12-2007

Biohydrogen Production by the Hyperthermophilic Bacterium Thermotoga neapolitana

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BIOHYDROGEN PRODUCTION BY THE HYPERTHERMOPHILIC BACTERIUM *THERMOTOGA NEAPOLITANA*

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Biosystems Engineering

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by
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December 2007

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ABSTRACT

Thermotoga neapolitana can use different sources of carbon and nitrogen for growth and produces biological hydrogen. Sources of carbon **(**glucose, sucrose, xylose, xylan, cellulose, cellobiose, starch, corn starch, beet pulp pellet, and rice flour) and nitrogen (yeast extract, fish meal, cottonseed meal, canola meal, linseed meal, and soybean meal) were compared. In the carbon studies, glucose, sucrose, rice flour, and xylan produced similar levels of hydrogen. In the nitrogen studies, Trypticase combined with alternative nitrogen sources can efficiently increases the yield of hydrogen produced by *Thermotoga neapolitana*. Yeast extract with trypticase as the dual nitrogen sources produced significantly increased concentration of hydrogen than the other combinations tested. Soybean meal and canola meal were second choices as alternative nitrogen sources. Sucrose and rice flour were promising carbon sources to replace glucose, and soybean meals was a promising nitrogen source to replace yeast extract for *Thermotoga neapolitana*.

Thermotoga neapolitana can utilize rice flour as sole carbon source, and soybean meal as one of nitrogen sources to produce hydrogen. Uniform design was used as experimental design to optimize the fermentation medium. The optimized medium was composed of 9 g/L rice flour, 4.5 g/L soybean meal, and 4.5 g/L trypticase. The hydrogen concentration for this optimized medium was 0.07083±0.006198 g H_2/L medium or 35.42±3.10 mmol H_2/L medium. The increased hydrogen concentration from control medium to optimized medium was 21.6%.

Thermotoga neapolitana ferments glucose as carbon source to produce acetate, carbon dioxide, and hydrogen as major products. The exponential phase of the bacterial growth was between 2 and 10 hrs of incubation time. The maximum cell mass concentration was reached after 10 hrs of incubation. The stationary phase lasted for 10hrs, and the death phase began at 20 hrs. The pH of broth decreased during the bacterium growth, which may inhibit hydrogen production. The maximum hydrogen partial pressure in this study was 45 kPa at 77 °C, and hydrogen partial pressure might inhibit the hydrogen production in this batch fermentation, an estimate of the critical hydrogen partial pressure of 38 kPa was calculated for the batch fermentation of *Thermotoga neapolitana* at 77 °C. The maximum specific growth rate (μ_{max}) of *Thermotoga neapolitana* with glucose as carbon source was 0.94 hr^{-1} at 77 °C. The Monod half saturation constant (K_S) was 0.57 g/L, the observed biomass yield from substrate was 0.25 g/g glucose or 44.59 g/mol glucose, the observed hydrogen yield from substrate was 0.028 g/glucose or 2.50 mol H_2 /mol glucose, and the observed hydrogen yield from biomass was 0.114 g/g dry weight. When glucose concentration was 5.0 g/L, the doubling time was 0.84 hr or 49 mins.

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DEDICATION

I dedicated this work to my wife, Zhaohui, my daughter, Yelia, and my family.

ACKNOWLEDGMENTS

I would like to thank my advisor, Dr. Caye M. Drapcho, for her assistance and support through this study. I am grateful to the other committee members: Dr. Joe E. Toler, Dr. Jeremy Tzeng, and Dr. Terry H. Walker for their guidance and activities in the completion of this dissertation.

I appreciate Dr. John Michael Henson and Dr. Zhaohui Xu (BGSU) for their discussions in this study. I would like to especially thank Jared Powell, Rongjuan Mi, and Fei Chen for their helps and suggestions in my research. I also extend my thanks to my other labmates and my classmates Kristy Conwell, Bhairavi D. Deshpande, Derek M. Little, Abhiney Jain, Cheng-Yi Kuan, and Meidui Dong for helping me in the lab and in class. I extend gratitude to the Clemson University, the College of Agricultural, Forestry and Life Science and the Department of Agricultural and Biological Engineering.

I especially want to thank my wife, Zhaohui, my daughter, Yelia, and my family. They support me without fail throughout my PhD graduate experience. My success is directly attributed to their understanding and support.

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CHAPTER I

INTRODUCTION

"To talk about "the hydrogen economy" is to talk about a world that is fundamentally different from the one we know now."

Spencer Abraham

Secretary of Energy

National Hydrogen Energy Roadmap, USDOE 2002

Energy plays an important role in global economic growth. The percentages of US energy that come from fossil fuels, nuclear, and renewable energy are 86.19%, 8.23%, and 6.12%, respectively in 2004 (DOE, 2004). But fossil fuel, as a non-renewable, limited energy resource, will become depleted in the not too far future. Crude oil production will approach a theoretical depletion near 2060-2070, and the theoretical depletion for natural gas is close for crude oil (Klass, 1998; Klass, 2003). In addition, the combustion of fossil fuels contributes to environmental problems such as global warming, acid rain, and health problems (Levin et al., 2004). The intergovernmental Panel on Climate Change (IPCC) reported that the emission of global greenhouse gas (GHG) increased 70% between 1970 and 2004 as the result of human activities. The increased atmospheric concentration of

carbon dioxide, the most important greenhouse gas, primarily comes from fossil fuels usage. The global warming, due to increased GHG, have made natural systems change, such as increasing in the temperature of global air and ocean, melting of snow, ice, and frozen ground , rising sea level, enlarging ice-lakes area and so on (IPCC Working Group I SPM, 2007; IPCC Working Group II SPM, 2007; IPCC Working Group III SPM, 2007). Since more than 60% crude oil in US is imported from other regions, energy security is another important issue needed be considered due to political instability in some oil exporting regions (DOE, 2004). For those reasons, renewable energy sources have been pursued for decades. Renewable energy sources include biomass, geothermal, wind, solar, and hydropower. In the United States, biomass energy ranks first, with 47% of the energy from renewable energy sources in 2004 (DOE, 2004).

Hydrogen is one of the most environmental friendly renewable energy sources, since the product of its combustion is water

$$H_2 + 1/2O_2 \rightarrow H_2O + 286\ kJ \qquad \text{(da Rosa, 2005)}$$

Hydrogen combustion has no contribution to environmental pollution and climate change (Levin et al., 2004). Therefore, hydrogen will play an important role in a low-carbon economy. Hydrogen also has the highest gravimetric energy density (122 kJ/g) among energy fuels, which is 2.75 times greater than hydrocarbon fuels (Han and Shin, 2004).

Currently, nine millions tons of hydrogen per year is produced in the US. Most of hydrogen is used in feedstock and intermediate chemical industries, such as for syntheses of ammonia or alcohols. Only a small portion of the hydrogen is used as an energy carrier today. DOE estimated that 40 million tons of hydrogen will be required to fuel about 100 million fuel-cell powered cars, or to provide electricity to about 25 million homes if the US would shift towards a hydrogen-economy (DOE, 2002; DOE, 2002; DOE, 2004).

Hydrogen can be produced through thermal, electrolytic, or biological methods. Steaming methane reforming, one of the thermal methods, produces 95 percent of the hydrogen in the United States today (DOE, 2002). This method is the most energy-efficient commercialized technology currently available, but catalyzes the reaction of steam with natural gas or other hydrocarbons to produce hydrogen and carbon dioxide (greenhouse gas). This gas mixture is then separated to produce high-purity hydrogen. Electrolysis of water needs a large amount of electricity and the cost of electricity accounts for 80% of the operating cost of hydrogen production.

Hydrogen production from biological systems is called biological hydrogen or biohydrogen (Kovacs et al., 2000). Today, this process is receiving more and more attention, because they can use renewable substrates, including agricultural wastes (Fan et al., 2006), or food processing waste (Fan et al., 2006; Wang et al., 2006), to produce hydrogen.

Microbes utilize agricultural and industrial waste or residues to produce hydrogen through dark-fermentation or light-driven process. Biohydrogen can be produced by algae, archaea, or bacteria. *Chlamydomonas reinhardtii* (Kosourov et al., 2005), and other green alga use direct biophotolysis to produce hydrogen. Cyanobacteria, such as *Anabaena variablilis* (Borodin et al., 2000; Lindblad et al., 2002), produce hydrogen through indirect biophotolysis. *Rhodobacter spheroids* (Koku et al., 2002; Zhu et al., 1999), and other purple non-sulfur bacteria produce hydrogen through photo-fermentation. Many researchers focus on algae and phototrophic bacteria, which utilize solar energy to produce hydrogen. However, light conversion efficiency, biogas (oxygen or hydrogen) inhibition, design of photo-bioreactors, and photoinhibation at high solar light intensities are limitations in photolytic biohydrogen production (Hallenbeck and Benemann, 2002; Levin et al., 2004; Melis, 2002).

Hydrogen can also be produced by anaerobic bacteria through dark-fermentation. This process does not need light as an energy source, and utilize various carbohydrates as an energy source and carbon source. Anaerobic bacteria capable of hydrogen production include species of *Enterobacter* (Nath et al., 2006), *Bacillus* (Kotay and Das, 2007), and *Clostridium* (Ferchichi et al., 2005; Zhang et al., 2006). Hydrogen production through dark-fermentation has benefits over other processes because it can use various renewable biomass materials from agriculture waste (Hussy et al., 2005; Logan et al., 2002), food processing waste (Van Ginkel et al., 2005), etc, and it can also use a wide range of microorganisms to continuously produce hydrogen.

Bacteria use different carbohydrates as carbon source and energy sources to grow and produce hydrogen, carbon dioxide, organic acid, alcohol, biomass and other products. The amount of hydrogen production from glucose by bacterium is affected by metabolic pathway and end-products.

$C_6H_{12}O_6 + 6H_2O \rightarrow 12H_2 + 6CO_2$ (1)

$\Delta G = -25.83$ kJ/mol (Thauer, 1976)

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

$\Delta G = -215.69$ kJ/mol (Thauer, 1976)

$C_6H_{12}O_6 + 2H_2O \rightarrow 2\ CH_3CH_2COOH + 2H_2 + 2CO_2$ (3)

$C_6H_{12}O_6 + 2H_2O \rightarrow CH_3CH_2OH + CH_3COOH + 2H_2 + 2CO_2$ (4)

According to reaction (1), theoretically the stoichiometric yield of hydrogen production is 12 mol H_2 /mol glucose when glucose is decomposed completely. If this reaction were possible, more than 99% of the combustion energy of glucose could be conserved in hydrogen. But no single microorganism known has the capability to produce hydrogen with this efficiency, because near 15 kcal energy per mol of ATP formed is required in the synthesis of ATP (Thauer, 1976). In this reaction, H^+ is the electron acceptor, and hydrogen gas is produced as the reduced form of electron acceptors.

The theoretical maximum yield for dark fermentation is 4 mol H_2 /mol glucose when acetic acid is the byproduct in reaction (2). 4 mol of hydrogen contains 33% of the combustion energy of glucose. The theoretical yield for dark fermentation is 2 mol H_2 / mol glucose when butyric acid is the byproduct in reaction (3) (Nandi and Sengupta, 1998). 2 mol of hydrogen contains 16.5% of the combustion energy of glucose. If ethanol and acetic acid are the end-products, 2 mol H_2/mol glucose is produced from reaction (4) (Hwang et al., 2004). If propionic acid is the end-product of dark fermentation, no hydrogen is produced (Hawkes et al., 2002; Ren et al., 2006). Practical biohydrogen production yield from dark fermentation is between 2 and 4 mol H_2/mol glucose. So hydrogen production from dark fermentation depends on the end-products and metabolic pathways. Selecting microorganism having the theoretical maximum hydrogen yield or close to it is very important, but there is very limited information related to the relationship between fermentation pathways and hydrogen production ability.

