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Improvements in the process of biohydrogen production by Thermotoga neapolitana

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IMPROVEMENTS IN THE PROCESS OF BIOHYDROGEN PRODUCTION BY *Thermotoga neapolitana*

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ABSTRACT

The production of biological hydrogen is an important process for the future of sustainability and alternative energies. *Thermotoga neapolitana* is a hyperthermophilic bacterium that produces H_2, CO_2 and acetate via fermentation. The goal of the research was to investigate the sustainable production of H_2 gas using waste agricultural feedstocks, recycled water and a simplified N_2 sparging method technique.

A limited sparging method was developed which includes 1 minute of N_2 gas sparging of the reactor headspace and 90 minutes of idle reaction time for cysteine-HCl to react with dissolved oxygen prior to inoculation. This method was found to increase the hydrogen percentage in the gas produced by *Thermotoga neapolitana* as compared to a 15 minute sparge, no-idle treatment. In the carbon and nitrogen source study, H_2 concentrations as high as 33.08 mmol H_2/L medium were achieved and yields as high as 0.38 g H_2/g substrate COD were achieved using cull peach medium Soybean or canola meal can act as carbon and nitrogen sources for this process. When waste water from a peach cooling process was used in medium, 35.04 mmol H_2/L medium resulted as compared to the 31.66 mmol H_2/L medium produced using distilled water. These results indicated that the hydrocooler water may be beneficial for the productivity of *Thermotoga neapolitana*. *Thermotoga neapolitana* was found to be able to grow in a CSABR reactor at a temperature of 77°C. It was found that when the pH was controlled at 7 and H_2 gas was collected the product concentrations were increased compared to the same treatments grown in serum bottles without pH control and H_2 gas removal. An alternative medium consisting of peaches and soybean meal as the carbon and nitrogen

sources, respectively, was successfully used to grow *T. neapolitana* in the CSABR system. It was also determined that *Thermotoga neapolitana* can thrive and product formation can be increased at substrate concentrations of 10 g/L compared to 5 g/L. The 10 g/L Standard medium treatment resulted in a H_2 concentration of 83.19 mmol H_2/L medium compared to 42.66 mmol/L medium for the 5 g/L standard medium.

DEDICATION

I would like to dedicate this thesis to my family. I would first like to thank my grandparents for all of their amazing generosity and support over the years because I would not be where I am today without them. I would like to thank my mother, father and brother as well, for their encouragement and support. I would also like to thank my lovely girlfriend, Whitney Blue Fraser, for her love and support throughout my collegiate years.

ACKNOWLEDGMENTS

I would first like to thank Dr. Caye Drapcho for her wonderful support over the last few years and for encouraging me to pursue this Masters degree. Her help and critical suggestions were invaluable. I would also like to thank Dr. Terry Walker and Dr. Nhuan Nghiem for their guidance as members of my committee. I am very grateful for the countless undergraduate and graduate students who helped me during my research, especially Karthik Gopalakrishnan.

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CHAPTER ONE

INTRODUCTION

For many years efforts have been made to find an alternative energy source that could replace fossil fuels. Fossil fuels are exhaustable as they may run out by 2060-2070 (Klass 2003) and the combustion of fossil fuels leads to the production of greenhouse gasses, which contributes to global climate change. Fossil fuels accounted for 83% of the total energy consumed in the United States in 2010 (EIA, 2012) so there is room for growth in the renewable energy sector. Hydrogen gas is primarily produced by steam reforming of methane which is a non-renewable, fossil fuel based process (Balat and Balat, 2009). The process of making hydrogen gas from fossil fuels produces the same amount of CO_2 as the direct combustion of the fossil fuels (Balat and Balat, 2009). Hydrogen is also commonly produced via the process of electrolysis in which an electric current used to separate water into H_2 and O_2 (Levin et al., 2004). The hydrogen in a combustion reaction contains 120kJ/g of energy compared to the 33 kJ/g of energy that carbon contains (Graetz, 2009). Hydrogen has the highest gravimetric energy density of any known fuel (Levin et al., 2004). When this is combined with the fact that hydrogen combustion with oxygen produces water, the prospects for hydrogen as a renewable energy source are very great if it can be produced sustainably.

Hydrogen gas can be a renewable energy source because it can be produced biologically and when it is combusted water is the only product (Hawkes et al., 2002). Hydrogen can be produced by photobiological processes or by fermentations processes (Hallenbeck and Benemann 2002). Photobiological hydrogen production can be by direct

biophotolysis, indirect biophotolysis or photofermentation. These photobilogical processes along with fermentation process provide possible energy solutions (Levin et al., 2004). A further discussion of photobiological production is beyond the scope of this paper so the focus will be on hydrogen production by fermentation.

Certain microorganisms in the Archaea and Bacteria Domains can produce hydrogen naturally through fermentation reactions (Huber and Hannig, 2006). These chemoorganotrophic organisms utilize an organic substrate under anaerobic conditions to form various products by using H^+ ions as electron acceptor. These organisms produce a biogas that contains primarily H_2 but also H_2S, CO and CH_4 (Levin et al., 2004). This is important because the membrane used in many hydrogen fuel cells can be poisoned by the presence of H_2S in this biogas (Levin et al., 2004). Along with these gasses, organic acids such as acetate, butyrate, lactate and propionate are produced via this fermentation process depending on which energy pathway is used by the organism. Many factors can influence how much of each organic acid is produced (Levin et al., 2004). These factors can also greatly affect the amount of H_2 produced by these organisms. These factors include various reactor environment conditions such as pH, hydraulic retention time and gas partial pressure (Levin et al., 2004). Equation 1 shows the balanced reaction for the fermentation when acetate is produced (Schroder et al., 1994) and Equation 2 shows the balanced reaction for the fermentation when butyrate is produced (Levin et al., 2004).

$$C_6H_{12}O_6 + 2H_20 \rightarrow 2CH_3COOH + 4H_2 + 2CO_2 \quad (1)$$

$$C_6H_{12}O_6 + 2H_20 \rightarrow 2CH_3CH_2CH_2COO^- + 2HCO_3 + 3H^+ + 2H_2 \quad (2)$$

Fermentation reactions can take place by organisms at various temperatures ranges: mesophilic (25 to 40°C), thermophilic (40 to 60°C), extreme thermophilic (60 to 75°C), and hyperthermophilic (>75°C) (Yu and Drapcho, 2011). Hyperthermophilic organisms have been shown to be as effective, or more effective, than mesophilic in fermenting myriad substrates including waste products (Sasaki, et al., 2011).

Thermotoga is a genus of fermentative hyperthermophiles that was found in the Bay of Naples in 1986 in underwater geothermal vents. (Jannasch et al., 1988). The Thermotoga genus is in the group Thermotogales which is the one group of H_2-producing, hyperthermophilic bacteria (Huber and Hannig, 2006). Nine species in the Thermotoga genus have been isolated and identified (Huber and Hannig, 2006). *Thermotoga neapolitana* is a Gram-negative, rod-shaped organism that is surrounded by an outer structure that is called a "toga" (Jannasch et al., 1988). *T. neapolitana* is an obligate anaerobe that primarily utilizes glucose for energy by fermenting it to acetate, CO_2 and H_2 (Huber and Hannig, 2006). Schroder et al. (1994) determined the Embden Meyerhoff (EM) pathway for the catabolism of glucose by *Thermotoga maritima*, which can be seen in Figure 1.1. Schroder et al. (1994) also determined that H^+ is reduced because of the transfer of electrons from ferredoxin using the hydrogenase enzyme. The conversion of pyruvate to acetyl-coenzyme A is catalyzed by the pyruvate: ferredoxin oxidoreductase enzyme (Schroder et al., 1994). is Selig et al (1997) determined that *Thermotoga maritima* fermented glucose via the EM pathway primarily (85%) but via the Entner Doudoroff (ED) to a lesser degree(15%). d'Ippolito et al. (2010), however, showed that *Thermotoga neapolitana* ferments glucose to pyruvate almost exclusively by

the EM pathway which results in acetate being the main organic acid produced, with a smaller amount of lactate. The theoretical amount of products formed per mol of glucose should be 4 mol H_2, 2 mol CO_2, 2 mol acetate and 2 mol H^+ (Thauer 1977).

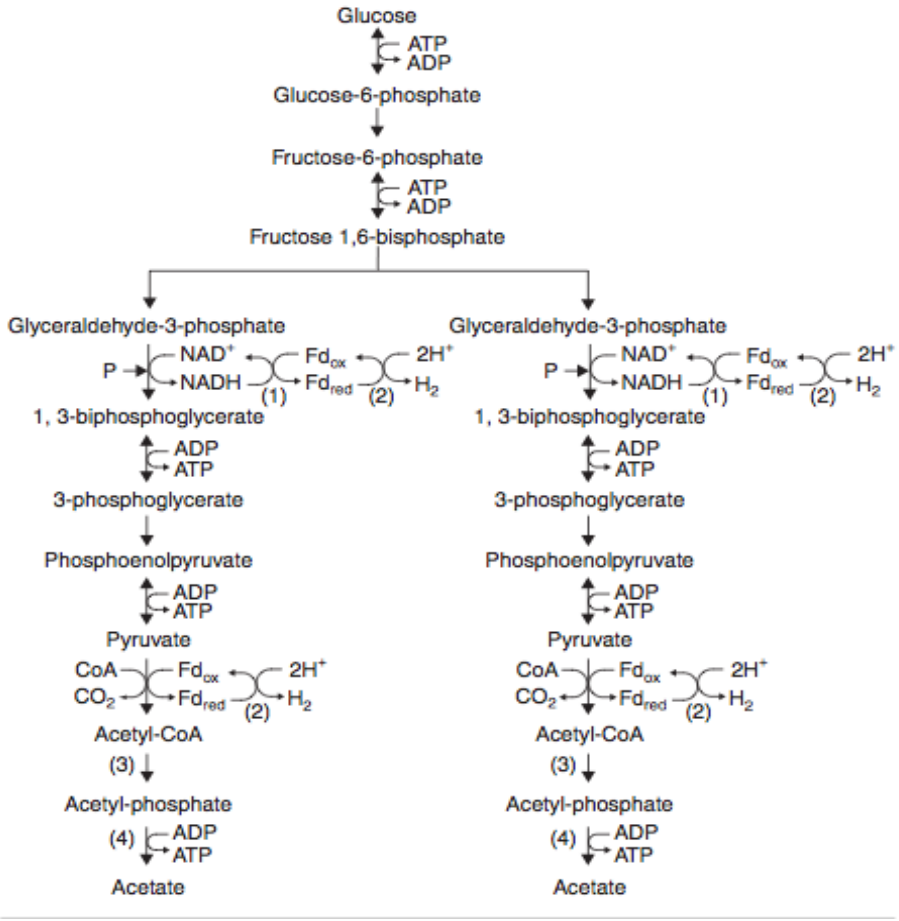

Figure 1.1: The Embden- Meyerhoff Pathway for the catabolism of glucose by *T. maritima*, adapted from Schroder, et al. (1994).

Equation 3, below, shows the overall reaction for the fermentation of glucose via the EM pathway (Schroder, et al., 1994).

$$C_6H_{12}O_6 + 2H_2O + 4ADP \rightarrow 2CH_3COO^- + 2H^+ + 2CO_2 + 4H_2 + 4ATP \qquad (3)$$

Hydrogen yields for *Thermotoga neapolitana* have been reported that come close to the maximum of 4 mol H_2/mol glucose but the theoretical has not yet been achieved. Munro et al, (2009) reported a hydrogen yield of 3.8 mol H_2/mol glucose. Munro et al, (2009) also reported yields of 2 mol CO_2, 1.8 mol acetate and 0.1 mol lactate per mol of glucose consumed. Eriksen et al. (2010) also reported a hydrogen yield of 3.8 mol H_2/mol glucose. Eriksen et al. (2010) reported a CO_2 yield of 2.4 mol CO_2/mol glucose which is greater than the theoretical yield.

Hydrogen gas is a growth associated product formed by *T. neapolitana* but it also acts as an inhibitor when it accumulates in the environment (Schonheit and Schafer, 1995). Research has shown that higher hydrogen concentrations can cause a shift in organic acid production from acetate to lactate, a more reduced acid (Kengen, et al., 1996). van Niel (2002) reported that a hydrogen partial pressure of less than 20 kPa is required in the reactor environment when the temperature is greater than 70°C. Studies have been done to try to find a method in which hydrogen gas can be removed from the headspace to prevent inhibtion.

Many researchers have found that pH decreases over time as *T. neapolitana* grows (Ravot et al., 1995; Van Ooteghem et al., 2004; Huber and Hannig, 2006). Van Ooteghem et al. (2004) reported that the a pH of 4.5 inhibits the growth of *T. neapolitana*. Yu and Drapcho (2011) reported in kinetics study that a pH of 5.1 was observed in the culture after 10 hours of growth and was maintained until the end of the study. Several studies have used base addition to control the pH within the reactor environment in order to utilize all of the glucose or other carbon source (Munro et al.,

2009; Ngo et al., 2011). The use of pH control may be necessary in order to maximize hydrogen production. The interaction between pH inhibition and hydrogen inhibition within a reactor environment is unclear. Another consideration is the possibility the formation of sodium acetate, which occurs after the production of acetate via fermentation, inhibits growth. Sodium acetate inhibited the growth of *Caldicellulosiruptor saccharolyticus*, which is an extreme thermophile, so it is possible this effect may also be seen with *Thermotoga neapolitana* (van Niel et al., 2002).

T. neapolitana is an obligate anaerobe. A method to remove the oxygen from the reactor environment is necessary. Many researchers have used N_2 sparging (Erikesen et al., 2010; Ngo et al., 2011; Yu and Drapcho, 2011) to remove the oxygen along with the addition of cysteine-HCl, an oxygen scavenger (Huber and Hannig, 2006). For future applications in larger scale bioprocessing an efficient method must be determined. It is important for the energy and material use to be decreased for the process.

Many studies of *Thermotoga neapolitana* have focused on determining the most suitable temperature for culture growth. *T. neapolitana* is grown commonly at temperatures between 70-80°C. An early study by Belkin et al, (1986) determined 77°C as the optimal temperature for *T. neapolitana* and a study by Munro et al. (2009) confirmed this finding.

Many researchers have made it a common practice to autoclave media before growing *Thermotoga* species (van Neil et al., 2002; van Ooteghem et al., 2004; Yu and Drapcho, 2011). In the Yu and Drapcho (2011) method the bottles were autoclaved at 121°C for 20 minutes and then sparged with N_2 gas for 1 minute prior to incubation.

However, due to the fact that *T. neapolitana* is a hyperthermophile, it may not be necessary to autoclave the media prior to incubation because mesophilic organisms are not able to grow in the same temperature range. In Jain (2009) the media were not autoclaved but were sparged with N_2 gas for 15 minutes.

T. neapolitana can ferment glucose and many other carbon sources. Yu and Drapcho (2011) compared several carbon sources including: glucose, xylan, sucrose, rice flour, starch, xylose, cellobiose, corn starch, beet pulp and cellulose. Medium containing glucose as the carbon source was found to result in higher hydrogen concentrations while sucrose had the third highest concentrations. It was also determined that a 36 hour fermentation time resulted in higher hydrogen concentrations in a medium containing more complex sugars such as sucrose (Yu, 2007). Jannasch et al. (1988) found that *T. neapolitana* would not utilize volatile acids or alcohols such as acetate, formate, pyruvate, propionate, ethanol, methanol, glycerol, glutamate, or glycine. An organic nitrogen source is needed for *T. neapolitana* to grow and often yeast extract is used for this purpose (Schroder, et al., 1994; Nguyen et al., 2008; d'Ippolito et al., 2010). Also, trypticase peptone is usually used along with the yeast extract as an organic nitrogen source (Van Ooteghem et al 2002). It was reported that when *Thermotoga elfii* was grown with yeast extract as the only nitrogen source, 26% of the glucose was turned into another product, which decreased the hydrogen yield (van Niel, et al., 2002). Table 1.1, below, shows the standard glucose medium used by Yu and Drapcho (2011) which is the standard medium used as the basis of comparison for all experiments.

Table 1.1: Standard glucose medium for growth of *T. neapolitana*

Standard Medium (weights per 1 liter of medium)	
5 g glucose	0.1 g $CaCl_2 \cdot 2H_20$
2 g yeast extract	10.0 g NaCl
2 g trypticase peptone	0.1 g KCl
1.0 g NH_4Cl	1.114 g cysteine-$HCl \cdot H_20$
0.3 g K_2HPO_4	0.121 g THAM
0.3 g KH_2PO_4	10 mL vitamin solution
0.2 g $MgCl_2 \cdot 2H_20$	10 mL trace element solution

Biomass feedstocks such as sugarcane, cull peaches, sugar beet and several cereal grains consist of high percentages of sugars. Lignocellulosic sources such as switchgrass and sugarcane bigasse contain cellulose and hemicelluloses but Yu and Drapcho (2011) found poor utilization of cellulose by *T. neapolitana.* Jain (2009) reported that *T. neapolitana* had been grown in media with peaches as the carbon source. For nitrogen sources, there are many alternatives. Yu and Drapcho (2011) studied the effect of several nitrogen sources on the growth of *T. neapolitana.* Yeast extract (YE), soybean meal, canola meal, cottonseed meal, linseed meal and fish meal were used as nitrogen sources with and without trypticase in the media. They reported that soybean meal and canola meal samples resulted in the highest hydrogen production after the yeast extract (Yu and Drapcho, 2011). Most other research concerning nitrogen sources has involved yeast extract and trypticase so further study in this area is needed to provide a viable option for use in a scaled-up reactor.

Available agricultural feedstocks may differ from location to location. In South Carolina, approximately 20 million pounds of cull peaches are discarded each year due to imperfections (SCDA, 2007). Colaric et al (2004) reported sugar contents across 19 different peach cultivars and reported total sugar concentrations as ranging from 62-91

g/kg fruit (wwb). Sucrose, fructose and glucose concentrations ranged from 46 and 70 g/kg, 7 to 13 g/kg fruit and 5 to 12 g/kg (wwb), respectively. Colaric et al. (2004) also reported that fructose and glucose concentrations varied more than sucrose concentration. In 2007 in South Carolina alone, 8,075,000 bushels of soybeans were produced (Clemson University, 2009). This value equates to 242,250 tons of soybeans. Soybean meal is about 78% (w/w) of the soybeans so soybean meal is readily available as nitrogen source (NCSPA, 2013).

It is also important that for this bio-process to move forward to a point where it can be utilized as a rural, clean energy solution the process must be as sustainable as possible. Studies are needed to learn how materials from other processes can be recycled. If this bio-process can be found to work efficiently using agricultural feedstocks then it may be possible to have a bio-refinery facility that produces H_2 gas for use as an energy source in rural areas. Recycle and re-use of as many resources as possible is necessary in order to decrease the cost of such a facility. Peaches are washed and sorted before they can be sold so it may be possible to integrate parts of this process with the biological H_2 production process.

Recent studies have begun focusing on scaling-up the fermentation process using larger reactors. Scale-up allows for better process control as most lab scale reactors have very intricate controls. Using these reactors, pH control as well as the removal and collection of H_2 gas from the reactor headspace is possible. Ngo, et al. (2011) used a continuously stirred anaerobic reactor to compare hydrogen production by *T. neapolitana* with and without pH control. H_2 gas was removed from the headspace in both pH control

and non pH control studies. The study reported 56% higher hydrogen gas yields for cultures grown with pH control compared to those grown without pH control (Ngo et al., 2011). It was also reported that a pH of 7 was the optimum pH for the reactor environment when compared to pH's of 6.5 and 7.5 (Ngo et al., 2011). There is another positive effect that H_2 collection and removal has on growth. The removal increases the $\%H_2$ gas in the headspace because the gas that is initially in the head space, which consists of mainly N_2, is removed which results in higher $\%H_2$ and $\%CO_2$ during the fermentation period. Also in this study, Ngo et al. (2011) kept a continuous flow of N_2 gas to sparge the headspace which would increase the $\%N_2$ in and therefore decrease $\%H_2$ and $\%CO_2$.

