280	5.4
4	12	Block 1	1396.00	884.00	0.00	0	0	0	5.5
13	13	Block 1	548.00	677.00	0.00	0	0	0	5.5
14	14	Block 1	548.00	677.00	10.00	875	75	282	5.4
2	15	Block 1	946.00	470.00	0.00	0	0	0	5.5
17	16	Block 1	548.00	677.00	5.00	805	65	273	5.2
16	17	Block 1	548.00	677.00	5.00	825	74	281	5.4
7	18	Block 1	150.00	884.00	10.00	875	70	280	5.5
18	19	Block 1	548.00	677.00	5.00	800	58	281	5.5
3	20	Block 1	150.00	884.00	0.00	0	0	0	5.5

Appendix D

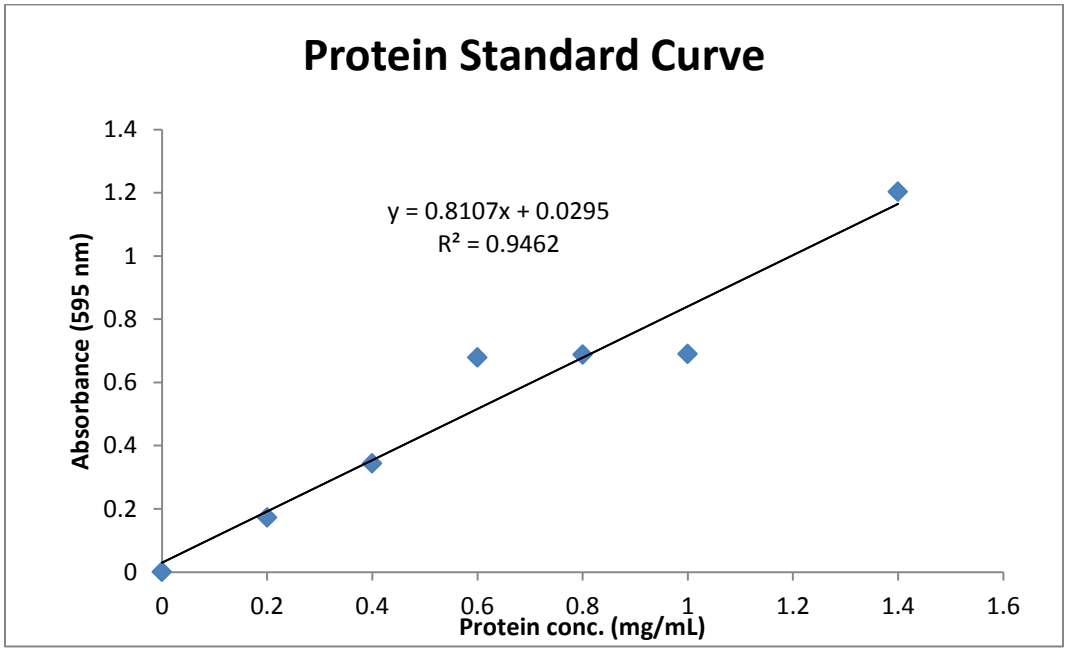
Carbohydrate and Protein Standard Curves

(A)



Glucose Standard Curve

(B)



Protein Standard Curve

Curriculum Vitae

Name: Emmanuel Andrew Tepari Jr.

Education:

Sept 2017- Aug 2019	Master of Engineering Science (Chemical & Biochemical Eng.) University of Western Ontario, London, Ontario, Canada
Sept 2010- June 2014	Bachelor of Science [Honours] Chemical Engineering Kwame Nkrumah University of Science and Technology, Ghana

Work History:

Sept 2017 – Aug 2019	Graduate Research and Teaching Assist Department of Chemical and Biochemical Eng. University of Western Ontario, London, Ontario, Canada
Sept 2015 -Aug 2017	Process Engineer Diageo Plc (Guinness Ghana Breweries Limited)
July 2014-Aug 2015	Teaching Assistant Kwame Nkrumah University of Science and Technology, Ghana