Hydrogen-producing microbes have been found in environments with a wide range of temperature, including mesophiles (25-40 °C) (Kotay and Das, 2007; Shin et al., 2007), thermophiles (40-65 °C), extreme thermophiles (65-80 °C), or hyperthermophiles (>80 °C) (van Niel et al., 2003). Among them, hyperthemophiles seem to be the promise for biohydrogen production in the future. Hydrogen, as the end-product of fermentation, inhibits the growth of hydrogen-evolving bacteria at high hydrogen partial pressure. In nature, hydrogen partial pressure need to be kept very low (<100 Pa or 10^{-3} atm) for many bacteria to ferment hexoses and form hydrogen as one of the end-products (Thauer,

1976; Thauer et al., 1977). The limit of hydrogen partial pressure for hydrogen-evolving bacteria growth can be increased at high temperatures although different species have different hydrogen partial pressure limits. The hydrogen partial pressure limit increases to 2,000 Pa for *Pyrococcus furiosus* growing without S° at the temperature 98 °C (Parameswaran et al., 1988), and the limit of hydrogen partial pressure could be increased to 10,000-20,000Pa for *Caldicellulosiruptor saccharolyticus* growing at the temperature 70 °C (van Groenestijn et al., 2002; van Niel et al., 2003). Hydorgenase catalyzes both the forward and reverse reaction of hydrogen production and consumption. The catalytic activity of hydrogenase is in favor of evolving hydrogen at high temperatures (Adams, 1990). The end products of fermentation hyperthermophiles fermentation have less variety. *Thermotoga maritima* can almost completely transfer glucose to acetate, CO_2 and H_2 (Schonheit and Schafer, 1995). So biogases produced by hypertheromophiles may be directly used as fuel for hydrogen fuel cells. Bacteria growing at high temperatures have less contamination than in low temperatures, since few bacteria can grow in the high temperature, and sterilization may be omitted if hyperthermophiles are used to produce hydrogen. As a consequence, a large amount of energy requirement for sterilization can be saved. In additional, energy requirement for cooling fermentation systems could be small if proper insulations are used in fermentation systems, because hydrogen produced by these bacteria still release heat, although hyperthemophiles grow at high temperatures.

The order of *Thermotogales* comprises a group of extremely thermophilic, Gram-negative, rod-shaped, nonsporulating bacteria with an outer sheath-like envelope or

"toga". Thermotoga belongs to a very deep branch with the bacterial phylogenetic tree (Jannasch et al., 1988). The genera *Thermotoga, Thermosipho, Fervidobacterium, Geotoga, Petrotoga, Marinitoga, Thermopallium* have been described within *Thermotogales (Conners et al., 2006; Huber and Hannig, 2006)*. The genera *Thermotoga* currently include 9 species. *Thermotoga maritima* was originally isolated from a geothermally heated, shallow marine sediment at Vulcano, Italy (Huber et al., 1986). *Thermotoga neapolitana* was first obtained from a submarine hot spring near Lucrino, the bay of Naples, Italy (Belkin et al., 1986; Jannasch et al., 1988). *Thermotoga elfii* was originally isolated from an Africa oil production well (Ravot et al., 1995).

Themotoga maritima completely ferments 1 mol glucose as carbon and energy sources to 2 mol acetate, 2 mol CO_2 and 4 mol H_2 through the "classical" Embden-Meyerhof pathway (Figure 1.1). The biomass yield from glucose is 45 g cell mass/mol glucose (Schroder et al., 1994). Most of the glucose –fermenting anaerobes produce less than 2 mol of acetate and 4 mol of hydrogen from 1mol of glucose, because various byproducts such as lactate, ethanol, or butyrate, etc are also produced (Schonheit and Schafer, 1995). The maximum rate of hydrogen production by *Thermotoga elfii* was 2.7-4.5 mmol H_2/ (L h) (van Niel et al., 2002). *Themotoga neapolitana* accumulated 25-30% hydrogen during its incubation (Van Ooteghem et al., 2002; Van Ooteghem et al., 2004).

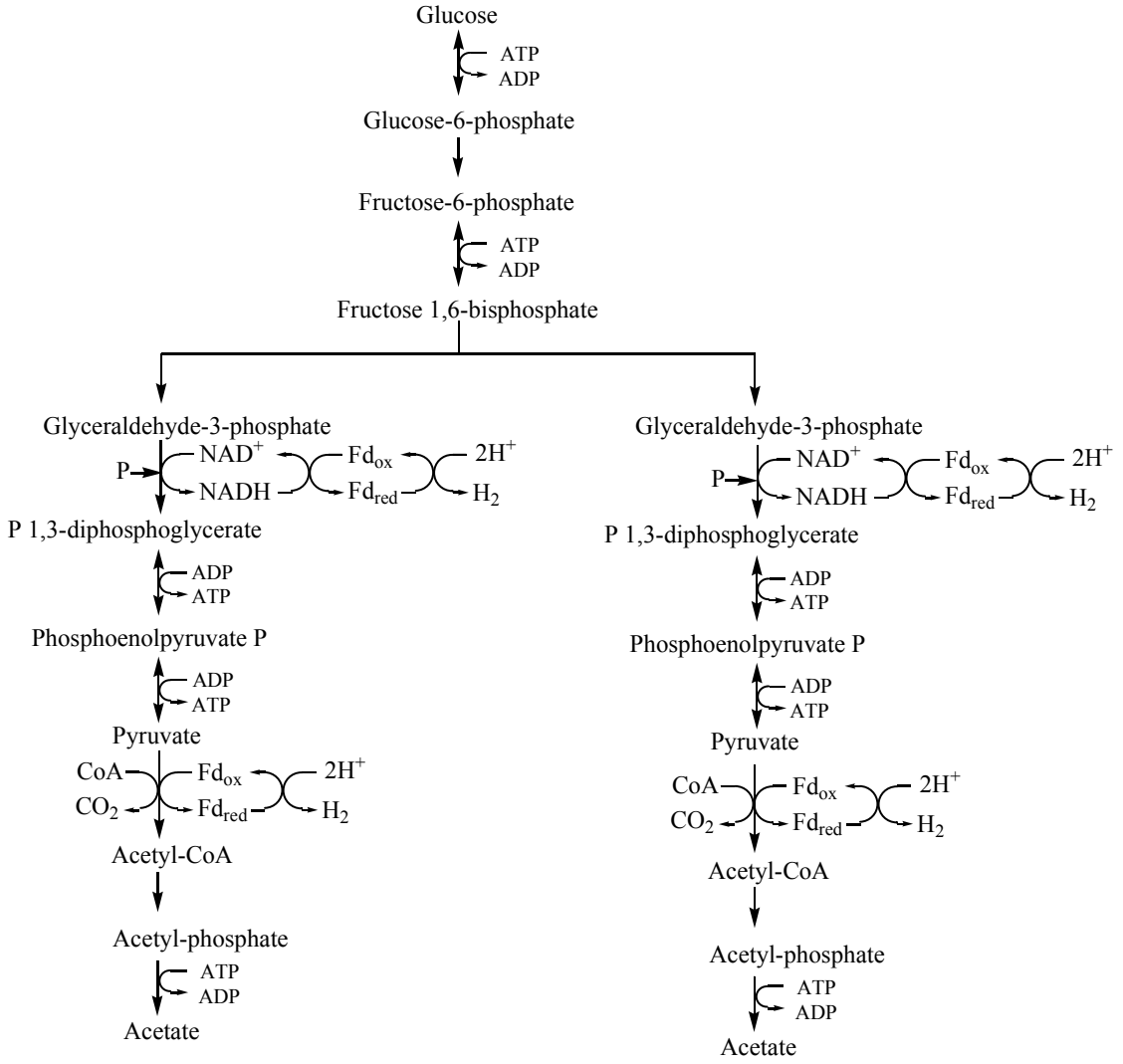

Figure 1.1. Metabolic pathway of 1 mol glucose being fermentation to 2 mol acetate, 2 mol CO_2 and 4 mol H_2 by *Thermotoga maritima (Schroder et al., 1994)*.

GOALS OF THIS STUDY

Little research data is available on the growth and hydrogen production by *Thermotoga neapolitana*. Only van Ooteghem et al. reported the biohydrogen produced by *Thermotoga neapolitana* with glucose as carbon and energy sources, and yeast extract and trypticase as double nitrogen sources (Van Ooteghem et al., 2002; Van Ooteghem et al., 2004). Limited research on the biohydrogen produced by *Thermotoga neapolitana* with agricultural feedstocks or agricultural wastes as carbon sources and nitrogen sources is available. Reports on kinetics studies on the biohydrogen produced by *Thermotoga neapolitana* were also not available. Therefore, the overall goal of this research is to find the kinetic parameters for this bacterium and look for the possibilities of using various carbon and nitrogen sources to produce hydrogen.

The main objectives of this dissertation are:

1. Establishing an efficient procedure for growing *Thermotoga neapolitana* and setting-up a basic fermentation condition for its biohydrogen production.
2. Screening carbon sources and nitrogen sources for biohydrogen production by *Thermotoga neapolitana*.
3. Optimizing the composite of carbon source and nitrogen sources of the medium.
4. Establishing the kinetic parameters of *Thermotoga neapolitana* involved in hydrogen production.

An overview of chapter II to VI follows:

Chapter II

In this chapter, the techniques for medium preparation, degassing, inoculation, and cultivation are described. Microscopic pictures of *Thermotoga neapolitana* are also presented.

Chapter III

Thermotoga neapolitana can use different sources of carbon and nitrogen for growth and biological hydrogen production. In this study, sources of carbon **(**glucose, sucrose, xylose, xylan, cellulose, cellobiose, starch, corn starch, beet pulp pellet, and rice flour) and nitrogen (yeast extract, fish meal, cottonseed meal, canola meal, linseed meal, and soybean meal) are compared. From these experiments, the most promising carbon sources and nitrogen sources for hydrogen production are determined.

Chapter IV

Results from the carbon and nitrogen experiments indicated that *Thermotoga neapolitana* can utilizes rice flour as carbon source, and soybean meal as one of nitrogen sources to

efficiently produce hydrogen. In this experiment, uniform design is used as an experimental set-up model to optimize the fermentation medium.

Chapter V

Thermotoga neapolitana utilized glucose as carbon source to produce acetate, carbon dioxide, and hydrogen as major fermentation products. In this chapter, growth curve of *Themotoga neapolitana* is described. The pH change with time is measured. Hydrogen partial pressure change with incubation time is also studied. Kinetic parameters are also calculated and possible equation describing this reaction is also predicated in this study.

Chapter VI

This chapter summarizes the conclusion of this study and offers suggestions for future work.

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CHAPTER II

INOCULATION TECHNIQUES

METHOD FOR INOCULATING HYPERTHERMOPHILE *THERMOTOGA NEAPOLITINA*

Important point: *Thermotoga neapolitana* is an anaerobic hyperthermophile. Oxygen will inhibit the growth of this bacterium.

This method is specifically developed for this bacterium only.

Autoclave

1. Make medium according to the requirements of the experiment and divide it into serum bottles.
2. Wrap aluminum foil around the mouths of serum bottles.
3. Autoclave the medium at 121 °C for 20 min.

Sparge

4. Take off aluminum foil, and put rubber stoppers in the mouth of medium bottles immediately at hot temperature (70-80 °C) in autoclave chamber.
5. Open the nitrogen valve and keep pressure at 0.5 psi.
6. Release nitrogen for 4 min (removing air in the tube)
7. Place one tube needle into liquid, and one into headspace of the bottle and sparge 1 min.
8. Apply a flange-type rubber stopper and seal it with an aluminum cap by crimper.

Inoculation

9. Sparge nitrogen into an empty bottle for 4 min to create a nitrogen bottle.
10. Seal this bottle with a flange-type rubber stopper and an aluminum cap.
11. Use needles to continuously sparge nitrogen into the nitrogen bottle
12. Use a syringe to take 5 ml nitrogen from the nitrogen bottle.
13. Use the syringe to inject nitrogen into seed bottle.
14. Syringe 5 ml of seed liquid and inject into the fresh medium bottles prepared in sparge phase.
15. Put inoculated bottle in shaker for incubation.

OXYGEN INHIBITS THE GROWTH OF *THERMOTOGA NEAPOLITANA*

van Ooteghem et al. has reported that *Thermotoga neapolitana* is a microaerobic bacterium which means that oxygen does not inhibit the growth of *Thermotoga neapolitana* (Van Ooteghem et al., 2002; Van Ooteghem et al., 2004). Others reported this bacterium is a strictly anaerobic bacterium (Jannasch et al., 1988). We found that *Thermotoga neapolitana* is an anaerobic bacterium, but it can tolerant oxygen under certain conditions. For example, *Thermotoga neapolitana* could grow at low concentration of oxygen when glucose but not xylose is used as the sole carbon source. However, *Thermotoga neapolitana* can utilize xylose very well under anaerobic conditions.