It is important to scale-up the substrate concentrations to determine the most suitable concentration for *Thermotoga neapolitana* growth and determine at what point substrate inhibition becomes a factor. Researchers have not focused on increasing growth medium constituent concentrations to determine the effects. (Nguyen et al., 2011) grew *T. neapolitana* in batch culture in serum bottles and found 7 g/L glucose resulted in the highest $\%H_2$ in the headspace when looking at a range of glucose concentrations from 1-10 g/L. This study was limited because of H_2 gas and pH inhibition so it is necessary to focus experiments on reactors which have greater process control. Another important step of this research is to utilize the advantages of larger reactor volumes along with the simplified medium using agricultural by-products.

The objectives of this study were: to measure hydrogen production by *T. neapolitana* in peach media and glucose media with three different nitrogen sources and

compare it to the standard glucose media; to modify existing techniques and processes in order to improve the efficiency of the batch fermentation of *T. neapolitana;* to measure hydrogen production by *T. neapolitana* in both standard and alternative media with hydrocooler water and compare it to distilled water; and to measure hydrogen production by *T. neapolitana* in both standard and alternative media using a scaled up batch reactor that utilizes pH control and hydrogen gas inhibition control.

CHAPTER TWO

THE EFFECT OF AGRICULTURAL-BASED CARBON AND NITROGEN SOURCES ON PRODUCTION OF BIOHYDROGEN BY *Thermotoga neapolitana*

Abstract

The production of biological hydrogen is an important process for the future of sustainability and alternative energies. *Thermotoga neapolitana* is a hyperthermophilic bacterium that produces H_2, CO_2 and acetate via fermentation. The goal of the research was to investigate the sustainable production of H_2 gas using waste agricultural feedstocks, recycled water and a simplified N_2 sparging method technique.

A limited sparging method was developed which includes 1 minute of N_2 gas sparging of the reactor headspace and 90 minutes of idle reaction time for cysteine-HCl to react with dissolved oxygen prior to inoculation. This method was found to increase the hydrogen percentage in the gas produced by *Thermotoga neapolitana* as compared to a 15 minute sparge, no-idle treatment. This limited sparging method was used in all subsequent studies. In the carbon and nitrogen source study, H_2 concentrations as high as 33.08 mmol H_2/L medium were achieved and yields as high as 0.038 g H_2/g substrate COD utilized were achieved using cull peach medium Soybean or canola meal can act as carbon and nitrogen sources for this process. When waste water from a peach cooling process was used in the medium, 35.04 mmol H_2/L medium resulted as compared to the 31.66 mmol H_2/L medium produced using distilled water. These results indicated that the hydrocooler water may be beneficial for the productivity of *Thermotoga neapolitana.*

This study showed that the peach variety is important and the variety can also effect the production of H_2, CO_2 and acetate.

Introduction

For many years efforts have been made to find an alternative energy source that could replace fossil fuels. Fossil fuels are exhaustable as they may run out by 2060-2070 (Klass 2003) and the combustion of fossil fuels leads to the production of greenhouse gasses, which contributes to global climate change. Fossil fuels accounted for 83% of the total energy consumed in the United States in 2010 (EIA, 2012) so there is room for growth in the renewable energy sector. Hydrogen gas is a renewable energy source because it can be produced biologically and when it is combusted water is the only product (Hawkes et al., 2002). Certain microorganisms in the Archaea and Bacteria Domains can produce hydrogen naturally through fermentation reactions (Huber and Hannig, 2006).

Thermotoga neapolitiana is a fermentative hyperthermophile that was found in the Bay of Naples over 25 years ago (Jannasch et al., 1988). *T. neapolitana* is a Gram-negative, rod-shaped organism that is surrounded by an outer structure that is called a "toga" (Jannasch et al., 1988). *T. neapolitana* comes from the Order Thermotogales, the one group of H_2-producing, hyperthermophilic bacteria (Huber and Hannig, 2006). *T. neapolitana* is an obligate anaerobe that primarily utilizes glucose for energy by fermenting it to acetate, CO_2 and H_2 (Huber and Hannig, 2006). d'Ippolito et al. (2010) showed that *T. neapolitana* ferments glucose to pyruvate primarily by the Emden-Meyerhoff Pathway. Schroeder et al. (1994) determined that H^+ is reduced because of the

transfer of electrons from ferredoxin. The theoretical ratio of products formed per mol of glucose is 4 mol H_2, 2 mol CO_2, 2 mol acetate and 2 mol H^+ (Thauer 1977).

Hydrogen gas is a growth associated product formed by *T. neapolitana* but it also acts as an inhibitor when it accumulates in the environment (Schonheit and Schafer, 1995). Many researchers have found that pH decreases over time as *T. neapolitana* grows (Ravot et al., 1995; Huber and Hannig, 2006). Van Ooteghem et al. (2004) reported that the a pH of 4.5 inhibits the growth of *T. neapolitana*. Yu and Drapcho (2011) reported in kinetics study that a pH of 5.1 was observed in the culture after 10 hours of growth and was maintained until the end of the study.

T. neapolitana is an obligate anaerobe. A method to remove the oxygen from the reactor environment is necessary. Many researchers have used N_2 gas sparging (Erikesen et al., 2010; Ngo et al., 2011; Yu and Drapcho, 2011) to remove the oxygen along with the addition to the culture medium of cysteine-HCl, an oxygen scavenger (Huber and Hannig, 2006). For future applications in larger scale bioprocessing an efficient method must be determined. It is important for the energy and material use to be decreased for the process.

Many researchers have made it a common practice to autoclave media before growing *Thermotoga* species (van Neil et al., 2002; van Ooteghem et al., 2004; Yu and Drapcho, 2011). In the Yu and Drapcho (2011) method the bottles were autoclaved at 121°C for 20 minutes and then sparged with N_2 gas for 1 minute prior to incubation. However, due to the fact that *T. neapolitana* is a hyperthermophile, it may not be necessary to autoclave the media prior to incubation because mesophilic organisms are

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nitrogen sources with and without trypticase in the media. They reported that soybean meal and canola meal resulted in the highest hydrogen production after the yeast extract (Yu and Drapcho, 2011). Most research concerning nitrogen sources has involved yeast extract and trypticase so further study in this area is needed to provide a viable option for use in a scaled-up reactor.

Available agricultural feedstocks may differ from location to location. In South Carolina, approximately 20 million pounds of cull peaches are discarded each year due to imperfections (SCDA, 2007). Peaches contain mostly sucrose, glucose and fructose. Colaric et al (2004) reported sugar contents across 19 different peach cultivars and reported total sugar concentrations as ranging from 62-91 g/kg fruit (wwb). Sucrose, fructose and glucose concentrations ranged from 46 and 70 g/kg, 7 to 13 g/kg fruit and 5 to 12 g/kg (wwb), respectively. Colaric et al. (2004) also reported that fructose and glucose concentrations varied more than sucrose concentration. In 2007 in South Carolina alone, 8,075,000 bushels of soybeans were produced (Clemson University, 2009). This value equates to approximately 242,250 tons of soybeans. Soybean meal is about 78% (w/w) of the soybeans so soybean meal is readily available as nitrogen source (NCSPA, 2013). If this bio-process can be found to work efficiently using agricultural feedstocks then it may be possible to have a bio-refinery facility that produces H_2 gas for use as an energy source in rural areas. Recycle and re-use of as many resources as possible is necessary to increase the sustainability of such a facility. Peaches are washed and sorted before they can be sold so it may be possible to integrate parts of the peach processing with the biological H_2 production process.

The goal of this research was to investigate a sustainable H_2 bioprocess using waste agricultural materials. The objectives of this study were to determine hydrogen production by *T. neapolitana* in peach media and glucose media with three different nitrogen sources and compare it to the standard glucose medium; to modify existing sparging techniques in order to improve the efficiency of the batch fermentation of *T. neapolitana;* and to determine impact on hydrogen production by *T. neapolitana* of using recycled process water in culture medium.

Materials and Methods

Culture Conditions

The bacterium *Thermotoga neapolitana* (DSM 4359) was obtained from DSMZ (the German Resource Centre for Biological Material) and was maintained in seed bottles on a standard glucose medium that was defined as the standard medium by Yu and Drapcho (2011). This standard medium contains all of the following components in dry weight per liter of medium: 5.0 g glucose, 2.0 g yeast extract (YE), 2.0 g trypticase, 1.0 g NH_4Cl, 0.3 g K_2HPO_4, 0.3 g KH_2PO_4, 0.2 g $MgCl_2 \cdot 2H_2O$, 0.1 g $CaCl_2 \cdot 2H_2O$, 10.0 g NaCl, 0.1 g KCl, 1.114 g cysteine-$HCl \cdot H_20$, 0.121 g trizma base(THAM), and 10.0 mL each of vitamin solution and trace element solution as outlined by DSMZ media 141. The pH of the medium was adjusted to 8 using 5N NaOH solution prior to inoculation. Bottles were sparged with N_2 (High Purity, Airgas Welders) for 1 minute prior to a 90 minute idle time at room temperature. Seed bottles were incubated for 20 hours at 77°C and then stored at room temperature before use. Cultures of *Thermotoga neapolitana* were kept in a 30% glycerol solution at -80°C.

The methods used in the experiments are based on the work done by Yu and Drapcho (2011). For fermentation trials, serum bottles (total volume = 565 mL) containing 200 mL of media were prepared. One liter of media was prepared for each treatment resulting in 4 replicate bottles per treatment. Each bottle was inoculated with 5 mL of active culture using a sterile syringe, capped with a butyl stopper and an aluminum crimp seal, and incubated in orbital shaker bed at 200 rpm and 77°C. Based on the findings of Jain (2009) the medium was not autoclaved prior to inoculation. Three studies were conducted.

Study One: Limited Sparging Study

An experiment was performed to compare two sparging treatments, each prepared using standard medium. For sparging treatment 1, the media and the headspace were sparged with N_2 gas for 15 minutes. Treatment 2 was a limited sparging treatment where only the headspace was sparged with N_2 gas for one minute. The Treatment 2 bottles then sat at room temperature for 90 minutes in order for the cysteine-HCl to sufficiently react with the oxygen. After this preliminary study the limited sparging technique was used in all subsequent studies.

Study Two: Carbon and Nitrogen Source Study

In order to determine the effect of varying carbon and nitrogen sources on the product yields of *Thermotoga neapolitana*, a seven treatment experiment was designed. Peaches and glucose were compared as carbon sources and both soybean meal and canola meal were compared as nitrogen sources to yeast extract/trypticase. Each treatment consisted of 5g/L (dwb) of carbon source and 4g/L (dwb) of nitrogen source while all

other media components were as listed in study 1. The following are the treatments 1-7, respectively: Glucose/Yeast Extract and Trypticase(Standard Medium), Glucose/Yeast Extract, Glucose/Canola Meal, Glucose/Soybean Meal, Peach/Yeast Extract, Peach/Canola Meal and Peach/Soybean Meal.

Red Prince variety peaches were obtained from Musser Farms, Clemson, SC. The pits were removed and the peaches were blended with skins together for 3 minutes. The peach slurry was poured into 0.5 liter bags and stored at -40° C.

The soybean meal and canola meal used in the experiment were obtained from Southern States (Pendleton,SC). Both were screened using a sieve and particles ≤ 1mm in diameter were used. Both were dried in an oven at 60°C before being added to the medium.

Study Three: Hydrocooler Water Study

A study was performed in order to determine the impact of using the recycled water from a peach hydrocooler--a cold water bath used to rapidly cool the fruit-- and using it in the growth medium instead of distilled (DI) water. A five treatment study was designed to study the effects of this hydrocooler (HC) water on the production of hydrogen gas by *T. neapolitana*. Each treatment contained 5g/L (dwb) carbon source and 4g/L (dwb) nitrogen source. The five treatments are: Standard Medium in DI water, Flavor Rich variety peaches/soybean meal in DI water, Standard Media in HC water, Flavor Rich variety peaches/soybean meal in HC water and Red Prince peaches/soybean meal in DI water. The Red Prince peaches were used in the carbon and nitrogen source study and this treatment was included to compare to the Flavor Rich peaches, the new

variety used for this experiment. No vitamins and trace elements were added to the three peach media treatments (Morris, 2013).

The Flavor Rich variety peaches were obtained from Titan Farms in Ridge Spring, SC. The pits were removed and the peaches were blended with skins together for 3 minutes. The peach slurry was poured into 0.5 liter bags and stored at -40° C. The soybean meal used is the same as used in the carbon and nitrogen source study and was processed in the same way before use.

The recycled hydrocooler water originally contained chlorine tablets at 600 ORP in order to disinfect the peaches during processing. The water was used for 2 days in the hydrocooler prior to being frozen for this study. The water was obtained from Titan Farms in Ridge Spring, SC and was frozen at -40°C prior to use.

Analytical Methods

After incubation the serum bottles were placed into a water bath for 30 minutes and cooled to 25°C. The pressure in the bottles was measured using a handheld digital manometer (Fisher Scientific). A 0.5 mL sample of headspace, with a glass syringe, was manually injected into a Gow-Mac Series 400 -gas partitioner with a thermal conductivity detector. The carrier gas was argon at a pressure of 22 psi. The column used was a 10’ X 1/8” packed Molecular Sieve 5A, alkali alumino silicate. The column temperatures were 40°C, 55°C and 40°C. The percent hydrogen gas and carbon dioxide in each sample were calculated using pure hydrogen and pure carbon dioxide standard curves. The absolute gas pressure was found by adding the atmospheric pressure to the gauge pressure reading. Then the hydrogen and carbon dioxide partial pressures were found by multiplying the

percent of each gas by the absolute total pressure. As reported by Yu and Drapcho (2011) the moles of each gas were calculated using the Ideal Gas Law which can be seen below (Equation 1):

$$n = \frac{P_1 V_1}{RT} \quad (1)$$

where n = mol in gas phase, P_1= partial pressure (kPa), V_1 = headspace volume (L), R = Universal gas constant (8.3145 L·kPa/mol·K), and T = temperature (K).

In order to take into account the dissolved H_2 and CO_2 in the medium Henry's Law was used, which can be seen in equation 2:

$$C_{aq} = k_h * p \quad (2)$$

where C_{aq}= concentration in mol/L, p= the partial pressure at 25°C and k_h= Henry's Law constant at 25°C.
The Henry's Law constant at 25°C for CO_2 is 3.36 x 10^{-4} M/kPa and for H_2 is 7.7 x 10^{-6} M/kPa (Sanders, 1999).

An HPLC with a refractive index detector was used for the determination of glucose, fructose and acetate concentrations. A Bio-Rad HPX-87H column was used along with a mobile phase of 0.1N H_2SO_4 at a flow rate of 0.6ml/min. The use of H_2SO_4 as mobile phase caused cleaving of sucrose to glucose and fructose which is why only glucose and fructose data is reported. Samples were filtered with 0.45µm filters prior to the analysis. Both initial and final samples were taken. Sample concentrations were compared to standards that were prepared in 10 g/L NaCl solution.

To determine solids content, triplicate samples of peach slurry were dried in an oven at 105°C for 48 hours. The percent solids was calculated as the: difference in weight / initial weight.

The experimental results were analyzed with SAS software (SAS, SAS Institute Inc., Cary, NC), with a 0.05 level of significance used for Tukey's Studentized range test.

Results and Discussion

Limited Sparging Study

It was found that *T. neapolitana* could produce H_2 gas without long periods of N_2 sparging prior to inoculation. Table 2.1 shows the mean H_2 gas concentrations for the treatments were not significantly different.

Table 2.1: H_2 production results for *Thermotoga neapolitana* grown in standard medium[1]

Treatment: Sparging Method	Mean H_2 concentration in headspace (%)[2]	Std. Dev.	Mean absolute total pressure at 25°C (kPa)[2]	Std. Dev.	Mean H_2 concentration (mmol H_2/L medium)[2]	Std. Dev.
Standard Sparging	35.43[b]	2.60	110.9[a]	5.8	29.28[a]	2.88
Limited Sparging	37.30[a]	2.32	111.6[a]	3.1	31.30[a]	1.71

[1] Means not sharing common letter are significantly different at ($p<0.05$) using Tukey's Studentized Range Test
[2] n=5

Both treatments showed H_2 gas production that compare to the 32.83 mmol H_2/L reported by Yu and Drapcho (2011) using the same standard medium. Statistical analysis showed that the two treatments were not significantly different. However, the limited sparging treatment showed slightly higher H_2 gas concentrations. Table 2.1 shows that the mean absolute total pressure for each treatment was nearly the same but the H_2 percentage was higher for the limited sparging treatment. Based on the results of this study, the limited sparging technique would be used in all subsequent studies, including the studies reported here.

Carbon and Nitrogen Source Study

The percent solids weight of the peaches was determined to be 13.09% so 38.19 g of peach slurry were added per liter of medium. The total sugars concentration added to the media was 4.3 g/L (2.2 g/L glucose and 2.1 g/L fructose).

The hydrogen production results for all seven treatments in the carbon and nitrogen source study can be seen in Table 2.2.

Table 2.2: Production of H_2 by *Thermotoga neapolitana* with various carbon and nitrogen sources[1,2]

Treatment: Carbon Source-Nitrogen Source	Mean H_2 concentration in headspace (%)	Std. Dev.	Mean absolute total pressure at 25°C (kPa)	Std. Dev.	Total Mean H_2 concentration (mmol H_2/L medium)	Std. Dev.	Mean H_2 Yield (g H_2/ g substrate COD utilized)	Std. Dev.
Glucose-YE/Trypticase[3]	24.68[b]	0.58	146.3[c]	0.9	26.88[b]	0.79	0.034[b]	0.001
Glucose-YE[4]	27.48[a]	0.49	152.8[b]	1.3	31.24[a]	0.81	0.023[c]	0.001
Glucose-Canola Meal[4]	22.07[c]	0.94	138.4[d]	2.9	22.74[c]	1.14	0.038[a]	0.002
Glucose-Soybean Meal[4]	21.43[c]	0.82	138.3[d]	3.2	22.07[c]	1.34	0.029[b]	0.002
Peach-YE[4]	28.06[a]	2.31	158.4[a]	0.9	33.08[a]	2.77	0.023[c]	0.002
Peach-Canola Meal[4]	27.91[a]	1.30	156.9[a]	3.8	32.63[a]	2.08	0.023[c]	0.001
Peach-Soybean Meal[3]	25.26[b]	1.21	148.8[b,c]	2.4	28.00[b]	1.79	0.021[c]	0.001

[1] Means not sharing common letter are significantly different at ($p<0.05$) using Tukey's Studentized Range Test
[2] Glucose medium containing 5 g/L glucose and peach medium containing 4.3 g/L total sugars (2.2 g/L glucose and 2.1 g/L fructose)
[3] n=3
[4] n=4

The standard medium treatment (Glucose-YE/Trypticase) resulted in a total mean H_2 concentration of 26.88 mmol H_2/L medium. This value is less than the 32.83 mmol H_2/L medium reported by Yu and Drapcho (2011) and is less than the value for the limited sparging treatment in the limited sparging study. The standard medium and peach-soybean meal treatments have smaller sample size because both treatments contained one bottle that resulted in an outlier data point so that bottle was disregarded. It

is possible that the bottle contained oxygen either from the inoculation procedure or from a possible loose aluminum crimp seal. It was found in this study that the Glucose-YE treatment produced a hydrogen gas concentration of 31.24 mmol H_2/L medium. This is higher than that of the standard glucose medium which includes trypticase.

The H_2 yield was calculated in terms of g H_2/ g substrate COD utilized. The COD was calculated from the glucose and fructose concentrations using the theoretical conversions of 1.067 g COD/g glucose or fructose. The theoretical yield for the production of H_2 by *T. neapolitana* is 0.042 g H_2/g substrate COD, based on Thauer (1977). The Glucose-Canola meal treatment had the highest yield which was 90.48% of the theoretical yield and the Peach-Soybean meal treatment had the lowest yield which was 50% of the theoretical yield. The high H_2 yield for the Glucose-Canola meal treatment may be an anomaly in the data or possibly due to sampling error.

Overall, the Peach-YE and Peach-Canola treatments resulted in higher H_2 concentrations. In general, the peach medium treatments did not result in significantly different total H_2 concentrations from the glucose medium treatments. These results indicate that alternative media sources can be used in future experiments in place of the standard media carbon and nitrogen sources.