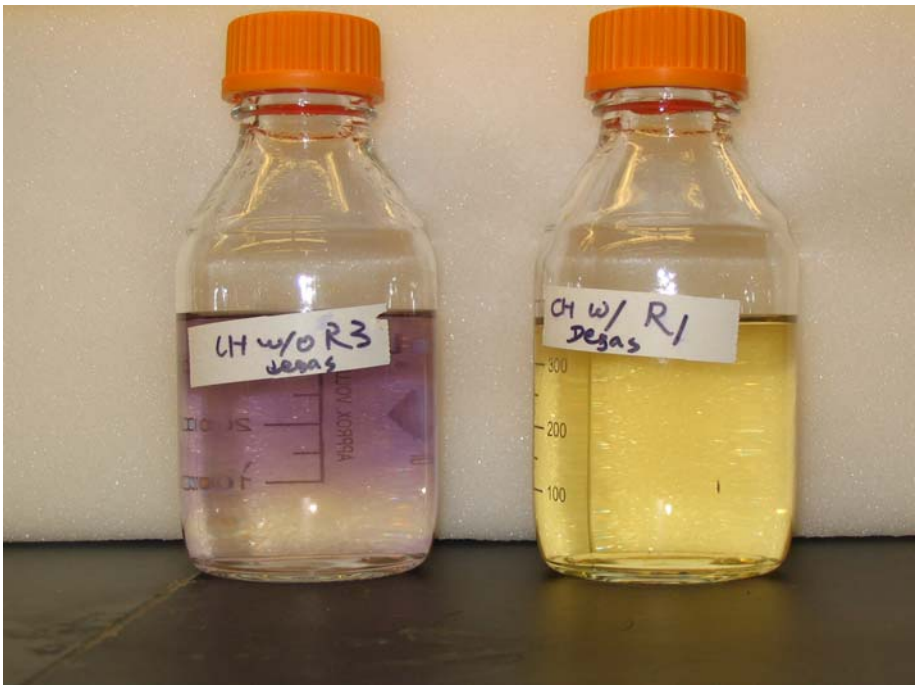

Figure 2.1. The color of medium changed with degassing procedure.
Left bottle- degassing procedure not used. Right bottle- degassing procedure used. Both bottles contained 0.1% resazurin as redox indicator. Pink or purple color indicates oxygen dissolved in liquid.

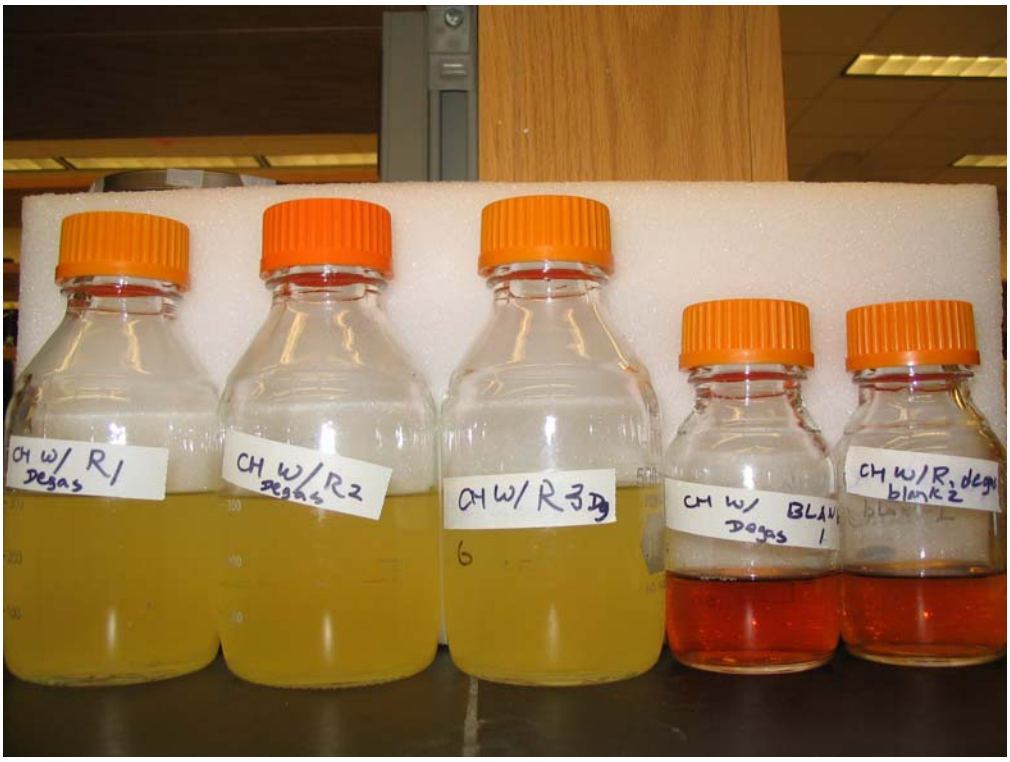

Figure 2.2. The colors of cultivated medium of *Thermotoga neapolitana* with degassing procedure.
Three large bottles on the left side are media inoculated with *Thermotoga neapolitana* with degassing procedure. Two small bottles on the right side are degassed medium without inoculation.

Figure 2.3. The colors of cultivated medium of *Thermotoga neapolitana* without degassing procedure.
Three large bottles on the left side are media inoculated *Thermotoga neapolitana* without degassing procedure. Two small bottles on the right side are media without inoculation and degassing procedure.

From Figure 2.1, Figure 2.2, and Figure 2.3, we concluded degassing procedure is important to the growth of *Thermotoga neapolitana*. Without degassing procedure, *Thermotoga neapolitana* did not grow well, and the colors of medium became dark due to Mailllard reaction (Fennema, 1997) when microbes did not consume sugars.

MICROSCOPIC PICTURES OF *THERMOTOGA NEAPOLITANA*

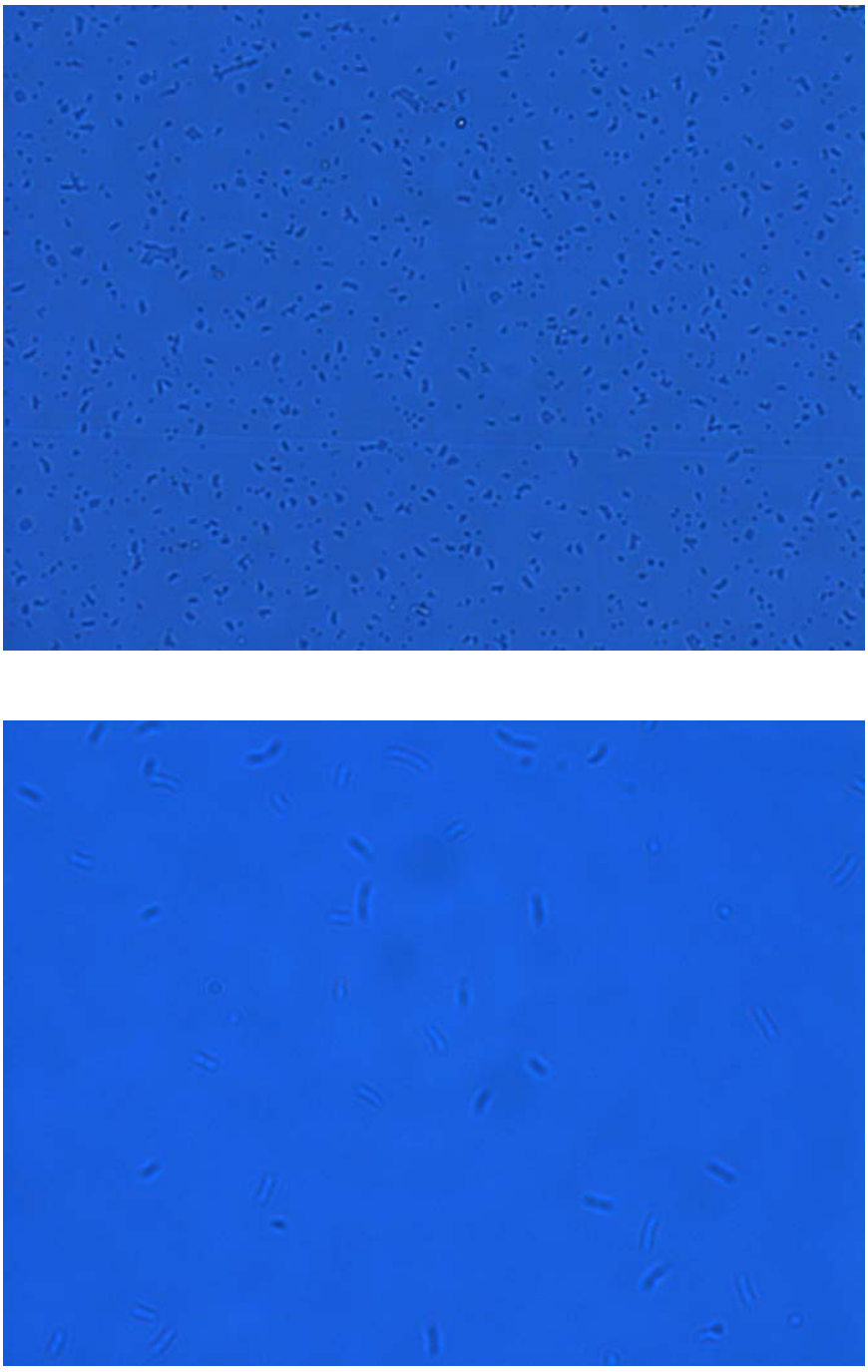

Figure 2.4. Pictures of *Thermotoga neapolitana* through phase contrast microscope. Top picture - 20X, Bottom picture – 100X

In Figure 2.4, *Thermotoga neapolitana* form the sheath-like envelop around the body of the bacterium.

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CHAPTER III

SCREEN CARBON SOURCES AND NITGROGEN SOURCES FOR BIOHYDROGEN PRODUCTION OF HYPERTHEMOPHILE *THERMOTOGA NEAPOLITANA*

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ABSTRACT

Thermotoga neapolitana can use different sources of carbon and nitrogen for growth to produce biological hydrogen. In this study, sources of carbon **(**glucose, sucrose, xylose, xylan, cellulose, cellobiose, starch, corn starch, beet pulp pellet, and rice flour) and nitrogen (yeast extract, fish meal, cottonseed meal, canola meal, linseed meal, and soybean meal) were compared. In the carbon studies, glucose, sucrose, rice flour, and

xylan produced similar levels of hydrogen. In the nitrogen studies, yeast extract with trypticase as the dual nitrogen sources produced significantly increased concentration of hydrogen than the other combinations tested. Soybean meal and canola meal were second choices as alternative nitrogen sources. Trypticase combined with alternative nitrogen sources can efficiently increases the yield of hydrogen produced by *Thermotoga neapolitana*. Combined with results of a complete randomized design experiments, sucrose and rice flour were selected as promising carbon sources to replace glucose, and soybean meal as a promising nitrogen source to replace yeast extract for future hydrogen production by *Thermotoga neapolitana*.

Keywords: biohydrogen; medium; carbon sources; nitrogen sources; screening; *Thermotoga neapolitana*

INTRODUCTION

Energy plays an important role in global economic growth. The percentages of US energy that come from fossil fuels, nuclear, and renewable energy are 86%, 8%, and 6%, respectively in 2004 (DOE, 2004). But fossil fuel, as a non-renewable and limited energy resource, will become depleted in the not too far future. Crude oil and nature gas production will approach a theoretical depletion near 2060-2070 (Klass, 1998; Klass,

2003). In addition, the combustion of fossil fuels contributes to environmental problems such as global warming, acid rain, and health problems (Levin et al., 2004).
Hydrogen is one of the most environmental friendly renewable energy sources, since the product of its combustion is water.

$$H_2 + 1/2O_2 \rightarrow H_2O + 286kJ \qquad \text{(da Rosa, 2005)}$$

Hydrogen combustion has no contribution to environmental pollution and climate change (Levin et al., 2004). Therefore, hydrogen will play an important role in a low-carbon economy. Hydrogen also has the highest gravimetric energy density (122 kJ/g) among energy fuels, which is 2.75 times greater than hydrocarbon fuels (Han and Shin, 2004).

Hydrogen can be produced through thermal, electrolytic, or biological methods. Hydrogen produced by biological systems is called biohydrogen (Kovacs et al., 2000). Today biohydrogen production is receiving more and more attention, because renewable substrates, including agricultural wastes (Fan et al., 2006), or food processing waste (Fan et al., 2006; Wang et al., 2006), can all be used to produce hydrogen.