When comparing the effect of nitrogen sources, it can be seen that the Peach/Canola treatment resulted in a higher total H_2 concentration than the Peach/Soybean treatment. The total H_2 concentrations from the Glucose/Canola and Glucose/Soybean treatments were not significantly different but they were both

significantly lower than the Glucose/YE-T treatment. That result was also observed by Yu and Drapcho (2011) in a comparable experiment.

Carbon dioxide is also produced as part of the growth of *Thermotoga neapolitana* and Table 2.3 shows the production of CO_2 for each treatment in the carbon and nitrogen source study.

Table 2.3: The production of CO_2 by *Thermotoga neapolitana* with various carbon and nitrogen sources[1,2]

Treatment: Carbon Source-Nitrogen Source	Mean Gas CO_2 concentration (mmol CO_2/L medium)	Std. Dev.	Mean Dissolved CO_2 concentration (mmol CO_2/L medium)	Std. Dev.	Total Mean CO_2 concentration (mmol CO_2/L medium)	Std. Dev.	Mean CO_2 Yield (g CO_2/ g substrate COD utilized)	Std. Dev.
Glucose-YE/Trypticase[3]	$11.28^{c,d}$	0.62	$5.14^{c,d}$	0.28	$16.60^{c,d}$	1.11	0.45^{a}	0.02
Glucose-YE[4]	$13.78^{a,b}$	0.97	$6.28^{a,b}$	0.44	$20.06^{a,b}$	1.41	0.32^{b}	0.02
Glucose-Canola Meal[4]	8.36^{e}	0.28	3.82^{e}	0.13	12.18^{e}	1.50	0.45^{a}	0.02
Glucose-Soybean Meal[4]	$9.67^{d,e}$	1.17	$4.41^{d,e}$	0.53	$14.08^{d,e}$	1.71	0.41^{a}	0.05
Peach-YE[4]	14.69^{a}	1.25	6.70^{a}	0.57	21.40^{a}	1.82	0.33^{b}	0.03
Peach-Canola Meal[4]	15.13^{a}	1.81	6.90^{a}	0.83	22.03^{a}	2.64	0.33^{b}	0.04
Peach-Soybean Meal[3]	$12.52^{b,c}$	1.16	$5.71^{b,c}$	0.53	$18.23^{b,c}$	1.69	0.30^{b}	0.03

[1] Means not sharing common letter are significantly different at ($p<0.05$) using Tukey's Studentized Range Test
[2] Glucose medium containing 5 g/L glucose and peach medium containing 4.3 g/L total sugars (2.2 g/L glucose and 2.1 g/L fructose)
[3] n=3
[4] n=4

Carbon dioxide has a much higher Henry's Law constant than H_2 because CO_2 is much more soluble in water. This difference causes the dissolved CO_2 concentration to have more of an effect on the total CO_2 concentration. Many researchers have not reported CO_2 concentrations and yields from similar studies because the focus has been on the H_2 and organic acid production. However, if this process is to be used in the future in a bio-refinery in the future the CO_2 must be quantified and re-used. For instance, studies have shown the potential of using CO_2 from waste streams such as those at a

wastewater treatment plant to grow photoautotrophic algae for further biofuel production (Wang, et al., 2009). The CO_2 produced in this process could be used in a similar way.

The theoretical yield for CO_2 for this fermentation process is 0.46 g CO_2/g substrate COD, based on Thauer (1977). The results of this study indicated yields from 0.3-0.45 g CO_2/g substrate COD which is a range of 65-98% of the theoretical yield. Eriksen et al (2010) reported an average CO_2 yield of 2.4 mol CO_2/mol glucose for *Thermotoga neapolitana* grown in batch culture with a standard glucose medium under similar conditions. The mol/mol yield is equivalent to 0.55 g CO_2/g substrate COD which is 20% higher than the theoretical yield and 22% higher than the yield reported for the standard medium treatment here. Here, as with the hydrogen yields, the peach medium treatments resulted in lower overall CO_2 yields than the glucose medium treatments. There also appears not to be a correlation between the nitrogen source used and the CO_2 yield achieved through fermentation.

Acetic acid is the main organic acid produced by *Thermotoga neapolitana* during its growth. Other organic acids such as lactic acid are produced via a different pathway and are not produced as readily as acetic acid. Table 2.4 is a table of the acetate production results for the carbon and nitrogen source study.

Table 2.4: The production of acetate by *Thermotoga neapolitana* with various carbon and nitrogen sources[1,2]

Treatment: Carbon Source-Nitrogen Source	Mean Final Acetate concentration (mmol acetate/L medium)	Std. Dev.	Mean Acetate Yield (g acetate/ g substrate COD utilized)	Std. Dev.	Mean Final pH of medium	Std. Dev.
Glucose-YE/Trypticase[3]	12.43[a,b]	4.02	0.46[a,b]	0.15	4.81	0.04
Glucose-YE[4]	11.84[a,b]	0.25	0.25[c]	0.01	4.73	0.07
Glucose-Canola Meal[4]	10.47[a,b]	2.64	0.52[a]	0.13	4.72	0.01
Glucose-Soybean Meal[4]	8.07[b]	1.55	0.31[b,c]	0.06	4.81	0.08
Peach-YE[4]	13.63[a]	0.24	0.28[b,c]	0.01	4.76	0.06
Peach-Canola Meal[4]	11.21[a,b]	2.49	0.23[c]	0.05	4.95	0.02
Peach-Soybean Meal[3]	10.01[a,b]	0.24	0.22[c]	0.01	4.92	0.08

[1] Means not sharing common letter are significantly different at ($p<0.05$) using Tukey's Studentized Range Test
[2] Glucose medium containing 5 g/L glucose and peach medium containing 4.3 g/L total sugars (2.2 g/L glucose and 2.1 g/L fructose)
[3] n=3
[4] n=4

The theoretical yield for acetate via the fermentation of glucose by *Thermotoga neapolitana* is 0.61 g acetate/g substrate COD, based on Thauer (1977). Actual yields were found to be between 0.22 and 0.52 g acetate/g substrate COD which is 36-85% of the theoretical yield. On average, the yields of the peach medium treatments were lower than the yields of the glucose medium treatments but this trend can be seen in all three product yields of this study. The fact that the average yield of the seven treatments is 0.33 g acetate/g substrate COD suggests that other organic acids, which were not analyzed for this study, are being produced at higher quantities than previously believed. Eriksen et al (2010) also reported acetate yield for *Thermotoga neapolitana* grown in batch culture with standard medium. The reported yield was 1.4 mol acetate/mol glucose (Eriksen, et

al., 2010). This is equivalent to 0.43 g acetate/g substrate COD consumed and this value is slightly less than value of 0.46 g acetate/g substrate COD reported in this study.

The pH of the medium decreases over time as the substrate is consumed and pH values between 4.5 and 5.1 have been noted as inhibitory for the growth of *Thermotoga neapolitana.* Studies have indicated that the accumulation of the organic acids is the cause of the drop in pH over time (Ngo, et al., 2011). The final pH of the medium for the seven treatments in this study ranged from 4.72-4.95 so it is possible that growth was being inhibited by pH by the end of the 40 hour incubation period.

Table 2.5 shows the residual concentrations for glucose and fructose at the end of the fermentation period. The residual concentrations indicate that not all of the substrate was consumed in the 40 hour fermentation period. This suggests that the growth of *T. neapolitana* was inhibited by some factor, probably a combination of low pH and high H_2 and CO_2 concentrations.

Table 2.5: Residual glucose and fructose concentrations in the media after 40 hour fermentation period

Treatment: Carbon Source-Nitrogen Source	Residual Glucose concentration (g glucose/L medium)	Std. Dev.	Residual Fructose concentration (g fructose/L medium)	Std. Dev.
Glucose-YE/Trypticase	2.78	0.54	-	-
Glucose-YE	2.45	0.02	-	-
Glucose-Canola Meal	3.86	0.61	-	-
Glucose-Soybean Meal	3.62	0.94	-	-
Peach-YE	0.58	0.04	1.82	0.10
Peach-Canola Meal	0.53	0.19	2.08	0.97
Peach-Soybean Meal	0.44	0.33	2.14	1.19

The theoretical molar ratio of products formed by the fermentation of glucose by *Thermotoga neapolitana* is 4 mol H_2, 2 mol CO_2, 2 mol acetate and 2 mol H^+, based on Thauer (1977). Figure 2.1 shows the actual molar ratios obtained in this experiment.

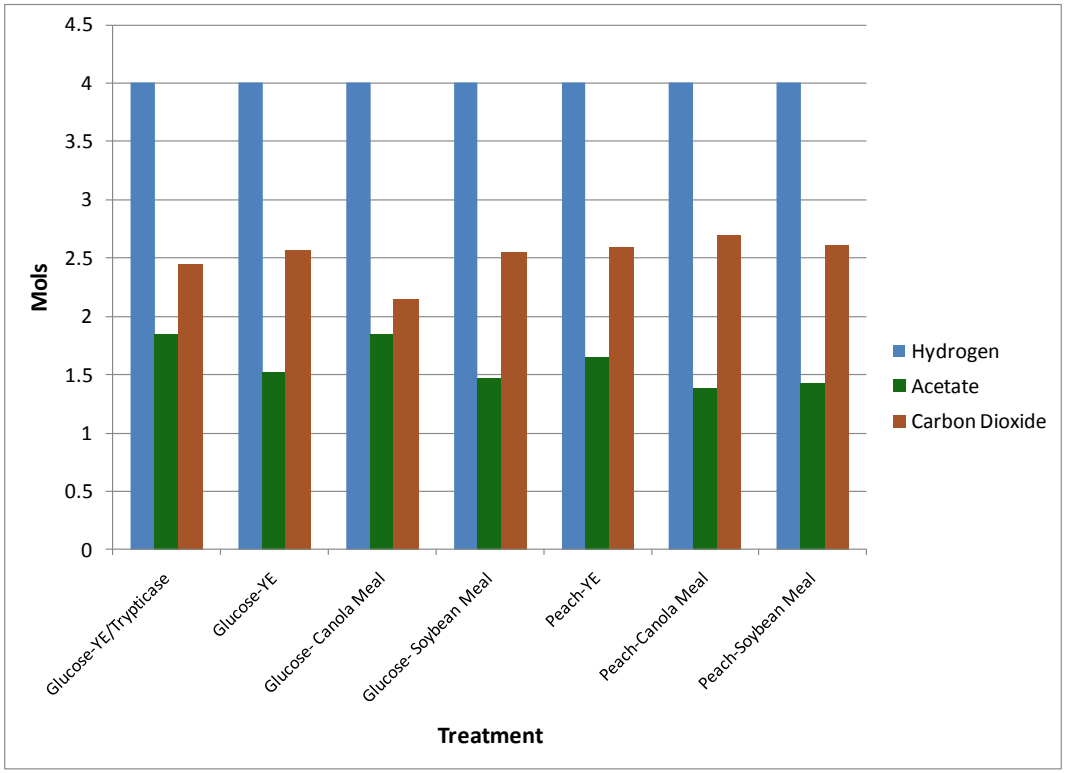

Figure 2.1: The actual molar ratios of the products of fermentation by *Thermotoga neapolitana.* Theoretical ratio is 4:2:2.

The ratios in Figure 2.1 were normalized to 4 mols of H_2 in order to directly compare to the theoretical ratio even though the yields per mol of glucose were lower than the theoretical. The ratios are similar to the theoretical ratio as none of the treatments have ratios beyond an acceptable range. It can be seen that overall the amount of acetate is lower than expected. This could be due to some other organic acid being produced or the acetate degradation. Also, the CO_2 ratio is higher than expected for most of the treatments. This could be due to the poor resolution of the CO_2 peak on the

chromatograph from the GC analysis. It is also possible that after incubation the bottles were not cooled for a long enough period to reach equilibrium which could have resulted in the dissolved CO_2 being lower than the calculated value. However, as stated earlier, Eriksen et al (2010) reported CO_2 yields of 2.4 mol CO_2/mol of glucose, which would correspond to a greater than 2 mol CO_2 in the product molar ratio. It is possible some phenomenon results in greater production of CO_2 but no hypothesis has been given on what the phenomenon might be. It can be seen from this data that overall the peach media treatments seem to produce less acetate and more CO_2 than the standard media treatments. This could be because the peach treatments may produce more lactate or other organic acids through other pathways or another process is releasing more CO_2.

Hydrocooler Water Study

From HPLC analysis it was found that residual sugars were present in the Hydrocooler water. It was found that the sugar concentration in the HC water was 0.1 g substrate COD/L medium. The percent solids weight for the Flavor Rich variety peaches was determined to be 7.74% so 64.6 g of peach slurry were added per liter of medium. The total sugars added to the Flavor Rich peach medium was 4.5 g/L (2.4 g/L glucose and 2.1 g/L fructose). For the Red Prince variety peaches the percent solids weight was determined to be 13.09% so 38.19 g of peach slurry were added per liter of medium. The total sugars added to the Red Prince peach medium was 4.3 g/L (2.2 g/L glucose and 2.1 g/L fructose).

Table 2.6 shows the H_2 results for the hydrocooler water study.

Table 2.6: The production of H_2 by *Thermotoga neapolitana* with DI water or HC water[1,2]

Treatment: Media/Water Type[3,4]	Mean H_2 concentration in headspace (%)	Std. Dev.	Mean absolute total pressure at 25°C (kPa)	Std. Dev.	Total Mean H_2 concentration (mmol H_2/L medium)	Std. Dev.	Mean H_2 Produced (g H_2/ g substrate COD utilized)	Std. Dev.
Standard Media/DI Water	26.58[a]	0.54	158.2[a]	1.0	31.66[b]	0.42	0.028[a]	0.000
FlavorRich Peach/DI Water	12.32[c]	3.97	116.3[c]	3.6	10.71[d]	3.55	0.026[a]	0.009
Standard Media/HC Water	29.22[a]	0.47	161.1[a]	0.8	35.04[a]	0.68	0.027[a]	0.001
FlavorRich Peach/HC Water	17.18[b]	0.91	118.8[c]	1.5	15.19[c]	0.85	0.029[a]	0.002
RedPrince Peach/DI Water	14.94[b,c]	1.64	130.7[b]	2.9	14.56[c]	1.90	0.032[a]	0.004

[1] Means not sharing common letter are significantly different at ($p<0.05$) using Tukey's Studentized Range Test

[2] Glucose medium containing 5 g/L glucose, Red Prince peach medium containing 2.2 g/L glucose and 2.1 g/L fructose and Flavor Rich peach medium containing 2.4 g/L glucose and 2.1 g/L fructose

[3] For peach treatments, nitrogen source was 4g/L soybean meal and no vitamins and trace elements were added

[4] n=4

The Standard Media/HC water treatment had the highest H_2 concentration and differed enough to be considered significantly higher than the Standard Media/DI water treatment. The H_2 concentration of 35.04 mmol H_2/L medium for the Standard Media/HC water treatment also surpassed the 32.83 mmol H_2/L medium reported by Yu and Drapcho (2011). The H_2 concentration of the FlavorRich Peach/HC water treatment was significantly higher than the FlavorRich Peach/DI water treatment. The RedPrince Peach/DI water treatment was similar to the FlavorRich Peach/HC water treatment. This could mean that the RedPrince variety contains more of other nutrients than the FlavorRich variety and is more suitable for use in this application. This is important because it shows that the variety of peaches can make a difference in the quality of this bio-process.

The actual yield for all the treatments was much lower than the theoretical yield of 0.042 g H_2/g substrate COD, based on Thauer (1977). The RedPrince Peach/DI water treatment had the highest yield of 0.032 g H_2/g substrate COD but there was no significant difference in the yields between any of the treatments. These yields compare, however, to the yields seen in the carbon and nitrogen source study. From these results it is possible that the HC water has a positive effect on H_2 production by *Thermotoga neapolitana.* This means that the chlorine originally put in the water may be reduced to a point that it does not negatively affect growth. Even though the glucose and fructose in the hydrocooler water only increased the substrate concentration by about 2%, the Standard/HC showed about a 10% increase in H_2 concentration so the hydrocooler water may have some effect on the process other than increased substrate concentration.

Table 2.7 shows the results of the production of CO_2 by *Thermotoga neapolitana* for the hydrocooler water study.

Table 2.7: The Production of CO_2 by *Thermotoga neapolitana* with DI water or HC water[1,2]

Treatment: Media/Water Type[3,4]	Mean Gas CO_2 concentration (mmol CO_2/L medium)	Std. Dev.	Mean Dissolved CO_2 concentration (mmol CO_2/L medium)	Std. Dev.	Mean Total CO_2 concentration (mmol CO_2/L medium)	Std. Dev.	Mean CO_2 Produced (g CO_2/ g substrate COD utilized)	Std. Dev.
Standard Media/DI Water	12.05[a]	1.76	5.50[a]	0.80	17.55[a]	2.57	0.34[b]	0.05
FlavorRich Peach/DI Water	2.72[c]	0.93	1.24[c]	0.43	3.96[c]	1.36	0.21[c]	0.07
Standard Media/HC Water	12.37[a]	1.20	5.64[a]	0.55	18.01[a]	1.74	0.31[b]	0.03
FlavorRich Peach/HC Water	6.13[b]	0.78	2.79[b]	0.36	8.92[b]	1.14	0.38[a,b]	0.05
RedPrince Peach/DI Water	6.34[b]	0.57	2.89[b]	0.26	9.23[b]	0.82	0.45[a]	0.04

[1] Means not sharing common letter are significantly different at ($p<0.05$) using Tukey's Studentized Range Test

[2] Glucose medium containing 5 g/L glucose, Red Prince peach medium containing 2.2 g/L glucose and 2.1 g/L fructose and Flavor Rich peach medium containing 2.4 g/L glucose and 2.1 g/L fructose

[3] For peach treatments, nitrogen source was 4g/L soybean meal and no vitamins and trace elements were added
[4] n=4

The Standard Media/HC water treatment produced the highest CO_2 concentration of 12.37 mmol CO_2/L medium which would be expected since that treatment also had the highest H_2 concentration. However, the Standard Media/HC water treatment was found not to have a significantly higher CO_2 concentration than the Standard Media/DI water treatment. As with the H_2 results, the CO_2 concentrations from the FlavorRich/DI water treatment and the RedPrince/DI water treatment were not significantly different. Also, the FlavorRich/DI water treatment had a significantly lower CO_2 concentration than the other treatments. The CO_2 concentrations of the standard medium treatments are comparable to the CO_2 concentrations for many of the treatments found in the carbon and nitrogen source study but the peach medium treatments had lower concentrations.

The RedPrince Peach/DI water treatment resulted in a CO_2 yield of 0.45 g CO_2/g substrate COD which is 98% of the theoretical yield of 0.46 g CO_2/g substrate COD, based on Thauer (1977). There was more range found among the CO_2 yields than was found among the H_2 yields which could be due to the difficulty of measuring the CO_2 peak on the GC chromatograph, which was mentioned previously. The CO_2 production results follow the same trend overall as the H_2 results, providing more evidence that the hydrocooler water has a positive effect on the growth of and productivity of *Thermotoga neapolitana.*

Acetate production by *Thermotoga neapolitana* is very important because it can be used by other organisms to produce more H_2 or it can be used to for methane

production in a wastewater treatment plant. Table 2.8 shows the acetate production results for the hydrocooler water study.