Microbes utilize agricultural and industrial waste or residues to produce hydrogen through dark-fermentation or light-driven process. Hydrogen can be produced by anaerobic bacteria through dark-fermentation which does not need light as energy source, and utilizes various carbohydrates as energy source and carbon source. Anaerobic bacteria capable of hydrogen production includes species of *Enterobacter* (Nath et al., 2006), *Bacillus* (Kotay and Das, 2007), and *Clostridium* (Ferchichi et al., 2005; Zhang et

al., 2006). Hydrogen production through dark-fermentation has benefits over other processes because it can use various renewable biomass materials from agriculture waste (Hussy et al., 2005; Logan et al., 2002), food processing waste (Van Ginkel et al., 2005), etc, and also, it can use a wide range of microorganisms to continuously produce hydrogen.

Bacteria use different carbohydrates as carbon and energy sources to grow and produce hydrogen, carbon dioxide, organic acid, alcohol, biomass and other products. The amount of hydrogen production from glucose by bacterium is affected by metabolic pathway and end-products.

$$C_6H_{12}O_6 + 6H_2O \rightarrow 12H_2 + 6CO_2 \quad (1)$$

ΔG = -25.83 kcal/mol (Thauer, 1976)

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

ΔG = -215.69 kcal/mol (Thauer, 1976)

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2\ CH_3CH_2CH_2COOH + 2H_2 + 2CO_2 \quad (3)$$

$$C_6H_{12}O_6 + 2H_2O \rightarrow CH_3CH_2OH + CH_3COOH + 2H_2 + 2CO_2 \quad (4)$$

According to reaction (1), theoretically the stoichiometric yield of hydrogen production is 12 mol H_2 /mol glucose when glucose is decomposed completely. If this reaction would be possible, more than 99% of the combustion energy of glucose could be conserved in

hydrogen. But no single microorganism known has the capability to produce hydrogen with this efficiency, because near 15 kcal energy /mol of ATP forming is required in the synthesis of ATP (Thauer, 1976). In this reaction, H^+ is the electron acceptor, and hydrogen gas is produced as the reduced form of electron acceptors.

The theoretical maximum yield for dark fermentation is 4 mol H_2 /mol glucose when acetic acid is the byproduct in reaction (2). 4 mol of hydrogen contains 33% of the combustion energy of glucose. The theoretical yield for dark fermentation is 2mol H_2/mol glucose when butyric acid is the byproduct in reaction(3) (Nandi and Sengupta, 1998). 2 mol of hydrogen contains 16.5% of the combustion energy of glucose. If ethanol and acetic acid are the end-products, 2 mol H_2/mol glucose is produced from reaction (4) (Hwang et al., 2004). If propionic acid is the end-product of dark fermentation, no hydrogen is produced (Hawkes et al., 2002; Ren et al., 2006). Practical biohydrogen production yield from dark fermentation is between 2 mol H_2/mol glucose and 4 mol H_2/mol glucose. Therefore, hydrogen production from dark fermentation depends on the end-products and metabolic pathways. Selecting microorganisms processing at or near the theoretical maximum hydrogen yield is important, but only limited information exists related to the relationship between fermentation pathways and hydrogen production ability.

Hydrogen-producing microbes have been found in environments with a wide range of temperatures, including mesophiles (25-40 °C) (Kotay and Das, 2007; Shin et al., 2007), thermophiles (40-65 °C), extreme thermophiles (65-80 °C), or hyperthermophiles (>80 °C) (van Niel et al., 2003). Among them, Hyperthemophiles seem to be the promise for biohydrogen production in the future. Hydrogen, as the end-product of fermentation, inhibits the growth of hydrogen-evolving bacteria at high hydrogen partial pressure. In nature, hydrogen partial pressure need to be kept very low (<100 Pa or 10^{-3} atm) for many bacteria to ferment hexoses and form hydrogen as one of the end-products (Thauer, 1976; Thauer et al., 1977). The limit of hydrogen partial pressure for hydrogen-evolving bacteria growth can be increased at high temperatures although different species have different hydrogen partial pressure limits. The hydrogen partial pressure limit increases to 2,000 Pa for *Pyrococcus furiosus* growing without S° at 98 °C (Parameswaran et al., 1988), and the limit of hydrogen partial pressure could be increased to 10,000-20,000 Pa for *Caldicellulosiruptor saccharolyticus* growing at 70 °C (van Groenestijn et al., 2002; van Niel et al., 2003). Hydorgenase involves the hydrogen-evolving and hydrogen-consuming. The catalytic activity of hydrogenase is in favor of evolving hydrogen at high temperatures (Adams, 1990). The end products of hyperthermophilic fermentation also have less variety. *Thermotoga maritima* can almost completely transfer glucose to acetate, CO_2 and H_2 (Schonheit and Schafer, 1995). So biogas produced by hypertheromophiles may be directly used as fuel for hydrogen fuel cells. In additional, bacteria growing at high temperatures have less contamination than at low temperatures, since few bacteria can grow in the high temperature, and sterilization can be omitted if

hyperthermophiles are being used to produce hydrogen. As consequences, a large amount of energy required for sterilization can be saved.

Originally isolated around the bay of Naples, Italy (Belkin et al., 1986), *Thermotoga neapolitana* is a Gram-negative, rod-shaped, obligate anaerobic, fermentative extreme thermophile surrounded by a bag-shaped sheath-like outer structure called "toga" (Huber et al., 1986). The optima growth temperature of *Thermotoga neapolitana* is 77 °C (Jannasch et al., 1988). Most species of *Thermotogales* produce certain amount of hydrogen during their growth (van Niel et al., 2002; Van Ooteghem et al., 2002; Van Ooteghem et al., 2004; Vrijie et al., 2002). The maximum rate of hydrogen production by *Thermotoga elfii* was 2.7-4.5 mmol H_2/ (L h) (van Niel et al., 2002). *Themotoga neapolitana* accumulated 25-30% hydrogen during its incubation (Van Ooteghem et al., 2002; Van Ooteghem et al., 2004).

The composition of the medium plays an important role in affecting the growth of bacteria, the yield of products, and other characteristics. The carbon sources have a very important impact on the bacterial growth and product yield. The product yields from the bacterium digesting different carbon sources can vary significantly.

Thermotoga neapolitana can use simple or complex carbohydrates or complex organic matter as carbon source or nitrogen source(Huber and Hannig, 2006). Biolog anaerobic microtiter plate system screened a series of carbon sources for utilization by the

bacterium (Van Ooteghem et al., 2004). A total of 57 carbon sources gave high scores with N_2 atmosphere, while 14 carbon sources gave high score with H_2 atmosphere.

The objective of this study is to screen a variety of carbon and nitrogen sources and find the potential sources that can be used for the future biohydrogen production by *Thermotoga neapolitana*. In the carbon studies, glucose, sucrose, xylose, xylan, cellulose, cellobiose, starch, corn starch, beet pulp pellet, and rice flour were investigated. Yeast extract and trypticase were used as nitrogen sources in these trials. In the nitrogen studies, yeast extract, fish meal, cottonseed meal, canola meal, linseed meal, and soybean meal were studied with glucose as the carbon source. Then, a three by three factorial design was used to find the most promising alternative carbon and nitrogen sources.

MATERIALS AND METHODS

Organism

Thermotoga neapolitana was obtained from DSMZ (the German Resource Centre for Biological Material).

Materials

For carbon sources, glucose, cellulose, xylose, starch, cellobiose, and sucrose were purchased from Fisher Scientific, xylan was purchased from TCI (Tokyo KASEI, Tokyo, Japan), corn starch, rice flour, and beet pulp pellet were purchased from Labuddle Group Incorporated (Wisconsin).

For nitrogen sources, yeast extract, and trypticase were purchased from Fisher Scientific, while all of other alternative nitrogen resources, such as cottonseed meal, fish meal, canola meal, linseed meal, and soybean meal, were purchased from Labuddle Group Incorporated (Wisconsin).

Culture Maintenance

Thermotoga neapolitana was maintained on the medium described by Van Ooteghem et al (Van Ooteghem et al., 2002) . The composition of medium is following as: 1.0 g of NH_4Cl, 0.3 g of K_2HPO_4, 0.3 g of KH_2PO_4, 0.2 g of $MgCl_2{\cdot}2H_2O$, 0.1 g $CaCl_2$, 10.0 g of NaCl, 0.1 g of KCl, 1.0 g of Cysteine HCl, 2.0 g of yeast extract, 2.0 g of Trypticase, 10.0ml of vitamin solution (DSM media 141), 10.0 ml of trace element solution (DSM media 141), 0.121 g of trizma base, 5 g of glucose, and 1.0 L of H_2O. The initial pH of the medium was adjusted to 8.5 with NaOH. The organism was preserved at 4 °C.

Cultivation Medium and Conditions

The composition of medium used for screening the carbon sources was described in culture maintenance, except that the carbon sources were changed. The composition of media used for screening the nitrogen sources was described in culture maintenance, except that the nitrogen sources were changed. As a first step in the screening of nitrogen sources, 2 g/L of the alternative nitrogen source was used to replace yeast extract. As the second step of screening, 4 g/L of the selected nitrogen source was used to replace both yeast extract and trypticase. In both sets of experiments, the third nitrogen sources (NH_4Cl) was included at 1.0 g/L.

In both the carbon and nitrogen source studies, the initial pH of medium was adjusted to 8.5 with NaOH addition before sterilization. Serum bottles (500ml) containing 200 ml medium were autoclaved in 121 °C for 20 min. The bottles were then boiled for 20 min and sparged with N_2 for 1 minute. The culture medium was inoculated with 5 ml volume using a sterile syringe. The culture was incubated on an orbital shaker bed at 200 rpm and 77 °C.

Analysis Methods

Hydrogen gas in the headspace was sampled using 1 ml tuberculin syringe, and 0.5 ml of gas was injected into a gas chromatograph (SRI 8610C, SRI Instruments, Torrance, CA

90503) with thermal conductivity detector at 100 °C and silicon column at 25 °C. The pressure of argon as a carrier gas was 22 psi.

The pressure of gas in the headspace of each reactor was measured with Traceable manometer (Fisher Scientific) after the reactor had cooled to room temperature. The volume of the headspace was 365 ml. The volume of culture medium was 200 ml. According to ideal gas law, the hydrogen concentration was calculated through the following equation

$$C_{H_2} = \frac{P_{H_2} V_1}{RT} * \frac{1}{V_2}.$$

Here, C_{H_2} is the hydrogen gas concentration (mol H_2 /L medium), P_{H_2} is the hydrogen partial pressure (atm), V_1 is the volume of headspace (L), T is the temperature (K), R is the universal gas constant (0.0821 L·atm/(mol·K)) , V_2 is the volume of medium (L).

The experimental results were analyzed with SAS software (SAS, SAS Institute Inc., Cary, NC), 0.05 level of significant was used.

RESULTS AND DISCUSSION

Incubation Time

The necessary incubation time varied with different carbon sources (Figure 3.1). When glucose, xylose, xylan, cellulose, beet pulp pellet or cellobiose was used as the carbon source, the hydrogen concentration did not differ between 20 hrs and 36 hrs of incubation. When sucrose, rice flour, starch and corn starch were used as carbon source, the hydrogen concentration increased with the incubation time from 20 to 36 hours.

Hydrogen production by *Thermotoga neapolitana* is affected by the types of carbohydrates. If monosaccharides, such as glucose, xylose, or cellobiose were used as carbon sources, an incubation time of 20 hrs is sufficient for biohydrogen production, while if polysaccharides, such as starch, rice flour, or corn starch were used as carbon sources, 36 hrs of incubation were necessary. Biohydrogen production levels were less consistent for polysaccharides using 30hrs incubation time. Since hydrogen production is growth-associated, we can concluded that the growth rates of *Thermotoga neapolitana* with oligosaccharides, such as glucose, xylose, and cellobiose were faster than that of the bacterium with polysaccharides, such as starch, and rice flour. The reason might be that the bacterium needs to take more time to synthesize enzymes that hydrolyze oligosaccharides and polysaccharides. The growth rate of the bacterium with xylan as a carbon source was very fast. The reason for this might be that the activity of the

microbial enzymes involving xylan utilization is very high. Another reason may be the structure of xylan used was degraded already, so it was easily accessed by the enzymes. Hydrogen production was very low when the bacterium utilized cellulose. This is because crystal cellulose was used, which is difficult to be accessed by the active sites of the enzymes. Based on these results, the incubation time for the rest of the study was set as 36 hrs.