Table 2.8: The production of acetate by *Thermotoga neapolitana* with DI water or HC water[1,2]

Treatment: Media/Water Type[3,4]	Mean Final Acetate Concentration (mmol acetate/L medium)	Std. Dev.	Mean Acetate Produced (g acetate/ g substrate COD utilized)	Std. Dev.	Mean Final pH of medium	Std. Dev.
Standard Media/DI Water	12.14[b]	0.21	0.31[b]	0.01	4.76	0.02
FlavorRich Peach/DI Water	1.05[d]	0.25	0.08[c]	0.02	5.06	0.07
Standard Media/HC Water	12.82[a]	0.19	0.30[b]	0.00	4.69	0.05
FlavorRich Peach/HC Water	0.69[d]	0.10	0.04[c]	0.01	4.89	0.03
RedPrince Peach/DI Water	6.17[c]	0.65	0.42[a]	0.04	4.96	0.06

[1] Means not sharing common letter are significantly different at ($p<0.05$) using Tukey's Studentized Range Test

[2] Glucose medium containing 5 g/L glucose, Red Prince peach medium containing 2.2 g/L glucose and 2.1 g/L fructose and Flavor Rich peach medium containing 2.4 g/L glucose and 2.1 g/L fructose

[3] For peach treatments, nitrogen source was 4g/L soybean meal and no vitamins and trace elements were added

[4] n=4

The Standard Media/HC water treatment produced the highest acetate concentration of 12.82 mmol acetate/L medium. This value was significantly different than the concentrations of the other treatments. This value is comparable to the acetate concentrations found in the carbon and nitrogen source study. The Standard Media/DI water treatment produced a high concentration of 12.14 mmol acetate/L medium but the next closest was the RedPrince Peach/DI water treatment with a concentration of 6.17 mmol acetate/L medium. The FlavorRich Peach/HC water treatment produced less acetate than expected based on the H_2 and CO_2 results. This could be explained by a measurement error or it is possible that the peaches provided an environment that favors

the production of other organic acids such as lactate, which was not measured in this study. Overall the peach medium treatments produced less acetate than expected.

The actetate yields were lower than expected overall and showed a wide range. The actual yields ranged anywhere from 6%-69% of the theoretical yield of 0.61 g acetate/g substrate COD, based on Thauer (1977). The RedPrince Peach/DI water treatment was significantly higher where as the standard medium treatments were not significantly different from each other and the FlavorRich peach treatments were not significantly different one another. The final pH values of the treatments are of the range expected based on the carbon and nitrogen source study and other previous studies. The mean pH of 4.89 for the FlavorRich/HC water treatment lends support to the idea that another organic acid was produced at a meaningful concentration or that some error in the acetate measurement occurred. The acetate results continue to support the hypothesis that the hydrocooler water is beneficial for this bioprocess in the future.

The residual glucose and fructose concentrations for the hydrocooler study can be seen in Table 2.9. As seen in the carbon and nitrogen source study, all of the substrate was not consumed during the 40 hour fermentation period which suggests inhibition.

Table 2.9: Residual glucose and fructose concentrations in the media after 40 hour fermentation period

Treatment: Media/Water Type[2,3]	Residual Glucose concentration (g glucose/L medium)	Std. Dev.	Residual Fructose concentration (g fructose/L medium)	Std. Dev.
Standard Media/DI Water	3.19	0.00	-	-
FlavorRich Peach/DI Water	2.06	0.00	1.75	0.01
Standard Media/HC Water	2.95	0.00	-	-
FlavorRich Peach/HC Water	2.09	0.00	1.73	0.02
RedPrince Peach/DI Water	1.61	0.00	1.88	0.01

As stated previously, the theoretical molar ratio of products formed by the fermentation of glucose by *Thermotoga neapolitana* is 4 mol H_2, 2 mol CO_2, 2 mol acetate and 2 mol H^+, based on Thauer (1977). Figure 2.2 shows the actual molar ratios obtained in the hydrocooler water study.

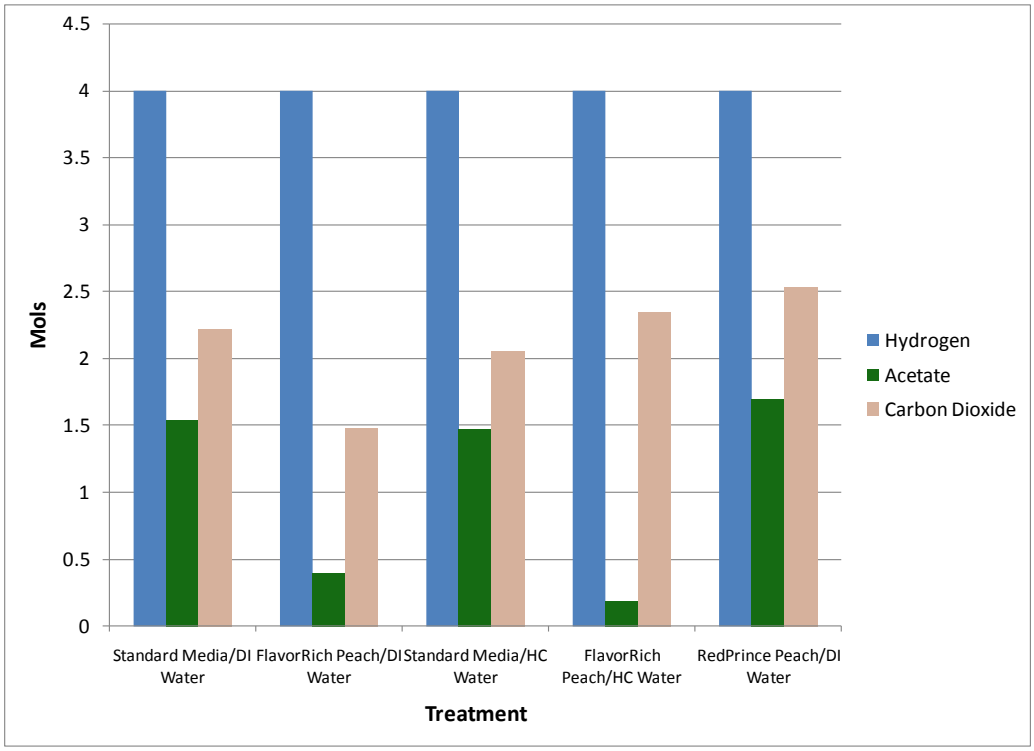

Figure 2.2: The actual molar ratios of the products of fermentation by *Thermotoga neapolitana* in the hydrocooler water study

The actual molar ratios found for this study have more variability than those found in the carbon and nitrogen source study. The ratios of the two FlavorRich Peach treatments are not very near the theoretical ratios but the other three treatments are closer to what is expected considering the previously mentioned limitations of this study: difficulty in CO_2 measurement and the lack of measurement of other organic acids.

Conclusions

Thermotoga neapolitana was found to be able to produce H_2 as effectively when the medium is not sparged with N_2 gas for an extended period of time. The limited sparging technique was found to be as effective as standard sparging technique at removing oxygen from the reactor environment and showed the possibility of improving H_2 production. This technique was found to be simpler and reduced the amount N_2 used. This technique was then used in all subsequent studies. *Thermotoga neapolitana* was found to be able to utilize agricultural based products as carbon and nitrogen sources in the growth medium. Sugars from cull peaches and nitrogen from both soybean meal and canola meal were utilized and H_2, CO_2 and acetate were produced. All of the agricultural based treatments did not perform as well as the standard medium treatment but there was strong evidence that in the future research could focus on these alternative carbon and nitrogen sources because of the sustainability of the process. The results indicate, as previous studies have shown, that pH and hydrogen gas inhibit the growth of *Thermotoga neapolitana* when grown in batch culture so it is important to find ways to control these factors in the future. *Thermotoga neapolitana* was found to produce higher concentrations of H_2, CO_2 and acetate when hydrocooler water from a peach processing

facility was used in the growth medium in the place of distilled water. The hydrocooler water study showed that the HC water could be recycled and used in a future biohydrogen facility. It was also learned from the hydrocooler study that the variety of peaches used as the carbon source has an impact on the production of H_2, CO_2 and acetate. Peach varieties can show great variation and it is possible that certain nutrients can have an effect on the process. Overall, the results show strong evidence that more sustainable and energy saving modifications to the growth medium and pre-incubation processing can be made to positively impact the productivity of *Thermotoga neapolitana*.

CHAPTER THREE

THE PRODUCTION OF BIOHYDROGEN BY *Thermotoga neapolitana* WITH pH CONTROL AND GAS COLLECTION

Abstract

The production of biological hydrogen is an important process for the future of sustainability and alternative energies. *Thermotoga neapolitana* is a hyperthermophilic bacterium that produces H_2 via fermentation along with CO_2 and acetate. Research has focused on using low volume serum bottles to grow *Thermotoga neapolitana* but further studies are needed to look at the feasibility of scaling-up the process in order to achieve greater process control. Inhibition caused by low pH and H_2 gas formation must be controlled to further increase the productivity of the *T. neapolitana*. The first goal of this research was to grow *Thermotoga neapolitana* in a continuously stirred anaerobic biological reactor (CSABR) for the production of H_2, CO_2 and acetate while collecting the off gas and controlling pH at 7. The next goal was to use media with alternative carbon and nitrogen sources for this scaled-up process. The third goal was to study the effects on product formation of doubling the carbon source concentration in both the standard and alternative media.

Thermotoga neapolitana was found to be able to grow in a CSABR reactor at a temperature of 77°C. It was found that when the pH was controlled at 7 and H_2 gas was collected the product concentrations were increased compared to the same treatments grown in serum bottles without pH control and H_2 gas removal. An alternative medium consisting of peaches and soybean meal as the carbon and nitrogen sources, respectively,

was successfully used to grow *T. neapolitana* in the CSABR system. It was also determined that *Thermotoga neapolitana* can thrive and product formation can be increased at substrate concentrations of 10 g/L compared to 5 g/L. The 10 g/L Standard medium treatment resulted in a H_2 concentration of 83.19 mmol H_2/L medium compared to 42.66 mmol/L medium for the 5 g/L standard medium over the same fermentation period of 40 hours. This indicates that the growth of *Thermotoga neapolitana* is not inhibited at the higher substrate concentrations and actually hydrogen concentrations are increased at 10 g/L.

Introduction

Thermotoga neapolitana is hyperthermophilic bacterium that can produce H_2, CO_2 and acetate as products of its fermentation of glucose. Over the last 25 years work has been done to maximize the production of H_2 by *T. neapolitana* by finding the most suitable culture conditions such as temperature and pH. Most of these studies have occurred in small serum bottles in batch culture.

Some studies have begun focusing on scaling-up the fermentation process using larger reactors with glucose as the carbon source. Scale-up allows for better process control as most lab scale reactors have very intricate controls. Using these reactors, pH control as well as the removal and collection of H_2 gas from the reactor headspace is possible. Ngo, et al. (2011) used a continuously stirred anaerobic reactor to compare hydrogen production by *T. neapolitana* with and without pH control. H_2 gas was removed from the headspace in both pH control and non pH control studies. The study reported 56% higher hydrogen gas yields for cultures grown with pH control compared to those

grown without pH control (Ngo et al., 2011). It was also reported that a pH of 7 was the optimum pH for the reactor environment when compared to pH's of 6.5 and 7.5 (Ngo et al., 2011). There is another positive effect that H_2 collection and removal has on growth. The removal increases the $\%H_2$ gas in the headspace because the gas that is initially in the head space, which consists of mainly N_2, is removed which results in higher $\%H_2$ and $\%CO_2$ during the fermentation period. Also in this study, Ngo et al. (2011) kept a continuous flow of N_2 gas to sparge the headspace which would increase the $\%N_2$ in and therefore decrease $\%H_2$ and $\%CO_2$.

The objectives of this research were: 1) to grow *Thermotoga neapolitana* in a CSABR for the production of H_2 while collecting the off gas and controlling pH at 7; 2) to use medium with alternative carbon and nitrogen sources for this scaled-up process and 3) to study the effects of doubling the carbon source concentration in both the standard and alternative media.

Materials and Methods

Culture Conditions

The bacterium *Thermotoga neapolitana* (DSM 4359) was obtained from DSMZ (the German Resource Centre for Biological Material) and was maintained in seed bottles on a standard glucose medium that was defined as the standard medium by Yu and Drapcho (2011). This standard medium contains all of the following components in dry weight per liter of medium: 5.0 g glucose, 2.0 g yeast extract (YE), 2.0 g trypticase, 1.0 g NH_4Cl, 0.3 g K_2HPO_4, 0.3 g KH_2PO_4, 0.2 g $MgCl_2 \cdot 2H_2O$, 0.1 g $CaCl_2 \cdot 2H_2O$, 10.0 g NaCl, 0.1 g KCl, 1.114 g cysteine-$HCl \cdot H_20$, 0.121 g trizma base(THAM), and 10.0 mL

each of vitamin solution and trace element solution as outlined by DSMZ media 141. The pH of the medium was adjusted to 8 using 5N NaOH solution prior to inoculation. The seed bottles were prepared in 565mL serum bottles and were grown for 20 hours at 77°C and 200rpm in shaker bed. The seed bottles were sparged with N_2 (High Purity, Airgas Welders) for 1 minute prior to an idle time of 90 minutes at room temperature. Along with the seed bottles, samples of *Thermotoga neapolitana* were kept in a 30% glycerol solution at -80°C.

For fermentation trials, *Thermotoga neapolitana* was grown in batch culture using a continuously stirred anaerobic bioreactor(CSABR). The reactor is the BIOSTAT® B Plus model manufactured by Sartorius Stedim (Germany). A high temperature pressure sensor (Ashcroft) was fitted to the thread port in the head plate of the reactor. The total reactor volume was 6.5L. Four liters of medium were prepared and placed into the bioreactor. The limited sparging technique was used (Louis Hill, unpublished, 2013) as well as a non-autoclave method (Jain, 2009). The headspace of the bioreactor was sparged with N_2 gas for 1 minute and the reactor was sealed to create an anaerobic environment. The medium was allowed to sit idle for 90 minutes in order for the cysteine-HCl·H_2O to react with the oxygen. After 90 minutes, a port was opened so that a seed bottle (200mL) could be poured into the reactor and the reactor was re-sealed. Additional N_2 gas sparging occurred during the inoculation process.

After inoculation, the reactor was operated at 77°C for 40 hours with a stirring speed of 200rpm. The pH was controlled at 7 using a 5N NaOH solution. In order to capture gas produced during fermentation, 565mL glass serum bottles were connected to

the reactor and changed during the 40 hours of fermentation to keep the pressure in the reactor < 20kPa. The bottles were prepared by using a robber stopper and an aluminum crimp to seal them and then creating a negative pressure in the bottles before they were connected to the reactors vessel. A bottle was connected to the reactor during the cool down period after the fermentation in order to analyze the reactor environment at 25°C.

Fermentor Study

In order to determine the feasibility in scaling-up this bio-process using agriculturally based carbon and nitrogen sources, a four treatment study was designed. The first treatment is the Standard medium treatment, the second treatment is a Peach/Soybean meal treatment, the third treatment is a 2x Standard medium treatment and the fourth treatment is a 2x Peach/Soybean meal treatment. The Peach/Soybean meal treatment contained 5 g/L (dwb) peaches and 4 g/L (dwb) soybean meal to replace the glucose and yeast extract/trypticase in the standard medium. The 2x treatments had 2 times the amount of carbon source so 10 g/L (dwb) of glucose or peach slurry was used. No vitamins or trace elements were added to the peach medium treatments (Morris, 2013). Two, 40 hour trials were run for each treatment and the results of the two trials were averaged to get the final results.

The Flavor Rich variety peaches were used for this experiment and they were obtained from Titan Farms in Ridge Spring, SC. The pits were removed and the peaches were blended with skins together for 3 minutes. The peach slurry was poured into 0.5 liter bags and stored at -40° C.

The soybean meal used in the experiment was obtained from Southern States (Pendleton, SC). The meal was screened using a sieve and particles ≤ 1mm in diameter were used. The meal was then dried in an oven at 60°C before being added to the medium.

Analytical Methods

After the 40 hour incubation the bottles were placed into a water bath to be maintained at 25°C. The pressure in the bottles was measured using a handheld digital manometer (Fisher Scientific). The pressure for each collected bottle was determined by taking the difference from the pressure read and the initial negative pressure that was in the bottle. A 0.5 mL sample of headspace gas, with a glass syringe, was manually injected into a Gow-Mac Series 400 -gas partitioner with a thermal conductivity detector. The carrier gas was argon at a pressure of 22 psi. The column used was a 10' X 1/8" packed Molecular Sieve 5A, alkali alumino silicate. The column temperatures were 40°C, 55°C and 40°C. The percent hydrogen gas and carbon dioxide in each sample were calculated using pure hydrogen and pure carbon dioxide standard curves. The absolute gas pressure was found by adding the atmospheric pressure to the gauge pressure reading. Then the hydrogen and carbon dioxide partial pressures were found by multiplying the percent of each gas by the absolute total pressure. As reported by Yu and Drapcho (2011) the mols of each gas were calculated using the Ideal Gas Law which can be seen below (Equation 1):

$$n = \frac{P_1 V_1}{RT} \quad (1)$$

where n = mol in gas phase, P_1= partial pressure (kPa), V_1 = headspace volume (L), R = Universal gas constant (8.3145 L·kPa/mol·K), and T = temperature (K).

The number of mols of each gas in each bottle was added together in order to determine the total number of mols produced during the fermentation period. In order to take into account the dissolved H_2 and CO_2 in the medium Henry's Law was used, which can be seen in equation 2:

$$C_{aq} = k_h * p \quad (2)$$

where C_{aq}= concentration in mol/L, p= the partial pressure at 25°C and k_h= Henry's Law constant at 25°C.
The Henry's Law constant at 25°C for CO_2 is 3.36 x 10^{-4} M/kPa and for H_2 is 7.7 x 10^{-6} M/kPa (Sanders, 1999).

An HPLC with a refractive index detector was used for the analysis of sugar and acetate. A Bio-Rad HPX-87H column was used along with a mobile phase of 0.1N H_2SO_4 at a flow rate of 0.6ml/min. The use of H_2SO_4 as mobile phase caused cleaving of sucrose to glucose and fructose which is why only glucose and fructose data is reported. Samples were filtered with 0.45µm filters prior to the analysis. Both initial and final samples were taken. Samples were compared to standards that were prepared in 10 g/L NaCl solution.

To determine solids content, triplicate samples of peach slurry were dried in an oven at 105°C for 48 hours. The percent solids was calculated as the: difference in weight / initial weight.

The experimental results were analyzed with SAS software (SAS, SAS Institute Inc., Cary, NC), with a 0.05 level of significance used for Tukey's Studentized range test.

Results and Discussion

The percent solids weight for the Flavor Rich peaches was determined to be 7.74% so 64.6 g of peach slurry were added per liter of medium. The total sugars added to the Flavor Rich peach medium was 4.5 g/L (2.4 g/L glucose and 2.1 g/L fructose).

It was found that *Thermotoga neapolitana* could be grown and could produce H_2 gas in a CSABR system. Table 3.1 shows the H_2 results for the fermentor study.

Table 3.1: Production of H_2 by *Thermotoga neapolitana* with various carbon and nitrogen sources[1,2]

Treatment: Media Type[3,4]	Mean Gas H_2 concentration (mmol H_2/L medium)	Std. Dev.	Mean Dissolved H_2 concentration (mmol H_2/L medium)	Std. Dev.	Total Mean H_2 concentration (mmol H_2/L medium)	Std. Dev.	Mean H_2 Produced (g H_2/ g substrate COD utilized)	Std. Dev.
Standard Media	41.86[b]	1.95	0.80[a,b]	0.03	42.66[b]	1.93	0.022[a]	0.000
Peach/Soybean	10.34[c]	4.94	0.47[b]	0.22	10.80[c]	5.16	0.022[a]	0.003
2x Standard Media	82.22[a]	0.08	0.97[a]	0.06	83.19[a]	0.01	0.024[a]	0.000
2x Peach/Soybean	16.69[c]	1.18	0.47[b]	0.04	17.15[c]	1.22	0.023[a]	0.000

[1] Means not sharing common letter are significantly different at ($p<0.05$) using Tukey's Studentized Range Test

[2] Glucose medium containing 5 g/L glucose, peach medium containing 2.4 g/L glucose and 2.1 g/L fructose

[3] For peach treatments, nitrogen source was 4g/L soybean meal and no vitamins and trace elements were added

[4] n=2

The Standard medium treatment produced 42.66 mmol H_2/L medium which shows that pH control and H_2 collection can increase the hydrogen production by *T. neapolitana.* That concentration is higher than the 32.83 mmol H_2/L medium and 26.88 mmol H_2/L medium reported by Yu and Drapcho (2011) and the carbon and nitrogen source study from Chapter 2 of this paper, respectively. The percent solids weight was determined to be 7.74% so 64.6 g of peach slurry were added per liter of medium for the

Peach/Soybean treatment and 129.2 g of peach slurry were added per liter of medium for the 2x Peach/Soybean treatment. The Peach/Soybean treatment produced a hydrogen concentration of 10.80mmol H_2/L medium which is comparable to the 10.60 mmol H_2/L medium that was found for the same treatment in the Hydrocooler Water Study in Chapter 2 of this document. This indicates that some other factor beyond pH and H_2 gas inhibition limits the production of H_2 by *T. neapolitana* when grown with these alternative media sources. The hydrogen concentration of the 2x Standard medium treatment was 83.19 mmol H_2/L medium which is almost double the concentration found from the standard medium treatment. This is important because it shows that the kinetics are greatly improved at 10 g/L than 5 g/L and there is a possibility of looking at higher substrate concentrations in the future. The 2x Peach/Soybean treatment resulted in a hydrogen concentration of 17.15 mmol H_2/L medium which was about a 70% increase from the normal Peach/Soybean treatment. This data shows that increasing the peach concentration does help but the process is still held back by some factor other than pH and hydrogen gas inhibition.

The actual yield for all the treatments was much lower than the theoretical yield of 0.042 g H_2/g substrate COD, based on Thauer (1977). In fact, the yields for the treatments were only just above 50% of the theoretical yield. The yields for these treatments are lower than those reported by Eriksen et al. (2010) and those reported for similar treatments in Chapter 2 of this manuscript. It is also interesting to note that the yields for the four treatments were not significantly different, indicating that substrate

concentration is not the only factor in the process and some other nutrients may be limiting.

In this study it is not possible to analyze a nitrogen source effect. This is because the two nitrogen sources, yeast extract/trypticase and soybean meal were used in media that included different carbon sources as well.

Figure 3.1 shows the $\%H_2$ over the duration of the 40 hour fermentation period for all four treatments of the fermentor study.

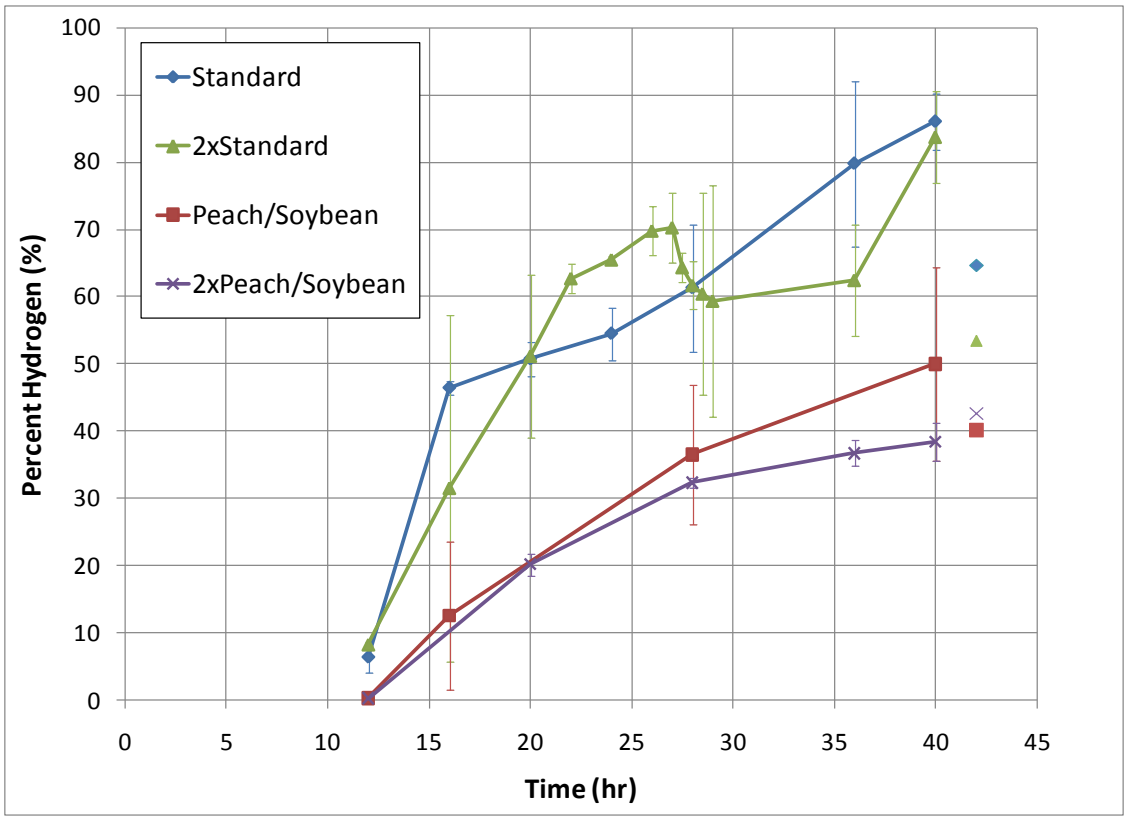

Figure 3.1: $\%H_2$ over time for all four treatments in the study

The $\%H_2$ for all treatments shows a general increase throughout the duration of the 40 hours of fermentation time. This result was expected due to the collection of the off gas. The $\%H_2$ increases because more of the nitrogen that was in the headspace at the

beginning of the trial is removed so that mostly H_2 and CO_2 are present. Each data point on the line represents the point at which the collection bottle was changed on the reactor. One more bottle was collected at the end of the run for each treatment. This final bottle was not measured until the reactor was cooled to 25°C and this measurement can be seen as the data point at hour 42. It is interesting to note that the 2xStandard treatment line does not completely follow the trend of the other three treatment's lines. The dip in $\%H_2$ may have been caused by a succession of bottles being changed in order to prevent a dangerous build up of pressure in the reactor overnight.

The cumulative gauge pressure in the reactor over the 40 hour fermentation can be seen plotted in Figure 3.2.

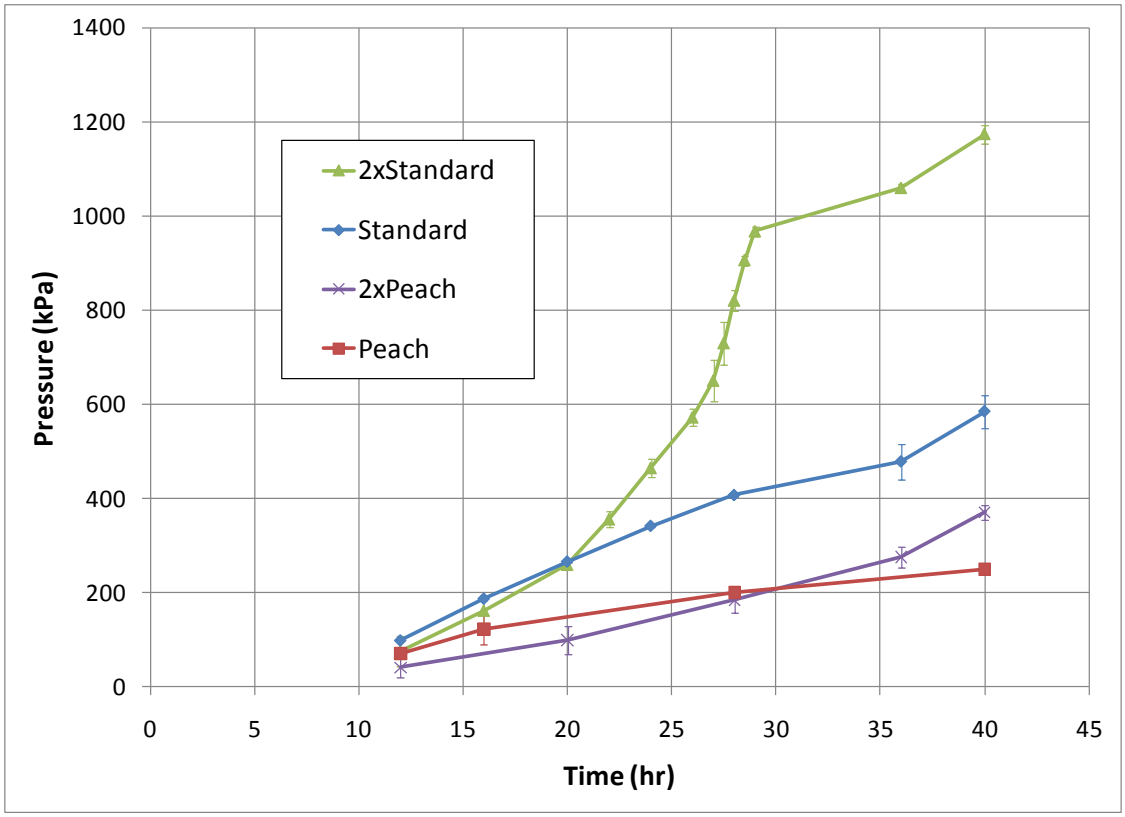

Figure 3.2: Graph of cumulative gauge pressure in bottles over 40 hour fermentation period

The cumulative pressure shows that gas was still being produced at 40 hours for all four treatments and was still increasing at the time when the fermentation was ended. This data suggests that 40 hours is not long enough for *T. neapolitana* to produce as much H_2 and CO_2 as possible with the amount of substrate in the medium.

The production of CO_2 by *Thermotoga neapolitana* was also studied and the results can be seen below in Table 3.2.

Table 3.2: The production of CO_2 by *Thermotoga neapolitana* with various carbon and nitrogen sources[1,2]

Treatment: Media Type[3,4]	Mean Gas CO_2 concentration (mmol CO_2/L medium)	Std. Dev.	Mean Dissolved CO_2 concentration (mmol CO_2/L medium)	Std. Dev.	Mean Total CO_2 concentration (mmol CO_2/L medium)	Std. Dev.	Mean CO_2 Produced (g CO_2/ g substrate COD utilized)	Std. Dev.
Standard Media	8.78[b]	0.22	8.99[a]	1.03	17.77[b]	1.25	0.21[a]	0.05
Peach/Soybean	1.41[d]	0.31	2.91[b]	0.08	4.31[c]	0.23	0.21[a]	0.07
2x Standard Media	18.11[a]	0.20	9.69[a]	0.98	27.80[a]	0.78	0.17[a]	0.03
2x Peach/Soybean	2.37[c]	0.17	3.46[b]	0.10	5.83[c]	0.27	0.18[a]	0.05

[1] Means not sharing common letter are significantly different at ($p<0.05$) using Tukey's Studentized Range Test

[2] Glucose medium containing 5 g/L glucose, peach medium containing 2.4 g/L glucose and 2.1 g/L fructose

[3] For peach treatments, nitrogen source was 4g/L soybean meal and no vitamins and trace elements were added

[4] n=2

The total CO_2 concentration produced by *T. neapolitana* for the Standard medium treatment was found to be 17.77 mmol CO_2/L medium which is higher than the 16.60 mmol H_2/L medium, the CO_2 concentration found in the carbon and nitrogen source study in Chapter 2. The 2x Standard medium treatment produced 27.80 mmol CO_2/L medium which is not quite double the concentration produced by the normal Standard

medium treatment. The Peach/Soybean and 2x Peach/Soybean treatments produced 4.31 mmol CO_2/L medium and 5.83mmol H_2/L medium, respectively, which is a similar ratio as the two Standard medium treatments. Both treatments resulted in a higher CO_2 concentration than was showed by the FlavorRich Peach/DI treatment (which is the same media as the Peach/Soybean treatment) in the Hydrocooler water study in Chapter 2. This result was expected due to the increased process control.

The theoretical yield for CO_2 for this fermentation process is 0.46 g CO_2/g substrate COD, based on Thauer (1977). In this study the actual yields for CO_2 ranged from 0.17-0.21 g CO_2/g substrate COD. This means that the actual yields were only about 37-46% of the theoretical yields which is lower than expected because Eriksen et al. (2010) reported CO_2 yields greater than the theoretical yield and yields of 65-98% of the theoretical yield were found in the carbon and nitrogen source study in Chapter 2. In this study, none of the CO_2 yields for any of the treatments were significantly different but overall the two normal treatments had higher yields than the two 2x treatments.

Figure 3.3 shows the percentage of CO_2 in the gaseous phase over the 40 hour fermentation period.

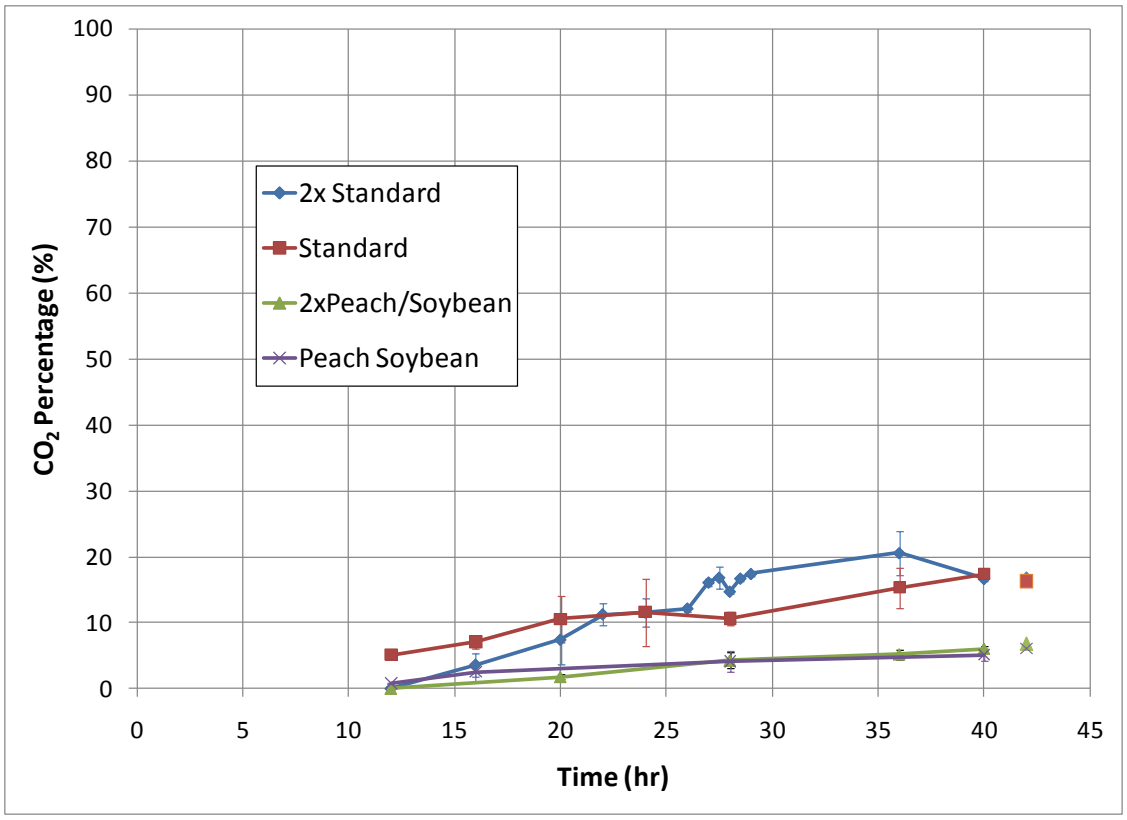

Figure 3.3: %CO_2 over time for all four treatments in the study

It can be seen in figure 3.3 the %CO_2 in the gaseous phase did not reach much greater than 20%. This was expected because more CO_2 is dissolved in the medium than H_2 based on Henry's law. The data point at 42 hours is representative of the final measurement taken from a bottle that collected gas after the fermentation period when the temperature reached 25°C. For the 2x Standard treatment is important to note that an increase in %CO_2 was seen when the %H_2 dipped in Figure 3.2. This change may have occurred due to the changing of collection bottles over a shorter period of time which could have affected the equilibrium of gasses in the headspace.

Table 3.3 shows the results for the production of acetate by *Thermotoga neapolitana* as found for the four treatments in this study. It also shows the residual glucose and fructose concentrations in the medium at the end of the fermentation period.

Table 3.3: The production of acetate by *Thermotoga neapolitana* and the residual glucose and fructose concentrations at the end of fermentation.[1,2]

Treatment: Media Type[3,4]	Mean Final Acetate Concentration (mmol Acetate/L medium)	Std. Dev.	Mean Acetate Produced (g Acetate/ g substrate COD utilized)	Std. Dev.	Residual Glucose concentration (g glucose/L medium)	Std. Dev.	Residual Fructose concentration (g fructose/L medium)	Std. Dev.
Standard Media	17.35[b]	0.52	0.27[b]	0.00	1.80	0.12	-	-
Peach/Soybean	6.60[c]	2.07	0.42[a]	0.02	1.95	0.16	1.99	0.08
2x Standard Media	30.91[a]	2.19	0.26[b]	0.02	3.81	0.08	-	-
2x Peach/Soybean	8.89[c]	0.03	0.36[a]	0.03	4.22	0.44	4.37	0.48

[1] Means not sharing common letter are significantly different at ($p<0.05$) using Tukey's Studentized Range Test

[2] Glucose medium containing 5 g/L glucose, peach medium containing 2.4 g/L glucose and 2.1 g/L fructose

[3] For peach treatments, nitrogen source was 4g/L soybean meal and no vitamins and trace elements were added

[4] n=2

The final acetate concentration produced by the Standard medium treatment was 17.35 mmol acetate/L medium. This is higher than the 12.43 mmol acetate/L medium that was produced in the standard medium treatment in the carbon and nitrogen source study in Chapter 2. As noted with H_2 and CO_2 this increase from the serum bottles indicates that pH and H_2 inhibition do negatively affect product formation and therefore growth of *T. neapolitana*. This data also indicates that pH control and H_2 gas collection can increase product formation. The Peach/Soybean treatment produced 6.60 mmol acetate/L medium compared to 1.05 mmol acetate/L medium produced by the same treatment in serum

bottles in the hydrocooler water study in Chapter 2. This continues to show the positive effects of pH control and H_2 gas collection. The 2x Standard medium treatment resulted in a acetate concentration of 30.91 mmol acetate/L medium, which as in the H_2 and CO_2 results, is not quite twice the concentration produced by the normal Standard medium treatment. The 2x Peach/Soybean treatment produced a higher acetate concentration than that of the normal Peach/Soybean treatment but this concentration was not significantly higher.

Overall the Peach/Soybean treatments showed higher acetate yields than the Standard medium treatments. This result is opposite from what was found in serum bottle trials. The theoretical yield for acetate via the fermentation of glucose by *Thermotoga neapolitana* is 0.61 g acetate/g substrate COD, based on Thauer (1977). The actual yields in this study ranged from about 44-69% of the theoretical yield which is lower than expected. It is possible that these values are lower because other organic acids such as lactic acid and butyric acid were not measured because acetic acid is the predominant acid produced. It is important to note that final pH values for each treatment were not reported because the pH was controlled at 7 throughout the fermentation process.

The theoretical molar ratio of products formed by the fermentation of glucose by *Thermotoga neapolitana* is 4 mol H_2, 2 mol CO_2, 2 mol acetate and 2 mol H^+(Thauer, 1977). Figure 3.3 shows the actual molar ratios obtained in this experiment.

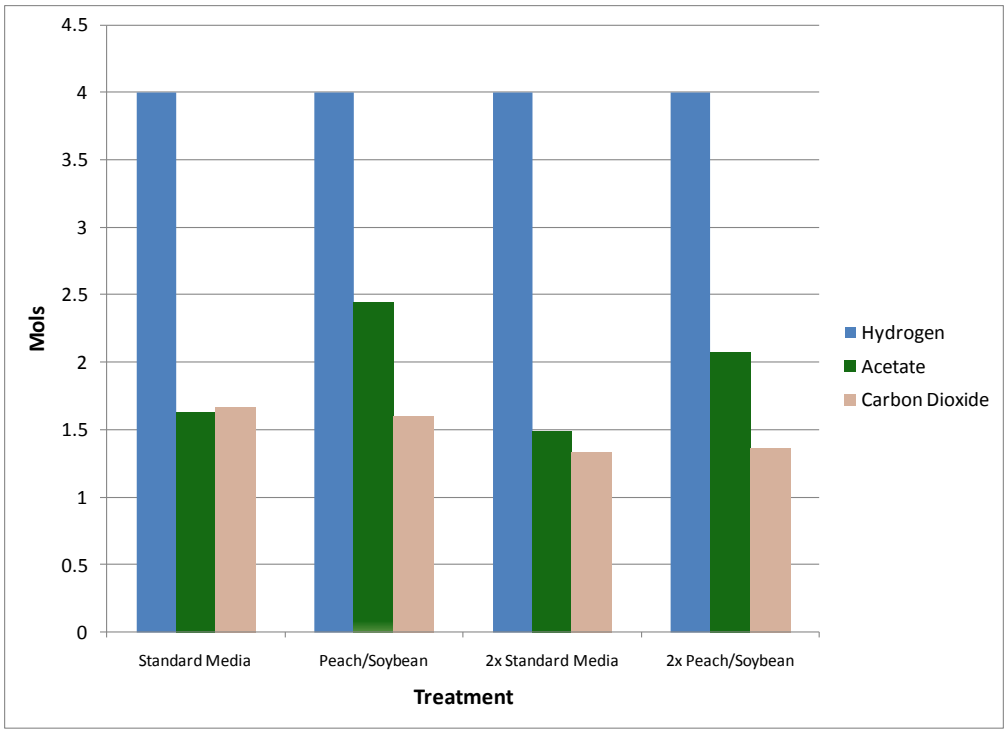

Figure 3.4: The actual molar ratios of the products of fermentation by *Thermotoga neapolitana.* Theoretical Ratio is 4:2:2.