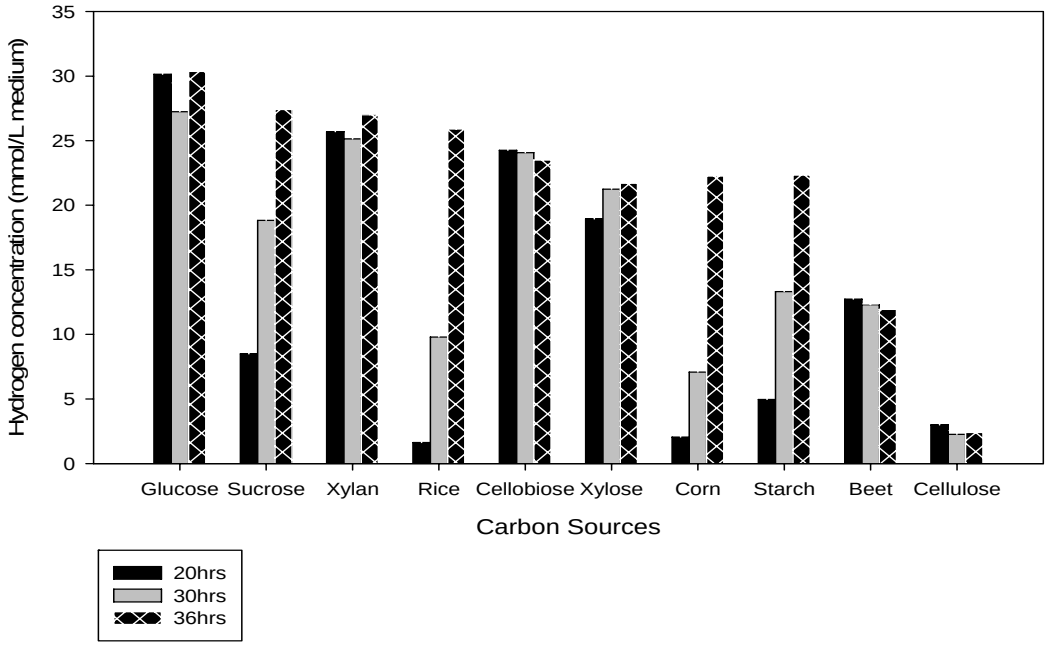

Figure 3.1. The hydrogen concentration produced by *Thermotoga neapolitana* with different carbon sources and various incubation times.

Screening Carbon Source

Bashed on results in Table 3.1, *Thermotoga neapolitana* used almost all carbon sources to grow and produce hydrogen. Among these carbon sources, the hydrogen concentration with glucose (30.36 mmol H_2/L medium) was not significantly different from sucrose (27.42 mmol H_2/L medium) or xylan (27.00 mmol H_2/L medium) ($p > 0.05$). The hydrogen concentrations for sucrose or xylan were not significantly different from that of rice flour (25.89 mmol H_2/L medium) or cellobiose (23.49 mmol H_2/L medium). Hydrogen produced by the bacterium with beet pulp pellet as the carbon source (11.94 mmol H_2/L medium) was the second lowest among the carbon sources due to its a lower concentration of carbohydrates. Hydrogen produced by the bacterium with cellulose as the carbon source (2.41 mmol H_2/L medium) was the lowest among all carbon sources tested, which had also been described by van Ooteghem et al (2002). However, cellobiose, a disaccharide subunit of cellulose, was a good carbon source for the bacterium. Cellulose is an unbranched linear polymer of glucoses with ß-1,4-glycoside-linkages, and the structure of cellulose is highly ordered and resists enzymatic degradation. Pretreatment of *Miscanthus* produced high yield of hydrogen by *Thermotoga elfii* (Vrijie et al., 2002). So if cellulose materials are to be used as a carbon source, pretreatment of cellulose will be necessary.

Table 3.1. Hydrogen concentrations of *Thermotoga neapolitana* with different carbon sources[1].

Carbon Sources	Percentage of hydrogen concentration (%)	Absolute total Pressure (kPa) at 25°C	Hydrogen concentration (mmol H_2/L medium)[2]
Glucose	28.95	142	30.36[a]
Sucrose	26.41	141	27.42[ab]
Xylan	26.29	139	27.00[ab]
Rice	25.67	137	25.89[bc]
Cellobiose	24.80	128	23.49[bc]
Xylose	24.42	120	21.69[c]
Corn	23.40	129	22.26[c]
Starch	22.70	130	22.34[c]
Beet	14.96	108	11.94[d]
Cellulose	3.03	108	2.41[e]

1. Base medium (as described at page 35) contains 2 g/L yeast extract and 2 g/L trypticase, and 5 g/L alternative carbon source.
2. Not sharing common letters are significant difference at LSD = 0.05.

Screening Nitrogen Sources

Screening Dual Nitrogen Sources

Two nitrogen sources, yeast extract and trypticase, were utilized in the original medium. In the first experiment yeast extract were replaced with other nitrogen sources, and trypticase was kept as a second nitrogen source. Each nitrogen source which combined with trypticase was utilized by *Thermotoga neapolitana* to produce hydrogen (Table 3.2). However, the hydrogen concentration varied among nitrogen sources. Yeast extract was the best nitrogen source for hydrogen production by *Thermotoga neapolitana*. Soybean meal and canola meal were the second best nitrogen sources, followed by linseed meal, and then fish meal. Cottonseed meal was the least favorable nitrogen source for hydrogen production from the bacterium.

Table 3.2. Hydrogen concentration with different dual nitrogen sources[1].

Nitrogen Source + Trypticase	Percentage of hydrogen concentration (%)	Absolute total Pressure (kPa) at 25°C	Hydrogen concentration (mmol H_2/L medium)[2]
Yeast	29.98	128	28.21[a]
Soybean meal	25.97	119	22.77[b]
Canola meal	22.47	126	20.98[b]
Linseed meal	21.00	113	17.47[c]
Fish meal	18.17	104	13.99[d]
Cottonseeds meal	3.68	110	2.95[e]

1. Base medium (as described at page 35) contains 4 g/L glucose and 2 g/L trypticase, and 2 g/L alternative nitrogen source.
2. Not sharing common letters are significant difference at LSD (α= 0.05).

Table 3.3. Hydrogen Concentration from different single nitrogen resource[1]

Nitrogen Source	Hydrogen Percentage (%)	Absolute total Pressure (kPa)	Hydrogen concentration (mmol H2/L medium)[2]
Yeast	0.0000	117	0.00 [d]
Soybean meal	23.0060	119	20.28 [a]
Canola meal	20.0351	110	16.28 [b]
Linseed meal	8.2877	109	6.59 [c]
Fish meal	0.0000	120	0.00[d]
Cottonseeds meal	0.0000	120	0.00[d]

1. Base medium (as described at page 35) contains 5 g/L glucose, and 4 g/L alternative nitrogen source.
2. Not sharing common letters are significant difference with LSD ($\alpha = 0.05$).

Screening Single Nitrogen Sources

In the second experiment, five alternative nitrogen sources at 4 g/L concentration were used to replace both yeast extract and trypticase. The results are shown in Table 3.3.

The yield of hydrogen production by *Thermotoga neapolitana* was affected by lack of trypticase (Table 3.3). The bacterium grown on soybean meal produced the highest amount of hydrogen among these nitrogen sources, followed by canola meal, and linseed

meal. Without trypticase, no hydrogen was produced by yeast extract, cottonseed meal, or fish meal. These results differ from the report of Schroder et al (1994), who found that yeast extract (0.5 g/L) was sufficient for growth and hydrogen production by *Thermotoga maritama*.

From these two experiments, yeast extract combined with trypticase as nitrogen source worked best for hydrogen produced with *Thermotoga neapolitana.* Cottonseed meal was not a good nitrogen source in this research. Soybean meal and canola meal appeared to be promising alternative nitrogen sources to replace yeast extract. Linseed meal and fish meal at the concentration tested were not as good as soybean meal and canola meal. Trypticase was essential for hydrogen production when yeast extract, cottonseed meal, and fish meal were used as nitrogen sources. Trypticase combined with alternative nitrogen sources efficiently increased the yield of hydrogen. Addition of bio-trypticase helped the growth of *Thermotoga elfii, Thermotoga hypogea*, and *Thermotoga lettingae* (Huber and Hannig, 2006). Trypticase can be supplied as a large quantity of peptides from pancreatic digestion of casein. Peptides or other compounds are contributed to the activity of trypticase for recovery of E. coli stressed by freezing (Moss and Speck, 1966). The activity of trypticase may also contribute to the growth of *Thermotoga neapolitana*, but the mechanism is unknown.

Screening both Carbon Sources and Nitrogen Sources

From above experiments, glucose was the best carbon source among the carbon sources evaluated. Sucrose, xylan, rice flour, and cellobiose were good candidates to replace glucose. Sucrose and rice flour were selected for future research after economic evaluation. The primary focus of this research was to use agricultural feedstocks or agricultural waste to produce hydrogen. Yeast extract with trypticase as dual nitrogen sources was the best combination to provide nitrogen for hydrogen production by *Thermotoga neapolitana.* Soybean meal or canola meal, combined with trypticase, was also a good way to provide nitrogen, instead of using yeast extract. In order to examine the main and interaction effects between these carbon sources and nitrogen sources, a complete randomized experiment design (CRD) was carried out with glucose, sucrose, and rice flour as carbon sources, and yeast extract, soybean meal, and canola meal combined with trypticase as dual nitrogen sources.

Table 3.4. Hydrogen concentration from different combinations of carbon sources and nitrogen sources[1]

carbon	nitrogen	Percentage of hydrogen concentration (%)	Absolute Pressure (kPa)	Hydrogen concentration (mmol /L medium)[2]
Glucose	yeast	28.76	139	29.33[a]
Glucose	soybean	26.36	136	26.35[b]
Glucose	canola	22.72	129	21.64[b]
Sucrose	yeast	26.69	136	26.77[bc]
Sucrose	soybean	26.38	128	24.91[bcd]
Sucrose	canola	24.24	130	23.29[cde]
Rice	yeast	26.61	131	25.66[de]
Rice	soybean	24.66	126	22.92[e]
Rice	canola	20.22	126	18.80[f]

1. Base medium (as described at page 35) contains 5 g/L carbon source, and 2 g/L nitrogen source, and 2 g/L trypticase.
2. Not sharing common letters are significant difference with LSD ($\alpha = 0.05$).

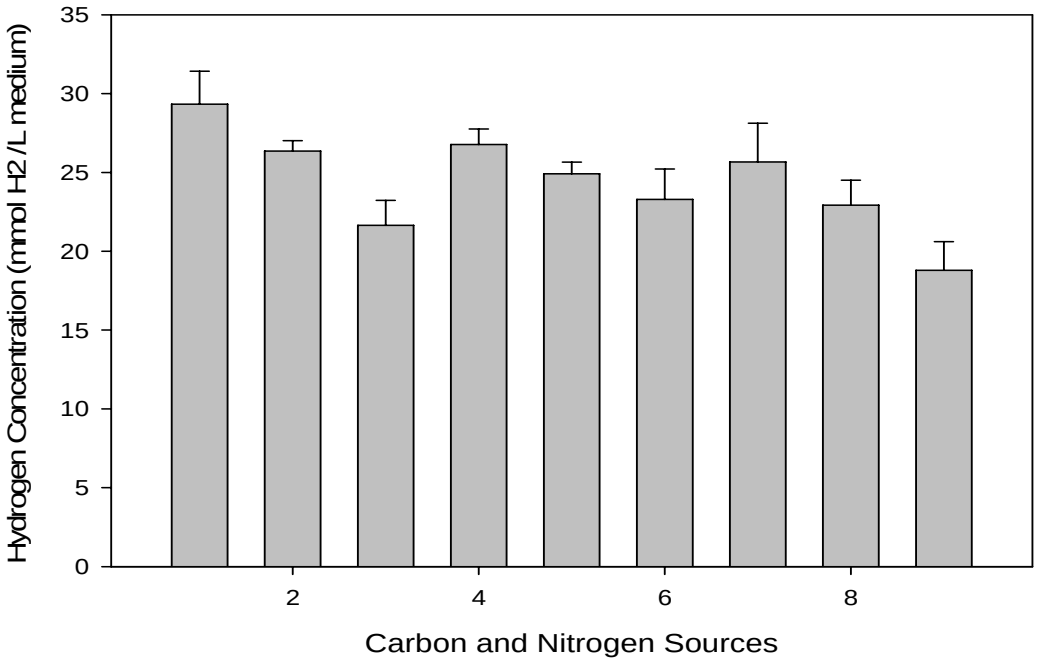

Figure 3.2. The concentration of hydrogen produced by *Thermotoga neapolitana* using different carbon and nitrogen sources combined with trypticase.
1- glucose and yeast extract, 2 – glucose and soybean meal, 3 – glucose and canola meal, 4 – sucrose and yeast extract, 5 – sucrose and soybean meal, 6 – sucrose and canola meal, 7 – rice flour and yeast extract, 8- rice flour and soybean meal, 9- rice flour and canola meal

In Table 3.4 and Figure 3.2, the yield of hydrogen produced by *Thermotoga neapolitana* with glucose as carbon source and yeast extract- trypticase as dual nitrogen sources was the highest in this experiment. The bacterium using sucrose and soybean meal produced the second highest amount of hydrogen. At a significant level of 0.05, the amount of hydrogen produced by the bacterium using glucose and soybean meal, rice flour and yeast extract, sucrose and soybean meal was not significantly different from that using sucrose and yeast extract as nutrients. Canola meal is not a very good nitrogen sources for hydrogen production by *Thermotoga neapolitana*.