The ratios in Figure 3.3 are normalized for 4 mols of H_2 to more easily understand the information even though 4mols of H_2 were not actually produced. Overall, the ratios are similar to the theoretical ratio of 4:2:2. It is interesting to notice that the acetate for the Peach/Soybean treatment is higher which was expected based on the acetate concentration data. However, this high value does not correlate to previous studies and may be higher than the true value. It is also interesting to note that the CO_2 values are lower than the values found in the serum bottle trials which were >2 in most of the treatments. The high CO_2 yields found in these previous experiments and by Eriksen et al. (2010) are caused by some unknown phenomenon but the results are repeatable. The lower values found here could be due to the difficulty in measuring CO_2 in lower

concentrations with gas chromatography or possibly the difference in application of Henry's Law with serum bottles compared to the CSABR in this study.

Conclusions

Thermotoga neapolitana was found to be able to grow in a CSABR reactor in order to achieve greater process control. It was found that when the pH was controlled at 7 and H_2 gas was collected the product concentrations were increased compared to the same treatments grown in serum bottles without pH control and H_2 gas removal. Both the Standard medium treatment and Peach/Soybean treatment resulted in higher H_2, CO_2 and acetate concentrations with the greater process control. These findings are in concordance with many previous studies. It was also determined that *Thermotoga neapolitana* can thrive and product formation can be increased at substrate concentrations of 10 g/L compared to 5 g/L. The 10 g/L Standard medium treatment resulted in nearly twice as high H_2 concentration over the same fermentation period of 40 hours. This indicates that the growth of *Thermotoga neapolitana* is not inhibited at these substrate concentrations, in fact the process kinetics are increased at 10 g/L, as expected. These results indicate the possibility of further studies in order to determine the optimal concentration for this process. The 2x Peach/Soybean treatment did not quite result in a doubling of product concentrations even though the concentrations did increase from the normal Peach/Soybean treatment. This suggests that there is some factor affecting the product formation other than pH and H_2 inhibition when *T. neapolitana* is grown in this alternative media. Further studies are needed in order to determine what factors are affecting this process. The data from these studies also show that this process can be

scaled-up successfully beyond small batch cultures so in the future larger reactors and higher substrate concentrations can be used in order to further increase the efficiency of the process of producing H_2, CO_2 and acetate via *Thermotoga neapolitana.*

CHAPTER FOUR

CONCLUSIONS AND IDEAS FOR FURTHER RESEARCH

Thermotoga neapolitana is a hyperthermophilic bacterium that produces H_2 via fermentation along with CO_2 and acetate. In this research, many studies were performed that looked at the production of H_2, CO_2 and acetate by *Thermotoga neapolitana.* The research was divided into 2 studies: one study using serum bottles for growth and one using a CSABR for growth. The first goal of the first study was to measure H_2, CO_2 and acetate production by *T. neapolitana* in peach media and glucose media with three different nitrogen sources and compare it to the standard glucose media. Another goal of the first study was to modify existing techniques and processes in order to improve the efficiency of the batch fermentation of *T. neapolitana*. The third goal of the first study was to measure H_2, CO_2 and acetate production by *T. neapolitana* in both standard and alternative media with hydrocooler water and compares it to distilled water. The first goal of the second study was to measure H_2, CO_2 and acetate production by *T. neapolitana* in both standard and alternative media using a scaled up batch reactor that utilizes pH control and hydrogen gas inhibition control. The second goal of the second study was to measure the effect of doubling substrate concentration on product formation using both standard alternative media sources.

Thermotoga neapolitana was found to be able to produce H_2 as effectively when the medium is not sparged with N_2 gas for an extended period of time. The “limited sparging” technique was found to be just as or more effective than a standard sparging technique at removing oxygen from the reactor environment and was found to be a

simpler method. This technique was then used in all subsequent studies. *Thermotoga neapolitana* was found to be able to utilize agricultural based products as carbon and nitrogen sources in the growth medium. Sugars from cull peaches and nitrogen from both soybean meal and canola meal were utilized and H_2, CO_2 and acetate were produced. All of the agricultural based treatments did not perform as well as the standard medium treatment but there was strong evidence that in the future research could focus on these alternative carbon and nitrogen sources because of the sustainability and economics of the process. *Thermotoga neapolitana* was found to produce higher concentrations of H_2, CO_2 and acetate when hydrocooler water from a peach processing facility was used in the growth medium in the place of distilled water. The hydrocooler water study showed that the HC water could be recycled and used in a future biohydrogen facility. It was also learned from the hydrocooler study that the variety of peaches used as the carbon source has an impact on the production of H_2, CO_2 and acetate. Peach varieties can show great variation and it is possible that certain nutrients can have an effect on the process.

Thermotoga neapolitana was found to be able to grow in a CSABR reactor in order to achieve greater process control. It was found that when the pH was controlled at 7 and H_2 gas was collected the product concentrations were increased compared to the same treatments grown in serum bottles without pH control and H_2 gas removal. It was also determined that *Thermotoga neapolitana* can thrive and product formation can be increased at substrate concentrations of 10 g/L compared to 5 g/L. The 10 g/L Standard medium treatment resulted in nearly twice as high H_2 concentration over the same fermentation period of 40 hours. This indicates that the growth of *Thermotoga*

neapolitana is not inhibited at these substrate concentrations, in fact the process kinetics are increased at 10 g/L, as expected. These results indicate the possibility of further studies in order to determine the optimal concentration for this process. The 2x Peach/Soybean treatment did not quite result in a doubling of product concentrations even though the concentrations did increase from the normal Peach/Soybean treatment. This suggests that there is some factor affecting the product formation other than pH and H_2 inhibition when *T. neapolitana* is grown in this alternative media. Further studies are needed in order to determine what factors are affecting this process. The data from these studies also show that this process can be scaled-up successfully beyond small batch cultures so in the future larger reactors and higher substrate concentrations can be used in order to further increase the efficiency and the economics of the process of producing H_2, CO_2 and acetate via *Thermotoga neapolitana.*

Many studies other than those mentioned in the above paragraphs can provide more vital information about this process. One interesting study could be to look at the possibility of re-using spent media after incubation without the addition of media components other than carbon source. Another important study could focus on determining whether acetate, by formation of sodium acetate, can inhibit the growth of *T. neapolitana.* Lastly, further studies could determine the effects of reducing or removing other media components such as cysteine-HCl, THAM and any of the many salts added to the growth medium. These process simplifications could be used to help develop a plan for a bio-refinery for this process, which is the ultimate goal of the research in this area.

APPENDICES

Appendix A

GC Data

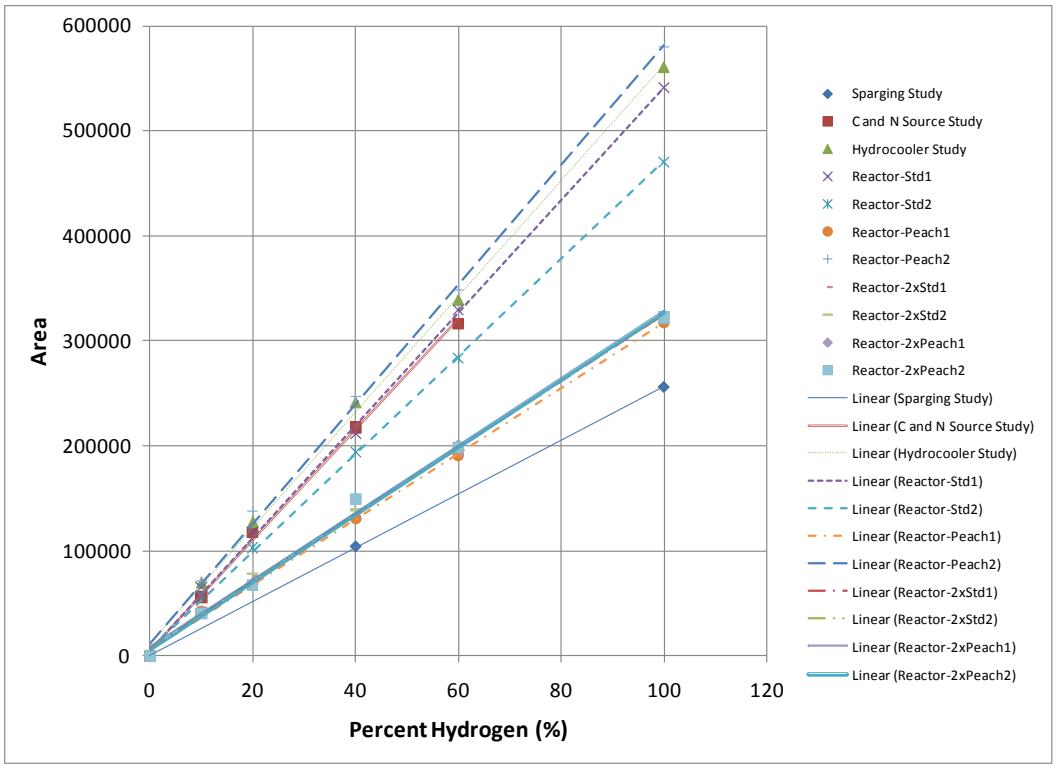

Figure A1: H_2 standard curves for all studies and treatments

Table A1: H_2 standard curve regression equations, average slope and R^2 values for all studies and treatments

Study/Trial	Equation	R^2
Sparging Study	y = 2554.6x + 937.5	0.9999
C and N Source Study	y = 5258.8x + 4813.2	0.9983
Hydrocooler Study	y = 5523.6x + 11400	0.9987
Reactor-Std1	y = 5349.1x + 5750.9	0.9991
Reactor-Std2	y = 4639.4x + 6834.1	0.9994
Reactor-Peach1	y = 3124.6x + 5105.9	0.9990
Reactor-Peach2	y = 5687.2x + 12427	0.9984
Reactor-2xStd1	y = 3175.8x + 7609.7	0.9975
Reactor-2xStd2	y = 3174x + 8188.2	0.9980
Reactor-2xPeach1	y = 3182.4x + 8590.5	0.9954
Reactor-2xPeach2	y = 3202x + 6854.4	0.9960
Average slope (Std dev)	4079.2 (1201.4)	0.9982

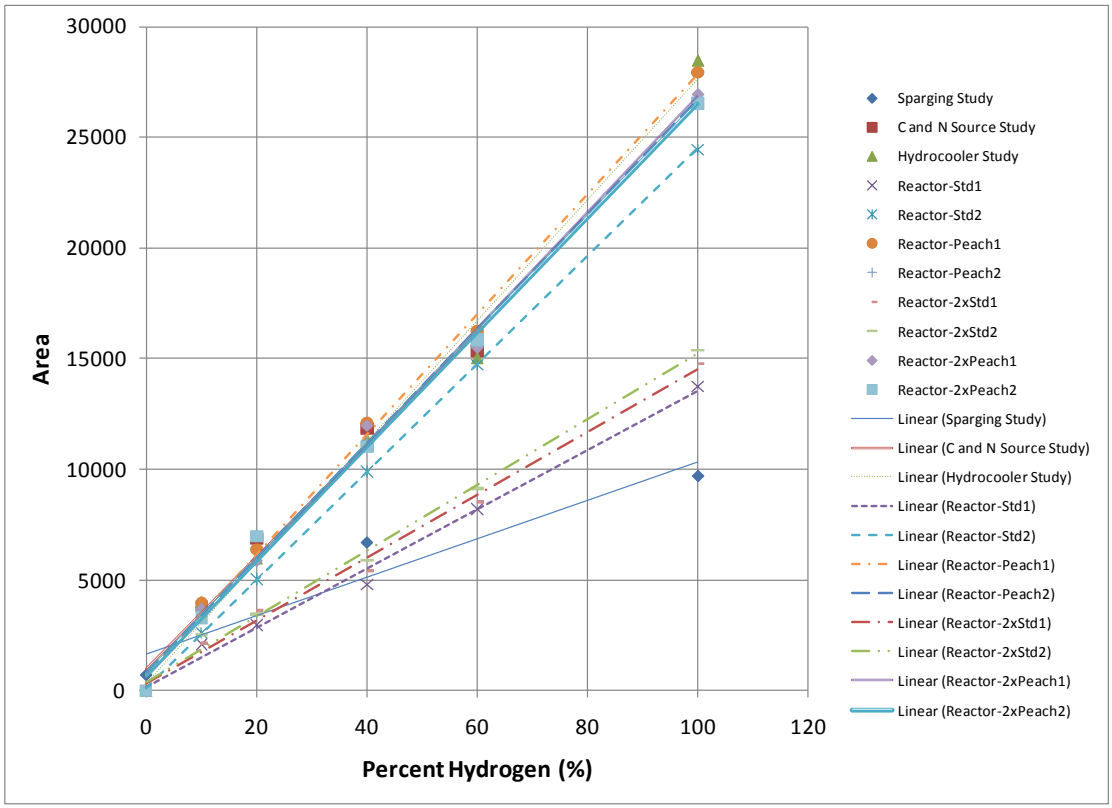

Figure A2: CO_2 standard curves for all studies and treatments

Table A2: CO_2 standard curve regression equations, average slope and R^2 values for all studies and treatments

Study/Trial	Equation	R^2
Sparging Study	y = 91.028x + 845.53	0.9946
C and N Source Study	y = 91.4x + 80.182	0.9937
Hydrocooler Study	y = 272.77x + 348.27	0.9912
Reactor-Std1	y = 133.74x + 173.76	0.9922
Reactor-Std2	y = 243.65x + 113.9	0.9999
Reactor-Peach1	y = 270.89x + 712.49	0.9967
Reactor-Peach2	y = 258.71x + 806.44	0.9959
Reactor-2xStd1	y = 142.1x + 305.53	0.9928
Reactor-2xStd2	y = 148.36x + 378.03	0.9946
Reactor-2xPeach1	y = 262.07x + 607.69	0.9957
Reactor-2xPeach2	y = 258.04x + 720.07	0.9959
Average slope (Std dev)	197.5 (75.4)	0.9948

Table A3: H_2 area data for the sparging study

Bottle	Injection	Standard Sparge	Limited Sparge
1	1	80176	86746
1	2	79237	96255
1	3	78034	97453
2	1	93155	99854
2	2	93925	99054
2	3	94311	97071
3	1	92167	95834
3	2	92106	97270
3	3	95144	97654
4	1	91961	88257
4	2	94842	88753
4	3	94578	87486
5	1	96240	102992
5	2	96258	104416
5	3	99404	104299

Table A4: H_2 and CO_2 area data for all glucose medium treatments from carbon and nitrogen source study

Treatment	Bottle	Injection	H_2 Area	CO_2 Area
Standard	1	1	137089	3724
Standard	1	2	135703	3556
Standard	2	1	130747	3869
Standard	2	2	130661	3485
Standard	3	1	131406	3624
Standard	3	2	128674	3624
Standard	4	1	136087	3710
Standard	4	2	137219	3524
GlucoseYeast	1	1	148873	4058
GlucoseYeast	1	2	148420	4131
GlucoseYeast	2	1	147734	4041
GlucoseYeast	2	2	146056	3863
GlucoseYeast	3	1	153981	4396
GlucoseYeast	3	2	152396	4121
GlucoseYeast	4	1	148464	4330
GlucoseYeast	4	2	148524	3810
GlucoseCanola	1	1	116045	3201
GlucoseCanola	1	2	118057	3043
GlucoseCanola	2	1	126448	3039
GlucoseCanola	2	2	125157	3077
GlucoseCanola	3	1	115977	3085
GlucoseCanola	3	2	115534	3030
GlucoseCanola	4	1	123434	3082
GlucoseCanola	4	2	126345	3032
GlucoseSoy	1	1	112389	3010
GlucoseSoy	1	2	110811	3041
GlucoseSoy	2	1	116351	3404
GlucoseSoy	2	2	118741	3638
GlucoseSoy	3	1	119224	3657
GlucoseSoy	3	2	117231	3586
GlucoseSoy	4	1	122728	3314
GlucoseSoy	4	2	122672	3514

Table A5: H_2 and CO_2 data for peach medium treatments from carbon and nitrogen source study

Treatment	Bottle	Injection	H_2 Area	CO_2 Area
PeachYeast	1	1	154986	4487
PeachYeast	1	2	122451	3576
PeachYeast	2	1	154860	4298
PeachYeast	2	2	158308	4189
PeachYeast	3	1	156070	4188
PeachYeast	3	2	156691	4367
PeachYeast	4	1	158618	4211
PeachYeast	4	2	156810	4140
PeachCanola	1	1	145482	4012
PeachCanola	1	2	144059	3731
PeachCanola	2	1	158028	4495
PeachCanola	2	2	158505	4661
PeachCanola	3	1	155738	4731
PeachCanola	3	2	156276	4194
PeachCanola	4	1	141455	4199
PeachCanola	4	2	153340	4390
PeachSoy	1	1	130139	3754
PeachSoy	1	2	128769	3533
PeachSoy	2	1	144195	4362
PeachSoy	2	2	145574	4174
PeachSoy	3	1	142210	4151
PeachSoy	3	2	141527	4109
PeachSoy	4	1	141384	3836
PeachSoy	4	2	142008	3920

Table A6: H_2 and CO_2 data for all treatments from hydrocooler water study

Treatment	Bottle	Injection	H_2 Area	CO_2 Area
StandardDI	1	1	157949	3006
StandardDI	1	2	159685	3432
StandardDI	2	1	159772	3684
StandardDI	2	2	159669	3350
StandardDI	3	1	152242	3318
StandardDI	3	2	155984	2784
StandardDI	4	1	158266	3015
StandardDI	4	2	162107	2496
FlavorRichDI	1	1	55421	1363
FlavorRichDI	1	2	56475	1314
FlavorRichDI	2	1	25669	946
FlavorRichDI	2	2	26426	853
FlavorRichDI	3	1	41004	1305
FlavorRichDI	3	2	40940	1061
FlavorRichDI	4	1	47805	1075
FlavorRichDI	4	2	47576	1769
StandardHC	1	1	167337	3059
StandardHC	1	2	173595	3641
StandardHC	2	1	171665	3392
StandardHC	2	2	173489	3104
StandardHC	3	1	172543	2793
StandardHC	3	2	173192	2980
StandardHC	4	1	174324	3406
StandardHC	4	2	176287	3152
FlavorRichHC	1	1	61422	2404
FlavorRichHC	1	2	61470	2328
FlavorRichHC	2	1	58680	2576
FlavorRichHC	2	2	59224	2250
FlavorRichHC	3	1	60588	2087
FlavorRichHC	3	2	62422	1883
FlavorRichHC	4	1	64663	2060
FlavorRichHC	4	2	68368	2470
RedPrinceDI	1	1	79643	1976
RedPrinceDI	1	2	81020	2054
RedPrinceDI	2	1	101331	2064
RedPrinceDI	2	2	103010	2309
RedPrinceDI	3	1	93036	2150
RedPrinceDI	3	2	93840	2039
RedPrinceDI	4	1	99719	2247
RedPrinceDI	4	2	99660	2300

Table A7: H_2 and CO_2 data for both replicates of the standard treatment from the fermentor study