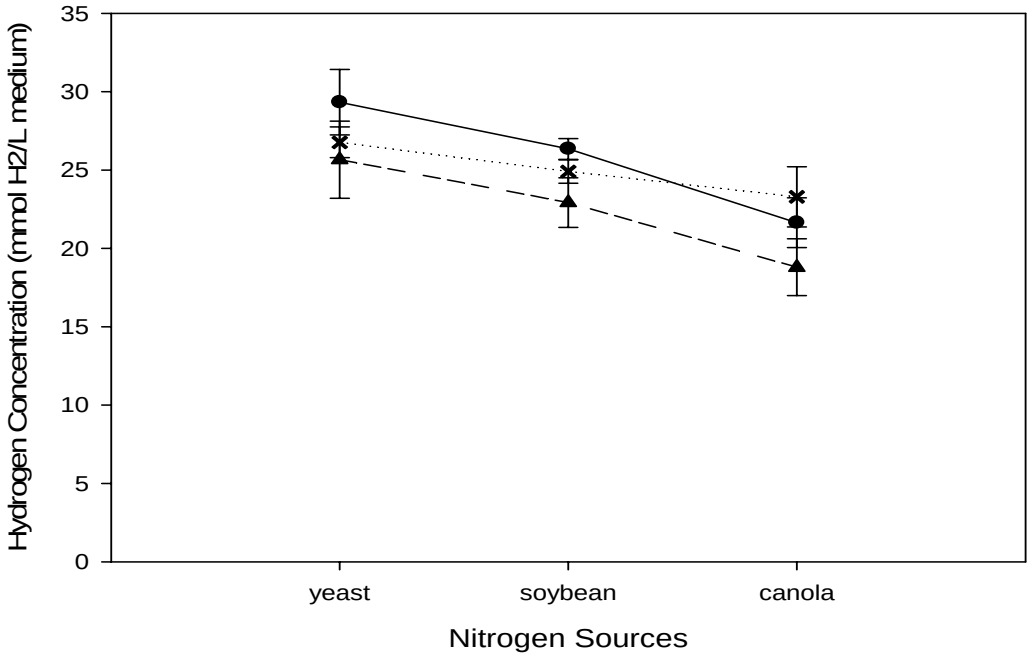

Figure 3.3. Hydrogen concentration changed with carbon sources when different nitrogen sources combined with trypticase were used as dual nitrogen sources.

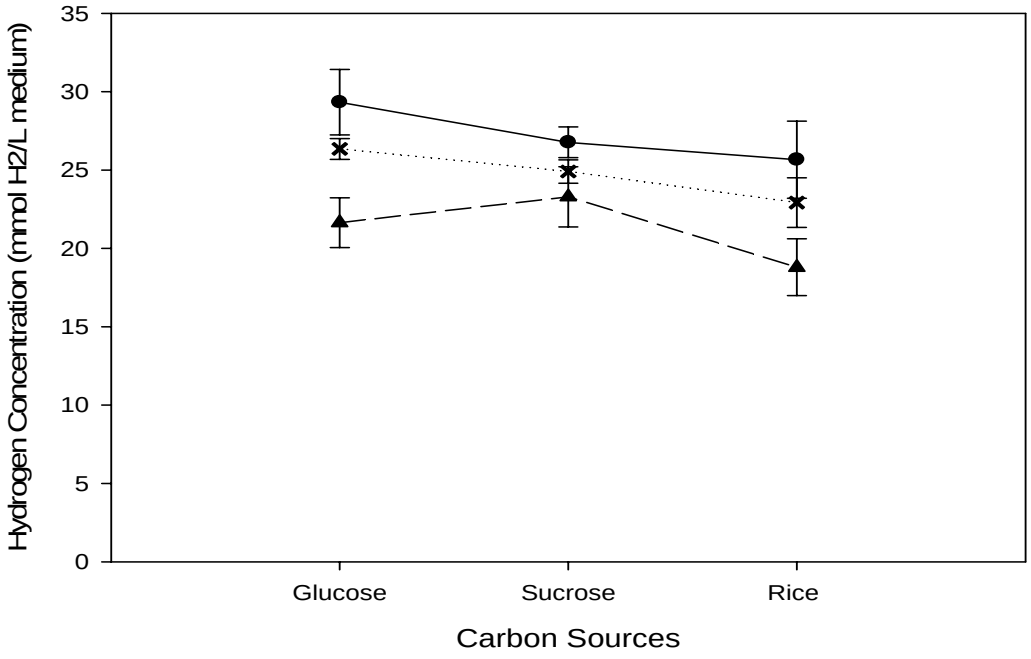

Figure 3.4. Hydrogen concentration changed with nitrogen sources combined with trypticase as dual nitrogen sources when different carbon sources were used.

There were not interactions between carbon sources and nitrogen sources, except for canola meal, as shown in Figure 3.3 and Figure 3.4. If yeast extract or soybean meal was combined with trypticase as dual nitrogen sources, glucose was the best carbon source, and sucrose was second best. But if canola meal combined with trypticase as dual nitrogen sources was used, sucrose became best carbon source.

From these experiments, sucrose and rice flour were promising carbon sources to replace glucose as the carbon source, soybean meal was a promising nitrogen source to replace yeast extract to provide nitrogen for the bacterium. Since yeast extract is more expensive than soybean meal, using the latter has an economic advantage.

CONCLUSION

Four conclusions can be drawn from this study.

Carbon source affects the incubation time of *Thermotoga neapolitana* for hydrogen production. For most monosaccharides or oligosaccharides, 20 hrs of incubation may be a

good choice. But for polysaccharides or agriculture waste, 36 hrs of incubation is necessary. The exact incubation time for each carbon source needs to be determined through experimentation.

Thermotoga neapolitana can utilize different carbon sources to produce hydrogen. Ten carbon sources, glucose, sucrose, xylan, rice flour, cellobiose, corn starch, starch, beet pulp pellet, and cellulose, were screened. Among those carbon sources, sucrose, rice flour, and xylan were almost as good as glucose as a carbon source for the bacterium to produce hydrogen.

Trypticase is essential for hydrogen production when linseed meal, yeast extract, cottonseed meal, and fish meal were used as nitrogen sources. Trypticase combined with alternative nitrogen sources efficiently increased the yields of hydrogen produced by *Thermotoga neapolitana*. Soybean meal and canola meal are promising nitrogen sources to replace yeast extract.

An experiment to evaluate all combination of three selected carbon sources-- glucose, sucrose, and rice flour, with three selected nitrogen sources-- yeast extract, soybean meal, and canola meal, was carried out. From this experiment, there was no interaction between carbon sources and nitrogen sources except for using canola- trypticase as a dual nitrogen

source. Sucrose and rice flour seem to be good carbon sources to replace glucose, and soybean meal appears to be a good nitrogen source to replace yeast extract.

Acknowledgments

This research was supported by funding of Department of Agricultural and Biological Engineering, Clemson University. Yu was partially supported by the Stackhouse fellowship of the College of Agricultural, Forest, and Life Science, Clemson University. We thank Dr. Joe E. Toler for the discussion of data analysis.

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CHAPTER IV

OPTIMIZATION OF FERMENTATION MEDIUM FOR BIOLOGICAL HYDROGEN PRODUCTION BY *THERMOTOGA NEAPOLITANA*

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ABSTRACT

Thermotoga neapolitana can utilize rice flour as a sole carbon source, and soybean meal as one of nitrogen sources to produce hydrogen. In this study, uniform design was used as experimental design to optimize the fermentation medium. The optimized medium was composed of 9 g/L rice flour, 4.5 g/L soybean meal, and 4.5 g/L trypticase. The hydrogen concentration for this optimized medium was 0.07083 ±0.006198 g H_2/L medium or

35.42 ±3.10 mmol H_2/L medium. The increased hydrogen concentration from control medium to optimized medium was 21.6%.

Keywords: biohydrogen; *Thermotoga neapolitana;* medium; optimization; uniform design

INTRODUCTION

Currently, more than 80% of US energy comes from fossil fuels (DOE, 2004). But fossil fuel, as a nonrenewable, limited storage energy resource, will become depleted in the near future. In addition, the combustion of fossil fuels contributes to environmental problems such as global warming and health problem. Hydrogen, as a energy carrier, has been considered as a promising alternately energy since its combustion has no contribution to the environmental pollution and climate change (Levin et al., 2004).

Hydrogen can be produced through physical, chemical, or biological methods. Currently, Steam methane reforming produce 95 percentage of hydrogen in USA (DOE, 2002), but this method is not environmental-friendly due to carbon dioxide as byproduct. Today hydrogen production from biological systems, call biohydrogen (Kovacs et al., 2000),

received a lot attention, because a lot of microbes can utilize agricultural and industrial waste or residues to produce hydrogen through dark-fermentation or light-driven process.

Anaerobic bacteria can produce hydrogen through dark-fermentation (Kotay and Das, 2007; Nath et al., 2006; Shin et al., 2007; Zhang et al., 2006). This process does not need light, instead, it uses carbohydrates as energy and carbon source. *Thermophiles* are also a good choice as hydrogen-producing microorganisms. *Thermotoga* belongs to a very deep branch in the bacteria phylogenetic tree (Jannasch et al., 1988). Most species of *Thermotogales* produce certain amount of hydrogen during their growth (van Niel et al., 2002; Van Ooteghem et al., 2002; Van Ooteghem et al., 2004). Originally isolated around the bay of Naples, Italy (Belkin et al., 1986), *Thermotoga neapolitana* is a Gram-negative, rod-shaped, obligate anaerobic, fermentative extreme thermophile surrounded by the bag-shaped sheath-like outer structure called "toga" (Huber et al., 1986). The optima growth temperature of *Thermotoga neapolitana* is 77 °C (Jannasch et al., 1988).

The composition of the medium plays an important role in affecting the growth of bacteria, the yield of products, and other characteristics. *Thermotoga neapolitana* can utilize different carbon sources and nitrogen sources to produce hydrogen (Huber and Hannig, 2006; Van Ooteghem et al., 2004).

The three common methods of experiment design are factorial design (including fractional factorial design and orthogonal design), D-optimal design and uniform design.

Factorial design generally needs many runs when the number of levels and/or the number of factors become large, so it is only optimal for two to three levels. D-optimal design need a pre-specified regression model and its search for optimal design is sometimes very time consuming and difficult.

Uniform design, based on quasi-Monte Carlo method or number-theoretic method, was proposed in by Kai-Tai Fang and Yuan Wang (Fang, 1980; Wang and Fang, 1981) in the early 1980s. In 1978, a Chinese industrial company proposed an experiment design problem which had six factors with at least 12 levels for each factor, but the total experimental runs could not exceed 50, which was impossible to use fractional factorial experiment design for this problem, so Kai-Tai Fang proposed Uniform Design and solved this problem with only 31 runs. Since then, it has been more and more popular in China, especially in agriculture, textile industry (Leung et al., 1998), science (Tang et al., 2004), chemistry/ chemical engineering (Liang et al., 2001), and fermentation industry (Zhang et al., 2007; Zhang et al., 1993). This experiment design is gradually accepted by researchers around world (Fang et al., 2000; Xu et al., 2006; Zhang et al., 2000). The attractive advantage of UD is using the fewest experimental trials to solve multiple-level experiment problem. UD selects experiment points that are most uniformly distributed in the experimental space according to number-theoretic method (Fang and Wang, 1994). UD method has advantages such as: space filling, robustness, and multiple levels. Samples produced by UD table are highly representative in the studied experimental domain. UD does not have strong assumption for the experimental model, and it is

against the change of model in certain sense. UD can have the largest possible number of levels for each factor in all experimental designs (Liang et al., 2001). Therefore, UD can dramatically decrease the number of experiment trials to achieve satisfactory results with minimal time consumption. But similar to orthogonal design, the specific designed tables and user tables are required for UD. The analysis of variance in common way is difficult to be used for UD, and regression analysis is the main method for data analysis due to a small number of experiments compared with the number of levels of factors in UD experiment (Fang and Wang, 1994). Discrepancy is used to measure the uniformity of experimental points distributed in experimental space. Smallest discrepancy means best uniformity of experimental points distributed in experimental space (Fang, 1994; Fang and Wang, 1994).