Bottle	Injection	Run 1 H_2 Area	Run 1 CO_2 Area	Run 2 H_2 Area	Run 2 CO_2 Area
1	1	56012	960	33262	1796
1	2	56316	760	34157	1089
2	1	268041	1275	221880	1062
2	2	275528	1187	224252	2398
3	1	303994	1812	236705	2143
3	2	299214	2034	239125	2149
4	1	325143	2455	248537	2150
4	2	330549	1972	252701	2089
5	1	385478	1630	265059	3057
5	2	388375	1362	263063	2854
6	1	501117	2601	337986	3669
6	2	498409	2437	342447	3100
7	1	374783	2062	407382	3886
7	2	373147	3068	410767	4909
8	1	-	-	310353	3731
8	2	-	-	300968	4149

Table A8: H_2 and CO_2 data for both replicates of the peach treatment from the fermentor study

Bottle	Injection	Run 1 H_2 Area	Run 1 CO_2 Area	Run 2 H_2 Area	Run 2 CO_2 Area
1	1	7177	1300	984	2929
1	2	7583	580	623	500
2	1	68133	1276	20166	1041
2	2	69398	980	20380	980
3	1	96721	2593	140532	1617
3	2	96398	1672	143640	1595
4	1	96487	2781	160682	2279
4	2	99624	2161	162100	2000
5	1	-	-	179049	1970
5	2	-	-	178029	2660

Table A9: H_2 and CO_2 data for both replicates of the 2xstandard treatment from the agricultural feedstock study

Bottle	Injection	Run 1 H_2 Area	Run 1 CO_2 Area	Run 2 H_2 Area	Run 2 CO_2 Area
1	1	32801	0	34624	0
1	2	34987	0	35412	0
2	1	51198	768	140419	1246
2	2	49088	517	192047	967
3	1	143112	1004	211555	1890
3	2	142938	954	184305	1867
4	1	202494	1681	195190	2282
4	2	201079	1794	229370	2186
5	1	215801	1540	198005	2345
5	2	213235	1927	236268	2302
6	1	220589	2054	217609	2181
6	2	221387	2107	258213	2089
7	1	218211	2495	228770	3171
7	2	220390	2577	256596	2500
8	1	203436	2229	211797	3179
8	2	210426	2844	223179	2927
9	1	196173	2372	212389	2549
9	2	195143	2381	211347	2632
10	1	165452	2973	222294	2830
10	2	166439	2483	244952	2798
11	1	164254	2956	226373	2861
11	2	151011	2677	243933	2998
12	1	204203	2485	248434	3753
12	2	207695	3312	238283	3815
13	1	285729	2332	136647	2978
13	2	261121	3020	140712	2894
14	1	233362	2568	-	-
14	2	200119	2409	-	-

Table A10: H_2 and CO_2 data for both replicates of the 2xpeach treatment from the agricultural feedstock study

Bottle	Injection	Run 1 H_2 Area	Run 1 CO_2 Area	Run 2 H_2 Area	Run 2 CO_2 Area
1	1	10107	100	5683	804
1	2	11219	580	5928	900
2	1	69234	1276	74653	1193
2	2	69218	1002	75890	1046
3	1	109214	2223	111875	1617
3	2	110718	1672	112344	1595
4	1	121823	2081	128956	1874
4	2	120495	2153	128569	1985
5	1	124844	2247	135984	1972
5	2	124755	2302	136782	2482
6	1	130894	2781	140765	2170
6	2	129764	2161	142987	2660

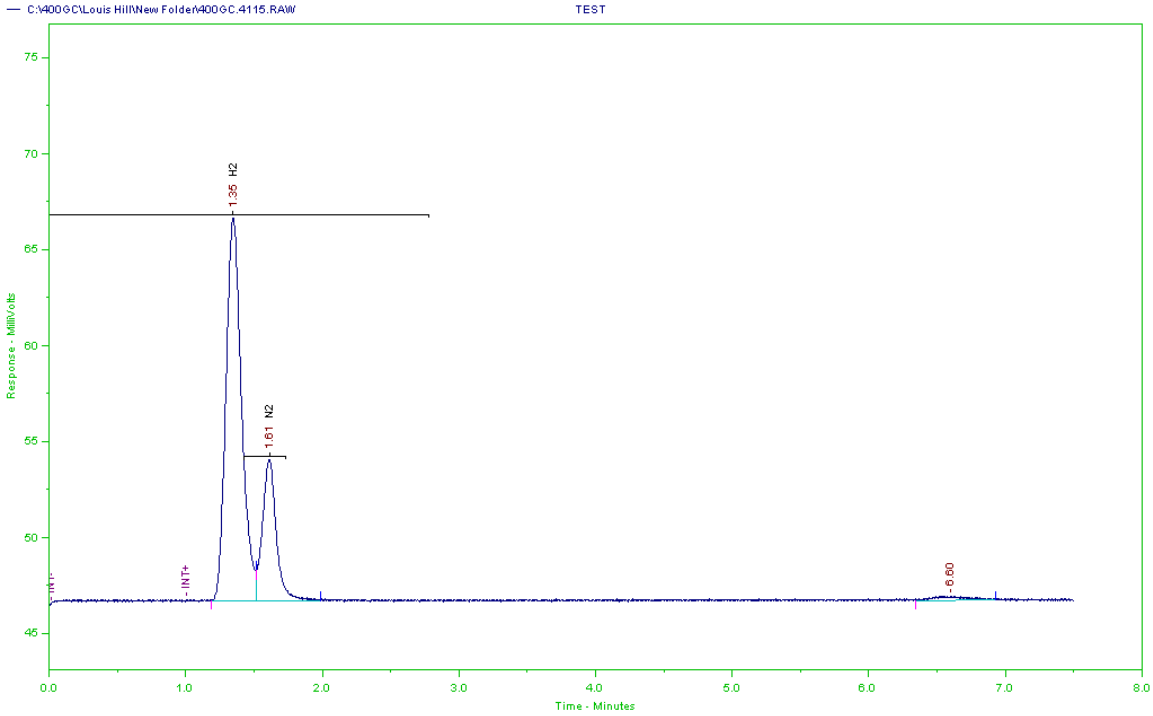

Figure A3: GC sample graph. H_2 peak at 1.35 minutes, N_2 peak at 1.61 minutes and CO_2 at 6.60 minutes

Appendix B

HPLC Data

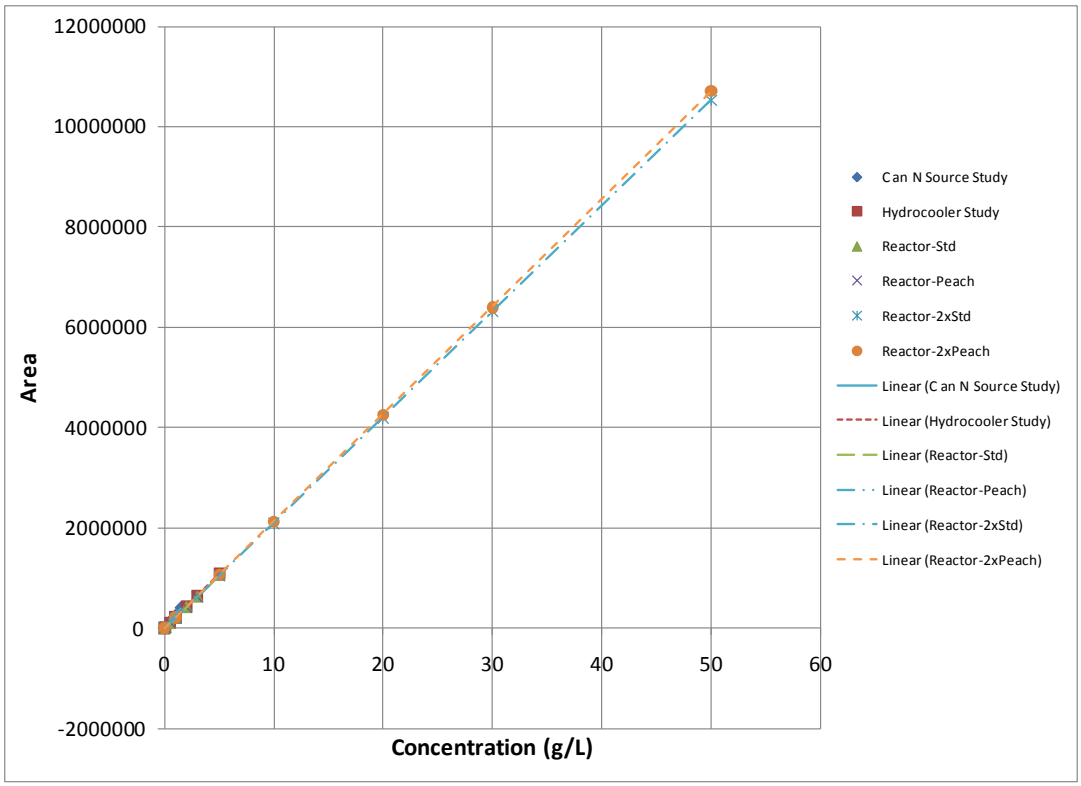

Figure B1: Glucose standard curves from HPLC for all studies and treatments

Table B1: Glucose standard curve regression equations, average slope and R^2 values for all studies and treatments

Study/Trial	Equation	R^2
C and N Source Study	y = 264792x - 3558.5	0.9983
Hydrocooler Study	y = 218470x - 3623.2	0.9999
Reactor-Std	y = 211001x + 39.104	0.9999
Reactor-Peach	y = 207832x - 298.11	0.9999
Reactor-2xStd	y = 210668x - 9679	1.0000
Reactor-2xPeach	y = 213997x - 11212	1.0000
Average slope (Std. dev)	221126.7 (21694.3)	0.9997

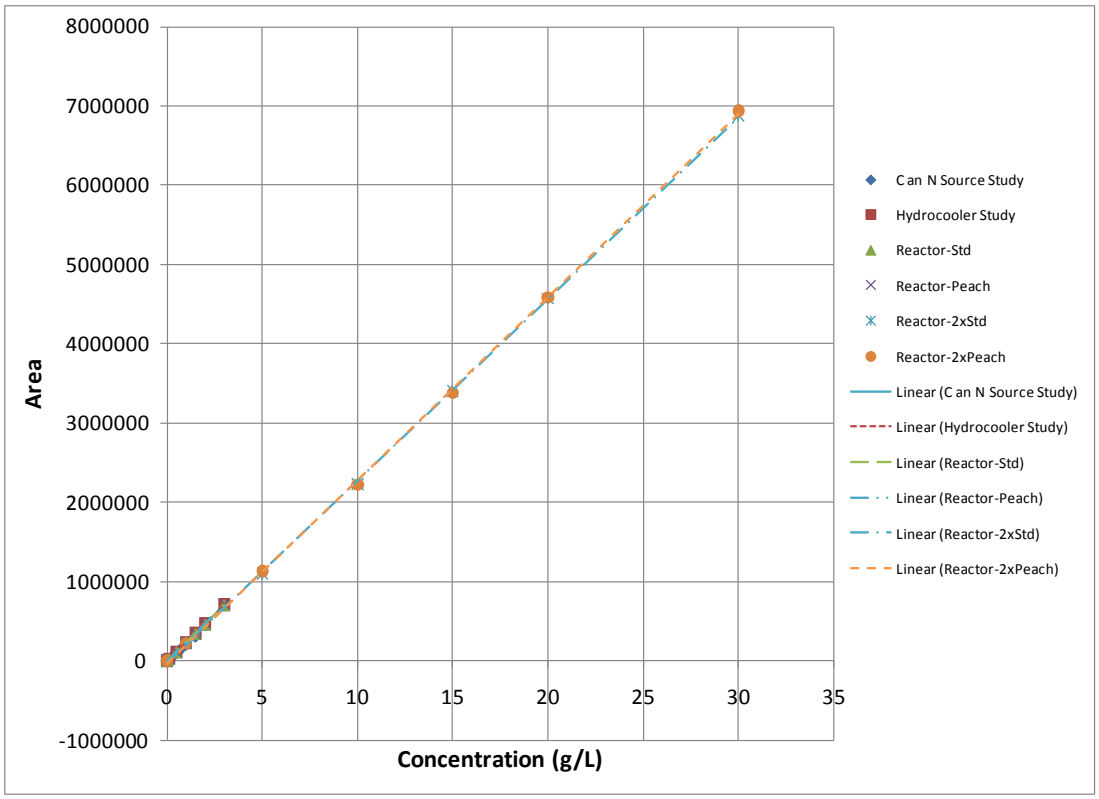

Figure B2: Fructose standard curves from HPLC for all studies and treatments

Table B2: Fructose standard curve regression equations, average slope and R^2 values for all studies and treatments

Study/Trial	Equation	R^2
C and N Source Study	y = 202137x - 1760.4	0.9997
Hydrocooler Study	y = 236325x - 3965.6	0.9997
Reactor-Std	y = 234182x - 2850	0.9997
Reactor-Peach	y = 240101x - 4401.6	0.9996
Reactor-2xStd	y = 229599x - 28243	0.9999
Reactor-2xPeach	y = 230959x - 32666	0.9998
Average slope (Std. dev)	228883.8 (13634.4)	0.9997

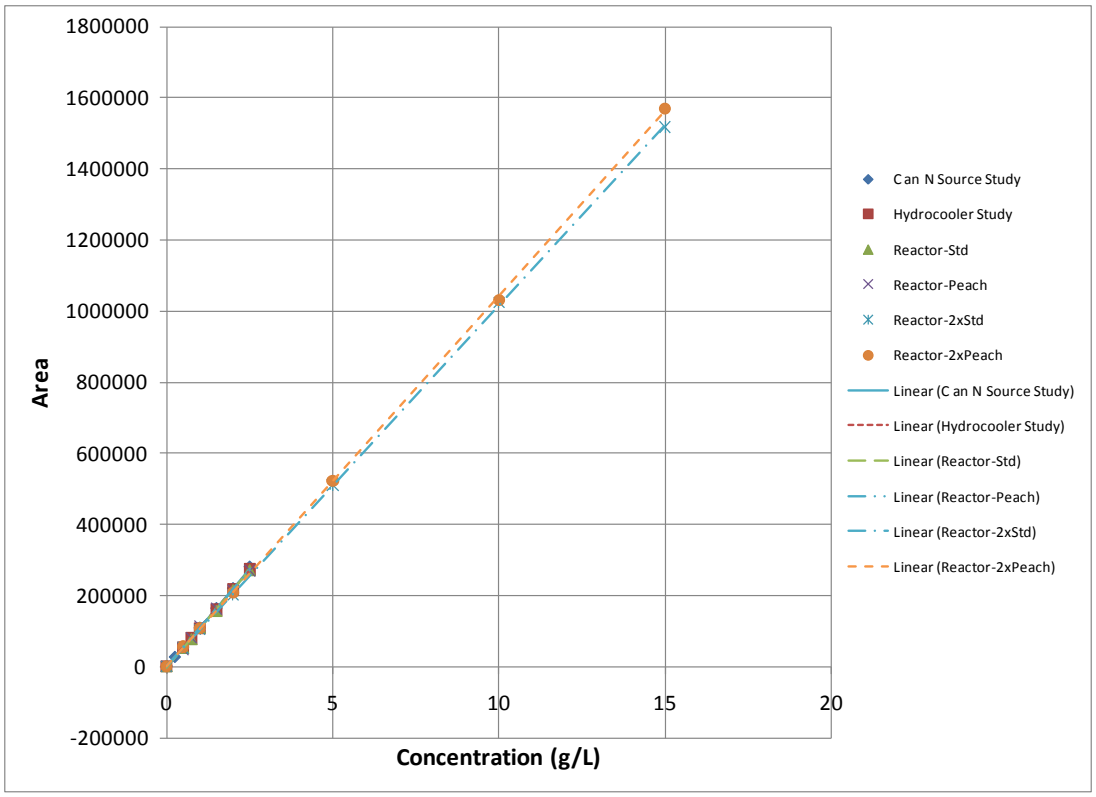

Figure B3: Acetate standard curves from HPLC for all studies and treatments

Table B3: Acetate standard curve regression equations, average slope and R^2 values for all studies and treatments

Study/Trial	Equation	R^2
C and N Source Study	y = 111433x - 2130.4	0.9996
Hydrocooler Study	y = 109141x - 934.64	0.9997
Reactor-Std	y = 108921x - 2153.2	0.9991
Reactor-Peach	y = 107947x + 235.53	0.9984
Reactor-2xStd	y = 101380x + 2212.8	0.9999
Reactor-2xPeach	y = 104091x + 1113	0.9999
Average slope (Std. dev)	107152.2 (3707.6)	0.9994

Table B4: Pre-fermentation glucose, fructose and acetate area data for all treatments from the carbon and nitrogen source study

Treatment	Replicate	Glucose	Fructose	Acetate
Standard	1	1489774	0	7386
Standard	2	1308281	0	5655
GlucoseYeast	1	1588800	0	4201
GlucoseYeast	2	1617995	0	8172
GlucoseCanola	1	1550355	0	0
GlucoseCanola	2	1623849	0	0
GlucoseSoy	1	1608495	0	0
GlucoseSoy	2	1606603	0	1499
PeachYeast	1	526086	614056	4025
PeachYeast	2	514634	626950	6086
PeachCanola	1	525550	662885	3128
PeachCanola	2	532506	675714	1431
PeachSoy	1	534688	646628	3728
PeachSoy	2	541755	656704	2010

Table B5: Post-fermentation glucose, fructose and acetate area data for all treatments from the carbon and nitrogen source study

Treatment	Replicate	Glucose	Fructose	Acetate
Standard	1	908201	0	57743
Standard	2	937095	0	102608
Standard	3	1180087	0	87028
Standard	4	1159473	0	104407
GlucoseYeast	1	910578	0	85370
GlucoseYeast	2	921688	0	82057
GlucoseYeast	3	911675	0	85363
GlucoseYeast	4	918312	0	83318
GlucoseCanola	1	1357448	0	62591
GlucoseCanola	2	1050605	0	59261
GlucoseCanola	3	1355144	0	58847
GlucoseCanola	4	1403037	0	94771
GlucoseSoy	1	1398553	0	55647
GlucoseSoy	2	855824	0	39248
GlucoseSoy	3	1344688	0	57176
GlucoseSoy	4	1310789	0	63081
PeachYeast	1	161491	337230	93463
PeachYeast	2	148674	387189	96993
PeachYeast	3	141272	374054	94488
PeachYeast	4	141209	365997	93731
PeachCanola	1	201635	442973	85000
PeachCanola	2	111660	242865	55364
PeachCanola	3	144862	305626	71003
PeachCanola	4	82868	684590	92583
PeachSoy	1	202851	399964	70540
PeachSoy	2	231128	0	78393
PeachSoy	3	68642	550436	67685
PeachSoy	4	65502	433892	67850

Table B6: Pre-fermentation glucose, fructose and acetate area data for all treatments from the hydrocooler water study

Treatment	Replicate	Glucose	Fructose	Acetate
StandardDI	1	1168354	0	0
StandardDI	2	1151908	0	0
FlavorRichDI	1	532113	510939	0
FlavorRichDI	2	527728	502935	0
StandardHC	1	1164268	0	0
StandardHC	2	1164078	0	0
FlavorRichHC	1	551540	527170	0
FlavorRichHC	2	554167	526316	0
RedPrinceDI	1	488708	499183	0
RedPrinceDI	2	486769	480939	0

Table B7: Post-fermentation glucose, fructose and acetate area data for all treatments from the carbon and nitrogen source study

Treatment	Replicate	Glucose	Fructose	Acetate
StandardDI	1	651615	0	85228
StandardDI	2	719953	0	87328
StandardDI	3	652252	0	84062
StandardDI	4	707542	0	88208
FlavorRichDI	1	451212	407136	4794
FlavorRichDI	2	444623	411646	7859
FlavorRichDI	3	444310	409790	8406
FlavorRichDI	4	442324	408086	8628
StandardHC	1	632638	0	91424
StandardHC	2	656136	0	93548
StandardHC	3	624631	0	90283
StandardHC	4	648675	0	91764
FlavorRichHC	1	452121	410894	4745
FlavorRichHC	2	453284	410340	4655
FlavorRichHC	3	454234	400276	4157
FlavorRichHC	4	450964	400992	5783
RedPrinceDI	1	344471	441443	38221
RedPrinceDI	2	345060	439730	48887
RedPrinceDI	3	356368	438480	43005
RedPrinceDI	4	343485	440557	46509