From previous experiments, we discovered that rice flour and soybean meal are good alternative agricultural carbon and nitrogen sources for hydrogen production by *Thermotoga neapolitana* (Yu and Drapcho). The objective of this study is to optimize the fermentation medium using UD when rice flour and soybean were the carbon and nitrogen source, respectively.

MATERIALS AND METHODS

Organism

Thermotoga neapolitana was obtained from DSMZ (the German Resource Centre for Biological Material).

Materials

For carbon sources, glucose was purchased from Fisher Scientific, and rice flour was purchased from Labuddle Group Incorporated (Wisconsin).

For nitrogen sources, yeast extract and trypticase were purchased from Fisher Scientific, and soybean meal was obtained from Labuddle Group Incorporated (Wisconsin).

Culture Maintenance

Thermotoga neapolitana was maintained on the medium described by Van Ooteghem (Van Ooteghem et al., 2002) . The composition of medium is as following: 1.0 g NH_4Cl, 0.3g K_2HPO_4, 0.3 g KH_2PO_4, 0.2 g $MgCl_2 \cdot 2H_2O$, 0.1 g $CaCl_2$, 10.0 g NaCl, 0.1 g KCl, 1.0 g Cysteine HCl, 2.0 g yeast extract, 2.0 g Trypticase, 10.0 ml vitamin solution (DSM media 141), 10.0 ml trace element solution (DSM media 141), 0.121 g trizma base, 5 g

glucose, and 1.0 L H_2O. The initial pH of the medium was adjusted to 8.5 with NaOH. The organism was preserved at 4°C.

Cultivation Medium and Condition

The composition of medium used for fermentation was same as the medium described for culture maintenance, except for the carbon and nitrogen sources. Rice flour was used to replace glucose, and soybean meal was used to replace yeast extract. The other components in the medium were the same as the medium for culture maintenance.

The initial pH of medium was adjusted to 8.5 with NaOH addition before sterilization. 500 ml serum bottles containing 200 ml medium were autoclaved in 121 °C for 20 min. Then, the bottles were boiled for 20 min and sparged with N_2 for 1 minute. The culture medium was inoculated with 5 ml inoculum with sterile syringe. The culture was incubated in an orbital shaker bed at 200 rpm and 77 °C. Four replicates were used for each experiment run.

Analysis Methods

Hydrogen gas in the headspace was sampled by collection with 1 ml tuberculin syringe. 0.5 ml of gas were injected into gas chromatograph (SRI 8610C, SRI Instruments,

Torrance, CA90503) with Thermal Conductivity Detector at 100 °C and Silicon Column at 25 °C. The pressure of Argon as carrier gas was 22 psi.

The pressure of the gas in the headspace of each reactor was measured with Traceable manometer (Fisher Scientific) after the reactor had cooled to room temperature. The volume of the headspace was 365 ml. According to the ideal gas law, the hydrogen concentration was calculated through this equation,

$$C_{H_2} = 2g/mol * \frac{P_{H_2} V_1}{RT} * \frac{1}{V_2}.$$

Here, C_{H_2} is the hydrogen gas concentration (g H_2 /L medium), P_{H_2} is the hydrogen partial pressure (atm), V_1 is the volume of headspace (L), T is the temperature (K), R is the universal gas constant (0.0821 L·atm/(mol·K)) , V_2 is the volume of medium (L)..

The experimental results were analyzed with SAS software (SAS, SAS Institute Inc., Cary, NC), 0.15 level of significant was used.

RESULTS AND DISCUSSION

Based on our previous results, rice flour was used as carbon source, combined soybean meal and trypticase were used as nitrogen sources in this study. Three factors were considered in this experiment. If second order and interactions between carbon source

and two nitrogen sources were considered, $U^*_9(9^4)$ table (Table 4.1) was used in this study, and columns 2, 3, and 4 were used to arrange rice flour, soybean meal and trypticase according to $U^*_9(9^4)$ user table (Table 4.2) (Fang, 1994).

Table 4.1. $U^*_9(9^4)$ Uniform Table

Run	X1	X2	X3	X4
1	1	3	7	9
2	2	6	4	8
3	3	9	1	7
4	4	2	8	6
5	5	5	5	5
6	6	8	2	4
7	7	1	9	3
8	8	4	6	2
9	9	7	3	1

Table 4.2. User Table of $U^*_9(9^4)$

Factors	Column	D
2	1 2	0.1574
3	2 3 4	0.1980

The ranges of rice flour, soybean meal, and trypticase used were from 1 g/L to 9 g/L, from 0.5 g/L to 4.5 g/L, and from 0.5 g/L to 4.5 g/L, respectively. The experimental table and results were listed in Table 4.3.

Table 4.3. The experimental arrangement and results

Group	Rice flour(g/L)	Soybean (g/L)	Trypticase (g/L)	Replicate	Hydrogen (g/L medium)
1	3.0	3.5	4.5	1	0.05538
1	3.0	3.5	4.5	2	0.06018
1	3.0	3.5	4.5	3	0.06775
1	3.0	3.5	4.5	4	0.06907
2	6.0	2.0	4.0	1	0.06154
2	6.0	2.0	4.0	2	0.06486
2	6.0	2.0	4.0	3	0.06307
2	6.0	2.0	4.0	4	0.05975
3	9.0	0.5	3.5	1	0.05156
3	9.0	0.5	3.5	2	0.05404
3	9.0	0.5	3.5	3	0.05696
3	9.0	0.5	3.5	4	0.05053
4	2.0	4.0	3.0	1	0.05344
4	2.0	4.0	3.0	2	0.05609
4	2.0	4.0	3.0	3	0.05799
4	2.0	4.0	3.0	4	0.06353
5	5.0	2.5	2.5	1	0.06401
5	5.0	2.5	2.5	2	0.05890
5	5.0	2.5	2.5	3	0.06004
5	5.0	2.5	2.5	4	0.06334
6	8.0	1.0	2.0	1	0.05049
6	8.0	1.0	2.0	2	0.04836
6	8.0	1.0	2.0	3	0.05845
6	8.0	1.0	2.0	4	0.05502
7	1.0	4.5	1.5	1	0.05316
7	1.0	4.5	1.5	2	0.05226
7	1.0	4.5	1.5	3	0.05149
7	1.0	4.5	1.5	4	0.05559
8	4.0	3.0	1.0	1	0.05504
8	4.0	3.0	1.0	2	0.05487
8	4.0	3.0	1.0	3	0.05686
8	4.0	3.0	1.0	4	0.05665
9	7.0	1.5	0.5	1	0.05169
9	7.0	1.5	0.5	2	0.05179
9	7.0	1.5	0.5	3	0.04820
9	7.0	1.5	0.5	4	0.04860

Table 4.4. The results of stepwise regression using SAS program

Stepwise Selection: Step 5

Variable nitrogen12 Removed: R-Square = 0.6553 and C(p) = 4.2161

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	0.00068227	0.00022742	20.28	<.0001
Error	32	0.00035888	0.00001121		
Corrected Total	35	0.00104			

The REG Procedure
Model: MODEL1
Dependent Variable: Hydrogen

Stepwise Selection: Step 5

Variable	Parameter Estimate	Standard Error	Type II SS	F Value	Pr > F
Intercept	0.04532	0.00239	0.00404	359.86	<.0001
nitrogen2	0.00255	0.00043234	0.00038941	34.72	<.0001
carbonnitrogen1	0.00073497	0.00019566	0.00015825	14.11	0.0007
sqcarbon	-0.00005510	0.00002162	0.00007285	6.50	0.0158

Bounds on condition number: 1.0513, 9.308

All variables left in the model are significant at the 0.1500 level.

No other variable met the 0.1500 significance level for entry into the model.

Summary of Stepwise Selection

Step	Variable Entered	Variable Removed	Number Vars In	Partial R-Square	Model R-Square	C(p)	F Value	Pr > F
1	nitrogen12		1	0.3921	0.3921	24.8184	21.93	<.0001
2	carbonnitrogen1		2	0.1399	0.5319	13.7466	9.86	0.0035
3	nitrogen2		3	0.0950	0.6269	6.8698	8.15	0.0075
4	sqcarbon		4	0.0424	0.6694	4.9030	3.98	0.0549
5		nitrogen12	3	0.0140	0.6553	4.2161	1.32	0.2599

Stepwise regression, the most widely used variable screening procedure, established a regression model for this experiment through five steps. $F = 20.18$, and $P < 0.0001$. The regression model was

$$Y = 0.04532 + 0.00255 * \text{Nitrogen2} + 0.00073497 * \text{Carbon} * \text{Nitrogen1} - 0.00005510 * \text{Carbon}^2$$

Where, Y is hydrogen concentration (g H_2/L medium), Carbon is rice flour concentration (g/L), Nitrogen 1 is the concentration of soybean meal (g /L), Nitrogen 2 is the concentration of trypticase (g/L).

Matlab® Optimization toolbox was used to optimize this equation. The maximum hydrogen concentration is 0.0821 g/L, when the concentration of rice flour, soybean meal, and trypticase were 9 g/L, 4.5 g/L, and 4.5 g/L, respectively.

Table 4.5. Hydrogen concentration for control group and optimized group

Group	Replicate	Absolute total Pressure(kPa at 25°C)	Percentage	Hydrogen Partial Pressure(kPa at 25°C)	Average Area	Hydrogen Concentration(g/L)	Average Hydrogen Concentration(g H2/L medium)	Average Hydrogen Concentration(mmol H2/L medium)
Control	1	129	32.27	42	1950.8532	0.06160	0.05825±0.006424	29.13±3.21
	2	121	35.68	43	2157.1512	0.06384		
	3	129	29.71	38	1796.2572	0.05654		
	4	119	27.19	32	1643.8190	0.04780		
	5	127	32.74	42	1979.3597	0.06147		
Optimized	1	140	34.41	48	2080.3458	0.07129	0.07083±0.006198	35.41±3.10
	2	127	34.00	43	2055.5047	0.06377		
	3	148	34.07	51	2060.0098	0.07460		
	4	134	33.13	45	2002.8152	0.06571		
	5	150	35.53	53	2148.2428	0.07878		

- Control group : rice flour 5 g/L, soybean meal 2 g/L, trypticase 2g/L
- Optimized group : rice flour 9 g/L, soybean meal 4.5 g/L, trypticase 4.5 g/L

The concentration of hydrogen produced by *Thermotoga neapolitana* for optimized and control groups in the verification experiments were 0.07083 g H_2/L medium (35.42 mmol H_2/L medium) and 0.05825 g H_2/L medium (29.13 mmol H_2/L medium) , respectively. Therefore, hydrogen concentration increased from the control to optimized group was 21.6%.

Hydrogen concentration by the bacterium with optimized medium increased significantly. To further increase the productivity, two aspects might be considered. One is the pH drop due to organic acid produced during the fermentation process, and the other is hydrogen partial pressure inhibition. The limit of hydrogen partial pressure on the gas phase for *Caldicellulosiruptor saccharolyticus* was 10 to 20 kPa at 70 °C (van Niel et al., 2002). From our previous study, the limited hydrogen partial pressure on the gas phase for *Thermotoga neapolitana* was 38kPa at 77 °C (Yu and Drapcho). In our experiments, the hydrogen partial pressure in the headspace was over the limit of hydrogen partial pressure calculated. So reducing hydrogen partial pressure and adding more buffer need to be considered in the future.

CONCLUSION

Thermotoga neapolitana can utilize rice flour as carbon source, and soybean meal as one of nitrogen sources to produce hydrogen. In this experiment, uniform design was used to optimize the fermentation medium. The optimized medium was 9 g/L rice flour, 4.5 g/L soybean meal, and 4.5 g/L trypticase. The hydrogen concentration for this optimized medium was 0.07083 ±0.006198 g H_2/L medium or 35.42 ±3.10 mmol H_2/L medium . The increased hydrogen concentration from control medium to optimized medium was 21.6%. Future experiment will be conducted to reduce hydrogen partial pressure for the and optimization of fermentation conditions.