Table B8: Pre-Fermentation (Initial) and Post-Fermentation (Final) data for glucose, fructose and acetate for both runs of the standard treatment from the fermentor study

Run	Replicate	Glucose	Acetate
Run1-Initial	1	1145540	0
Run1-Initial	2	1139748	0
Run1-Initial	3	1137602	0
Run1-Initial	4	1130674	0
Run1-Final	1	404248	105289
Run1-Final	2	400823	107497
Run1-Final	3	402724	108540
Run1-Final	4	406454	106583
Run2-Initial	1	1136479	0
Run2-Initial	2	1127276	0
Run2-Initial	3	1121518	0
Run2-Initial	4	1122409	0
Run2-Final	1	353282	110691
Run2-Final	2	356742	113130
Run2-Final	3	356282	114075
Run2-Final	4	357071	112006

Table B9: Pre-Fermentation (Initial) and Post-Fermentation (Final) data for glucose, fructose and acetate for both runs of the peach treatment from the fermentor study

Run	Replicate	Glucose	Fructose	Acetate
Run1-Initial	1	554529	497553	0
Run1-Initial	2	545210	495276	0
Run1-Initial	3	556624	525588	0
Run1-Initial	4	547385	492594	0
Run1-Final	1	432393	452193	31282
Run1-Final	2	436495	482100	31903
Run1-Final	3	437013	485575	31475
Run1-Final	4	442171	488346	31107
Run2-Initial	1	552966	497080	0
Run2-Initial	2	550153	501132	0
Run2-Initial	3	609325	563047	0
Run2-Initial	4	530091	475189	0
Run2-Final	1	388012	458309	42069
Run2-Final	2	371231	443525	47728
Run2-Final	3	368524	444841	43933
Run2-Final	4	366735	488346	50221

Table B10: Pre-Fermentation (Initial) and Post-Fermentation (Final) data for glucose, fructose and acetate for both runs of the 2sstandard treatment from the fermentor study

Run	Replicate	Glucose	Acetate
Run1-Initial	1	2243009	0
Run1-Initial	2	2188519	0
Run1-Initial	3	2183251	0
Run1-Initial	4	2171490	0
Run1-Final	1	787027	183872
Run1-Final	2	811310	201435
Run1-Final	3	815328	201429
Run1-Final	4	810304	198704
Run2-Initial	1	2158170	0
Run2-Initial	2	2171919	0
Run2-Initial	3	2160419	0
Run2-Initial	4	2132934	0
Run2-Final	1	785461	177566
Run2-Final	2	783115	177008
Run2-Final	3	771593	178073
Run2-Final	4	773802	178649

Table B-11: Pre-Fermentation (Initial) and Post-Fermentation (Final) data for glucose, fructose and acetate for both runs of the 2x peach treatment from the fermentor study

Run	Replicate	Glucose	Fructose	Acetate
Run1-Initial	1	1007249	1123370	0
Run1-Initial	2	993021	1111489	0
Run1-Initial	3	1001289	1186553	0
Run1-Initial	4	974277	1113670	0
Run1-Final	1	815314	1051492	55590
Run1-Final	2	800915	1024348	54052
Run1-Final	3	803796	1028782	55968
Run1-Final	4	800396	1043329	57730
Run2-Initial	1	1013428	1214743	0
Run2-Initial	2	1007421	1211730	0
Run2-Initial	3	1013855	1218658	0
Run2-Initial	4	1013927	1216314	0
Run2-Final	1	812955	1017838	52195
Run2-Final	2	835253	1111570	59591
Run2-Final	3	832654	1088676	58698
Run2-Final	4	845647	1091205	51931

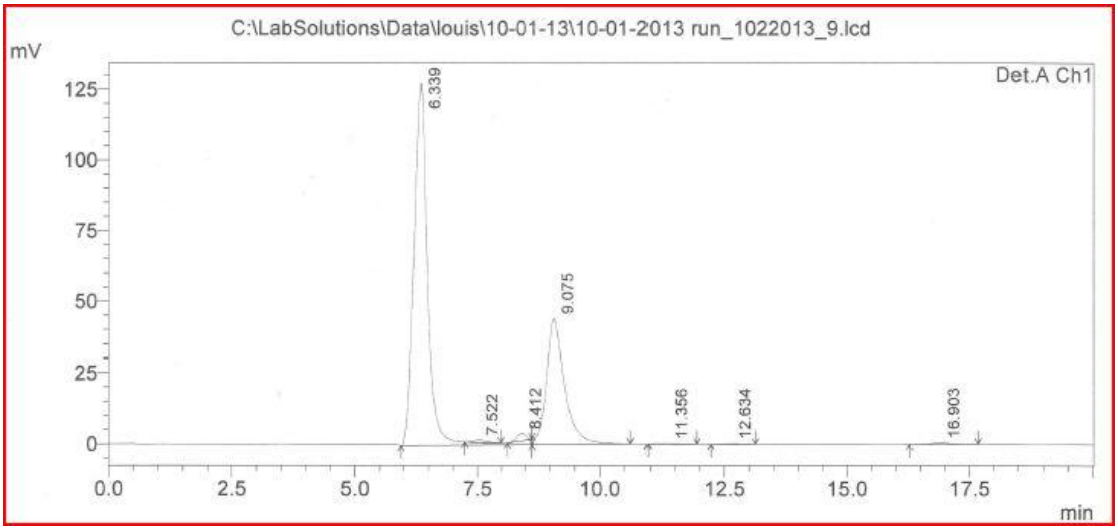

Figure B4: HPLC chromatograph of pre-fermentation standard glucose medium; glucose- 9.075 minutes

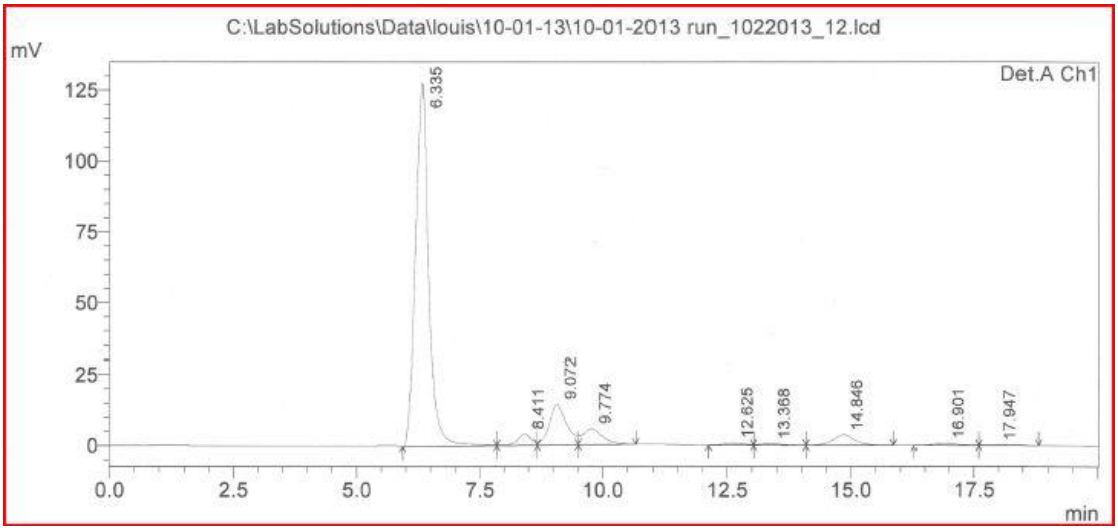

Figure B5: HPLC chromatograph of post-fermentation standard glucose medium; glucose- 9.072 minutes, acetate- 14.846 minutes.

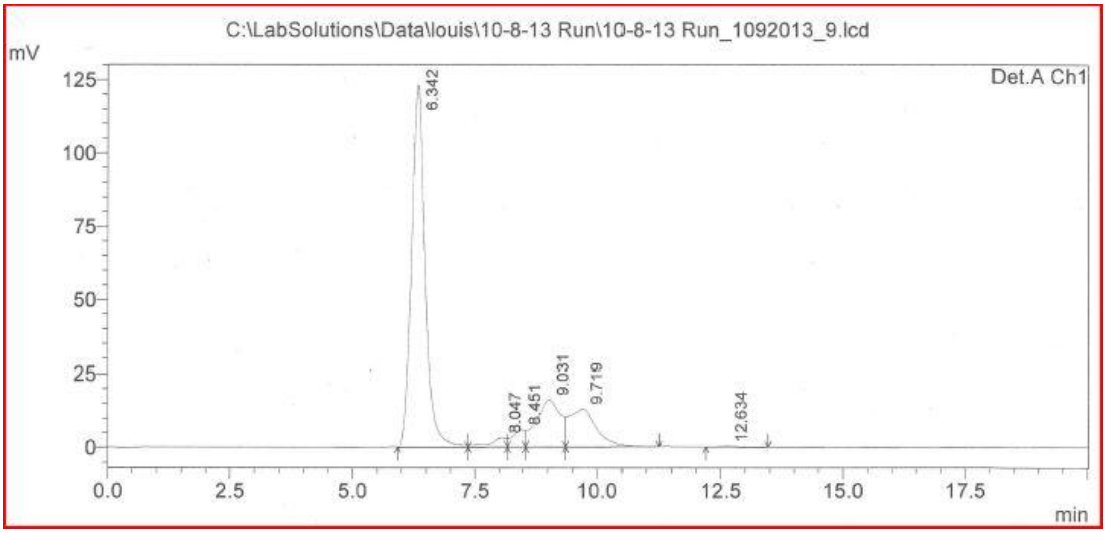

Figure B6: HPLC chromatograph of pre-fermentation peach medium; glucose- 9.031 minutes, fructose- 9.719 minutes

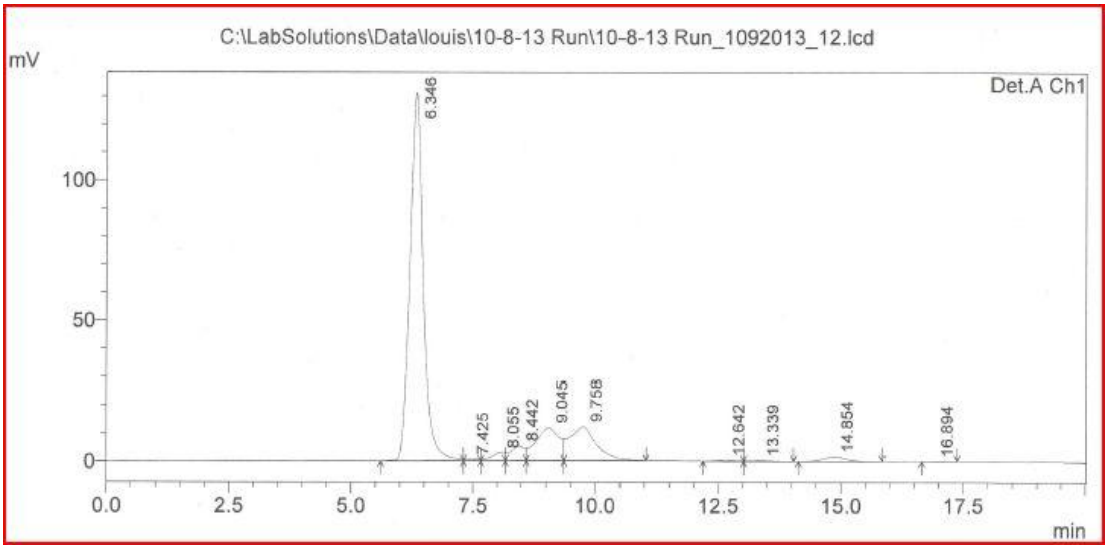

Figure B7: HPLC chromatograph of post-fermentation peach medium; glucose- 9.045 minutes, fructose- 9.758 minutes, acetate- 14.854 minutes.

REFERENCES

Balat, M. and M. Balat. 2009. Political, economic and environmental impacts of biomass-based hydrogen. *International Journal of Hydrogen Energy* 34(9): 3589-3603.

Belkin, S., C.O. Wirsen, and H.W. Jannasch. 1986. A new sulfur-reducing, extremely thermophilic eubacterium from a submarine thermal vent. *Applied Environ. Microbiology*, 51:1180-1185.

Clemson Univeristy, 2009. 2009 South Carolina Soybean Production guide http://www.clemson.edu/extension/rowcrops/soybeans/2009_South_Carolina_Soybean_Production_Guide.pdf.

Colarič M., F. Štampar , M. Hudina . Peach (Prunus persica L.) and nectarine (Prunus persica var. nucipersica Schneid.)2004. Acta agriculturae slovenica;83(1).

d'Ippolito, G., L. Dipasquale, F. M. Vella, I. Romano, A. Gambacorta, A. Cutignano, and A. Fontana. 2010. Hydrogen metabolism in the extreme thermophile *Thermotoga neapolitana*. Int. J. of Hydrogen Energy, 35:2290-2295.

Drapcho, C.M., N.P. Nhuan, T.H. Walker. 2008. *Biofuels Engineering Process Technology*. New York, NY: McGraw Hill.

Energy Information Agency. 2012. Official Energy Statistics from the US Government, Energy Information Administration, Report #DOE/EIA 0383(2012). http://eia.doe.gov/.

Eriksen, N.T., M.L. Riis, N.K. Holm and N. Iversen. 2011. H_2 synthesis from pentoses and biomass in *Thermotoga spp.* Biotechnology Letters, 33: 293-300.

Graetz, J. 2009. New approaches to hydrogen storage. Chem.Soc.Rev., 38: 73–82.

Hallenbeck, P.C. and J.R. Benemann. 2002. Biological hydrogen production; fundamentals and limiting processes. *International J. of Hydrogen Energy*, 27: 1185-1193.

Hawkes, F.R., R. Dinsdale, D.L. Hawkes, and I. Hussy. 2002. Sustainable fermentative hydrogen production: challenges for process optimization. *Intl. J. of Hydrogen Energy*, 27:1339-1347.

Huber, R., and M. Hannig. 2006. Thermotogales. in: M. Dworkin (Ed.) *Prokaryotes*. Springer New York.

Jain, A. 2009. Biohydrogen production from cull peach medium by hyperthermophilic bacterium *Thermotoga neapolitana.* MS thesis, Clemson, South Carolina: Clemson University, Department of Environmental Engineering and Earth Sciences.

Jannasch, H.W., R. Huber, S. Belkin, and K.O. Stetter. 1988. *Thermotoga neapolitana* sp. nov. of the extremely thermophilic, eubacterial genus *Thermotoga*. *Arch, Microbiol*, 150:103-4.

Kengen S.W.M., A.J.M. Stams , W.M. de Vos. 1996. Sugar metabolism of hyperthermophiles. FEMS Microbiol Rev 18:119–137.

Klass, DL. 2003. A critical assessment of renewable energy usage in the USA. Energy Policy. 353.

Levin, D. B., L. Pitt and M. Love. 2004. Biohydrogen production: prospects and limitations to practical application. *International Journal of Hydrogen Energy* 29(2): 173-185.

Morris,D. 2012 Biohydrogen production by *Thermotoga neapolitana* in Agricultural Media. MS thesis, Clemson, South Carolina : Clemson University, Department of Environmental Engineering and Earth Sciences.

Munro, S.A., S. H. Zinder, and L.P. Walker. 2009. The fermentation stoichiometry of Thermotoga neapolitana and influence of temperature, oxygen and pH on hydrogen production. Biotech Prog. 25(4):1034-1042.

NCSPA. 2013. http://www.ncsoy.org/ABOUT-SOYBEANS/Uses-of-Soybeans.aspx.

Ngo, T.A, M.S. Kim and S.J. Sim. 2011. High-yield biohydrogen production from biodiesel manufacturing waste by *Thermotoga neapolitana. Int J of Hydrogen Energy,* 36: 5836-5842.

Ngo, T.A, M.S. Kim and S.J. Sim. 2011. Thermophilic hydrogen fermentation using *Thermotoga neapolitana* DSM 4359 by fed-batch culture. *Int J of Hydrogen Energy,* 36:14014-14023.

Nguyen, T. A.D., J.P. Kim, M.S. Kim, Y.K. Oh, and S. J. Sim. 2008. Optimization of hydrogen production by hyperthermophilic eubacteria, *Thermotoga maritima* and *Thermotoga neapolitana* in batch fermentation. Int J of Hydrogen Energy. 33:1483-1488.

Ravot, G., M. Magot, M.L. Fardeau, B.K.C. Patel, G. Prensier, A. Egan, J.L. Garcia, and B. Ollivier. 1995. *Thermotoga Elfii* Sp-Nov, a novel thermophilic bacterium from an African oil-producing well. *Intl. J of Systematic Bacteriology*, 45, 308-314.

Sanders, Rolf. 1999. Compilation of Henry's Law Constants for Inorganic and Organic Species of Potential Importance in Environmental Chemistry. http://www.rolf-sander.net/henry/henry.pdf.

Sasaki, D., T. Hori, S. Haruta, Y. Ueno, M. Ishii and Y. Igarashi. 2011. Methanogenic pathway and community structure in a thermophilic anaerobic digestion process of organic solid waste. *Journal of bioscience and bioengineering* 111(1): 41-46.

SCDA.2007.http://www.scda.state.sc.us/relatedaglinks/associations/peach/consumerinformation/peachfacts.htm

Schonheit, P., and T. Schafer. 1995. Metabolism of hyperthermophiles. *World J of Microbiology & Biotechnology*, 11, 26-57.

Schroder, C., M. Selig, and P. Schonheit. 1994. Glucose fermentation to acetate, CO_2 and H_2 in the anaerobic hyperthermophilic eubacterium *Thermotoga maritima* - Involvement of the Embden-Meyerhof pathway. *Arch. Microbiology*, 161:460-470.

Selig M, Xavier KB, Santos H, Schonheit P. 1997. Comparative analysis of embden-meyerhof and entner-doudoroff glycolytic pathways in hyperthermophilic archaea and the bacterium thermotoga. Arch Microbiol 1997 APR ;167(4).

Thauer, R., 1977. Limitation of microbial H2-formation via ferementation. in: H.G. Schlegel, Barnea, J. (Ed.) *Microbial Energy Conversion*. Erich Goltze KG, Gottingen, pp. 201-4.

van Niel, E.W.J., M.A.W. Budde, G.G. De Haas, F.F. Van der Wal, P.A.M. Claassen and A.J.M. Stams. 2002. Distinctive properties of high hydrogen producing extreme thermophiles, Caldicellulosiruptor saccharolyticus and Thermotoga elfii. *International Journal of Hydrogen Energy* 27(11): 1391-1398.

van Niel, E.W.J., P.A.M. Claassen, A.J.M. Stams. 2002. Substrate and product inhibition of hydrogen production by the extreme thermophile *Caldicellulosiruptor saccharolyticus* Biotechnol Bioeng, 81:255–262.

Van Ooteghem, S.A., S.K. Beer, and P.C. Yue. 2002. Hydrogen production by the thermophilic bacterium *Thermotoga neapolitana*. *Appl Biochem Biotechnol*, 98-100: 177-89.

Van Ooteghem, S.A., A. Jones, D. Van Der Lelie, B. Dong, and D. Mahajan. 2004. H_2 production and carbon utilization by *Thermotoga neapolitana* under anaerobic and microaerobic growth conditions. *Biotechnol Lett*, 26, 1223-32.

Wang, L., M. Min, Y. Li, P. Chen, Y. Chen, Y. Liu, Y. Wang, R. Ruan. 2009. Cultivation of Green Algae Chlorella sp. in Different Wastewaters from Municipal Wastewater Treatment Plant. *Appl Biochem Biotechnol* (2010) 162:1174-1186.

Yu, X. 2007. Biohydrogen production by the hyperthermophilic bacterium *Thermotoga neapolitana*. PhD Dissertation, Clemson, South Carolina: Clemson University, Department of Environmental Engineering and Earth Sciences.

Yu, X., C.M. Drapcho. 2011. Hydrogen Production by the Hyperthermophilic Bacterium *Thermotoga neapolitana* using Agricultural-Based Carbon and Nitrogen Sources. Biological Engineering Transactions 2011;4:101-112.