Acknowledgments

This research was supported by funding of Department of Agricultural and Biological Engineering, Clemson University. Yu was partially supported by the Stackhouse fellowship of the College of Agricultural, Forest, and Life Science, Clemson University.

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CHAPTER V

KINETICS STUDY OF BIOHYDROGEN PRODUCTION BY THE HYPERTHERMOPHILIC BACTERIUM *THERMOTOGA NEAPOLITANA*

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ABSTRACT

Thermotoga neapolitana ferments glucose as carbon source to produce acetate, carbon dioxide, and hydrogen as major products. The exponential phase of the bacterial growth was between 2 and 10 hrs of incubation time. The maximum cell mass concentration was reached after 10 hrs of incubation. The stationary phase lasted for 10 hrs, and the death phase began at 20 hrs. The pH of broth decreased during the bacterium growth. When pH decreased to 5.0, it may inhibit hydrogen production. The maximum hydrogen partial

pressure in this study was 45 kPa, and hydrogen partial pressure might inhibit the hydrogen production in this batch fermentation due to the limit of hydrogen partial pressure of 38 kPa at 77 °C. The maximum specific growth rate (μ_{max}) of *Thermotoga neapolitana* with glucose as carbon source was 0.94 hr^{-1} at 77 °C. The substrate constant (K_S) was 0.57 g/L, the observed biomass yield from substrate was 0.25 g/g glucose or 44.59 g/mol glucose, the observed hydrogen yield from substrate was 0.028 g/glucose or 2.50 mol H_2 /mol glucose, and the observed hydrogen yield from biomass was 0.114 g/g dry weight. When glucose concentration was 5.0 g/L, the doubling time was 0.84 hr or 49 mins at 77 °C.

Keywords: biohydrogen; *Thermotoga neapolitana* ; batch growth; kinetic study

INTRODUCTION

Most hyperthermophiles, with a optimum growth temperature between 80 °C and 110 °C, belong to the Archaeal domain. A few hyperthermophiles belong to two bacterial orders, the *Thermotogales* and the *Aquificales (Stetter, 1996)*. The order of *Thermotogales* comprises a group of extremely thermophilic, Gram-negative, rod-shaped, nonsporulating bacteria with an outer sheath-like envelope or "toga". *Thermotoga* belongs to a very deep

phylogenetic branch with the bacterial phylogenetic tree (Jannasch et al., 1988). The genera *Thermotoga, Thermosipho, Fervidobacterium, Geotoga, Petrotoga, Marinitoga, Thermopallium* have been described within *Thermotogales (Conners et al., 2006; Huber and Hannig, 2006)*. The genera *Thermotoga* currently include 9 species (*T. maritima, T. neapolitana, T. thermarum, T. elfii, T. subterranea, T. hypogea, T. petrophila, T. naphthophila, T. lettingae*). On the basis of 16S rRNA gene sequence analysis, *Thermotoga marima* and *Thermotoga neapolitana* are closely related (Huber and Hannig, 2006).

Members of the *Thermotogales* are strictly anaerobic, fermentative, hyperthermophilic bacteria. *Thermotoga maritime* was originally isolated from a geothermally heated, shallow marine sediment at Vulcano, Italy (Huber et al., 1986). *Thermotoga neapolitana* was firstly obtained from a submarine hot spring near Lucrino , the bay of Naples, Italy (Belkin et al., 1986; Jannasch et al., 1988). And *Thermotoga elfii* was originally isolated from an Africa oil production well (Ravot et al., 1995). The optimal pH for their growth is in the range of neutral pH. The optimal temperature for *T. maritima* and *T. neapolitana* is around 80 °C (Huber et al., 1986; Jannasch et al., 1988). The optimal temperature for *Thermotoga elfii* is 66 °C (Ravot et al., 1995).

Thermotogales can use simple or complex carbohydrates or complex organic matter as a carbon source or nitrogen source. *Thermotogales* can produce lactate, acetate, ethanol,

L-alanine, carbon dioxide, and hydrogen when the bacteria utilize glucose as carbon source (Huber and Hannig, 2006).

Themotoga maritima completely ferments 1 mol glucose as carbon and energy sources to 2 mol acetate, 2 mol CO_2 and 4 mol H_2 through the "classical" Embden-Meyerhof pathway. The biomass yield from glucose is 45 g cell mass/mol glucose (Schroder et al., 1994). Most of the glucose –fermenting anaerobes produce less than 2 mol of acetate and 4 mol of hydrogen from 1 mol of glucose; because a various of byproducts, such as lactate, ethanol, or butyrate, etc are also produced (Schonheit and Schafer, 1995). *Thermotoga martima* degrades glucose through simultaneous operation of both conventional Embdem-Meyerhof glycolytic pathway (85% relative contribution) and conventional phosphorylated Entner-Doudoroff glycolytic pathway (15% relative contribution) (Selig et al., 1997). Compared to other completely sequenced microbial species, 24% of predicated coding sequences of *Thermotoga maritima*'s genome sequence are homologous to that of archaeal species, which indicate that hyperthermophilic archaea and bacteria exchange gene through lateral gene transfer and *Thermotoga maritima* is the most archaea-like bacteria (Nelson et al., 1999).

Most species of *Thermotogales* produce a certain amount of hydrogen during their cultivation. The maximum rate of hydrogen production by *Thermotoga elfii* was 2.7-4.5 mmol H_2/ (L h) (van Niel et al., 2002). *Themotoga neapolitana* accumulated 25-30% hydrogen during its incubation (Van Ooteghem et al., 2002; Van Ooteghem et al., 2004;

Yu and Drapcho). But hydrogen, as the end-product of fermentation, inhibits the growth of *Themotogales* at high hydrogen partial pressure. For example, hydrogen inhibits the growth of *Thermotoga maritima* on glucose when hydrogen concentration in gas phase is greater than 2% (Schroder et al., 1994). This inhibition can be overcome by adding sulfur or inorganic sulfur-containing compounds ("detoxification"), flushing with nitrogen, co-cultivating with hydrogen-consuming hyperthermophiles (Huber et al., 2000; Huber et al., 1986).

Most researches focus on the biochemistry of *Thermotogales*, few researches focus on the biohydrogen production by these bacteria (van Niel et al., 2002; Van Ooteghem et al., 2002). *Thermotoga neapolitana* have a very strong ability to produce hydrogen and biogases produced by this bacterium have less variety (Van Ooteghem et al., 2002). The object of the present research is studying the kinetic parameters and physiology of this bacterium. Through those researches, we aim to establish an unstructured model for future research.

MATERIALS AND METHODS

Organism

Thermotoga neapolitana was obtained from DSMZ (the German Resource Centre for Biological Material).

Culture Maintenance

Thermotoga neapolitana was maintained on the medium described by Van Ooteghem (Van Ooteghem et al., 2002) . The composition of medium is as follows: 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 $CaCl_2$, 10.0 g NaCl, 0.1 g KCl, 1.0 g Cysteine HCl, 2.0 g yeast extract, 2.0 g Trypticase, 10.0 ml vitamin solution (DSM media 141), 10.0 ml trace element solution (DSM media 141), 0.121 g trizma base, 5 g glucose, and 1.0 L H_2O. The initial pH of the medium was adjusted to 8.5 with NaOH. The organism was preserved at 4 °C.

Cultivation Medium and Condition

The initial pH of medium was adjusted to 8.5 with NaOH addition before sterilization. 500 ml serum bottles containing 200 ml medium were autoclaved in 121 °C for 20 min. The bottles were then boiled for 20 min and sparged with N_2 for 1 minute. The medium

was inoculated with 5 ml volume with sterile syringe. The culture was incubated on an orbital shaker bed at 200 rpm and 77 °C.

Analysis Methods

Hydrogen gas in the headspace was sampled by collection with 1 ml tuberculin syringe. 0.5 ml of gas was injected into gas chromatograph (SRI 8610C, SRI Instruments, Torrance, CA 90503) with Thermal Conductivity Detector at 100 °C and Silicon Column (25 °C). The pressure of Argon as carrier gas was 22 psi.

The pressure of the gas in the headspace of each reactor was measured with Traceable manometer (Fisher Scientific) after the reactor had cooled to room temperature. The volume of the headspace was 365 ml. According to ideal gas law, the hydrogen concentration was calculated through the equation (1)

$$C_{H_2} = \frac{P_{H_2} V_1}{RT} * \frac{1}{V_2} \qquad (1)$$

Here, C_{H_2} is the hydrogen gas concentration (mol H_2 /L medium), P_{H_2} is the hydrogen partial pressure (atm), V_1 is the volume of headspace (L), T is the temperature (K), R is the universal gas constant (0.0821 L·atm/(mol·K)) , V_2 is the volume of medium (L). The Hydrogen partial pressure in 77 °C was also calculated through equation (2).

$$\frac{P_1}{T_1} = \frac{P_2}{T_2} \qquad (2)$$

Where, P1 is the pressure of the specific gas pressure of at temperature T1 (K), P2 is the press of that gas at temperature T2 (K).

The optical density of the culture was measured at 600 nm with spectrophotometer Spectronic $20D^{+}$ (Thermo Fisher Scientific, Inc, Waltham, MA 02454).

The measurement of reduced-sugar is modified methods according to the methods described by Miller (Miller, 1959) .

Dry weight was measured as following: 30ml of cell culture was centrifuged at 25,000 g for 20 min, pellet was washed with 30 basal salt solution, recentrifuged, and heat at 105 °C until reached constant weight.

The experimental results were analyzed with SAS software (SAS, SAS Institute Inc., Cary, NC), 0.05 level of significant was used.

The dissolved concentration of hydrogen was calculated according to Henry's law,

$$k_H = C_a/P_g \qquad (3)$$

Here, k_H is the Henry's law constant, C_a is the concentration of the specific gas in the aqueous phase, P_g is the partial pressure of the specific gas in the gas phase. Because Henry's law constant dependent on temperature, Henry's law constant can be predicted by the following equation.

$$k_H = k_H^{\vartheta} * \exp(\frac{-\Delta_{solv}H}{R}(\frac{1}{T} - \frac{1}{T_{\vartheta}})] \quad \text{(Sander, 1999)} \qquad (4)$$

Where, k_H is the Henry's constant at Temperature T ; k_H^{ϑ} is the Henry's constant at 298 K, equal to $7.2*10^{-9}$ M/Pa for hydrogen; T is the temperature (K); T_{ϑ} is 298 K; and $-\frac{\Delta_{solv}H}{R}$ equal to 500.

A general applicable growth inhibition equation described by Han and Levenspiel can be used to calculate hydrogen's inhibiting effect (Han and Levenspiel, 1988). The equation is

$$r(H_2) = r(H_2)_{MAX} * (1 - C / C_{CRIT})^n * \frac{S}{(S + K_S)} \qquad (5)$$

Equation (5) can be simplified to

$$r(H_2) = r(H_2)_{MAX} * (1 - P / P_{CRIT})^n \qquad (6)$$

Where,

$r(H_2)$ is the hydrogen production rate,

n is the degree of inhibition,

$r(H_2)_{MAX}$ is the maximum hydrogen production rate,

P is hydrogen partial pressure, and P_{CRIT} is the limit of hydrogen partial pressure.

RESULTS AND DISCUSSION

Batch Growth

pH values changed during incubation time (Figure 5.1). pH was 7.74 at the beginning of incubation and then rapidly dropped during the exponential phase, until reaching 5.06 around 20 hrs of incubation.

Thermotogales form acetate, lactate, L-alanine, ethanol, carbon dioxide, and hydrogen gas as the products from fermentation with glucose as growth carbon sources and energy sources (Huber and Hannig, 2006; Ravot et al., 1995; Van Ooteghem et al., 2002; Van Ooteghem et al., 2004). The organic acids cause the pH to drop, and the accumulation of organic acids also inhibited the reaction of evolving hydrogen. Jannasch et al reported that the pH range for growth is between 5.5 and 9 (Jannasch et al., 1988). Van Ooteghem also reported that pH of medium dropped from 7.5 to 4.5 within 20 hrs; but if itaconic acid was added, the pH decreased from 7.5 to 5.9 and hydrogen production reached a maximum plateau value after 20 hrs (Van Ooteghem et al., 2004). The proton concentration affects the yield and rate of hydrogen production, acid pH favors hydrogen production (Mu et al., 2006; Nath and Das, 2004), and the range of pH favorable to hydrogen production is narrow (Lay, 2000). Therefore, optimizing pH or blocking the formation of organic acids becomes necessary.

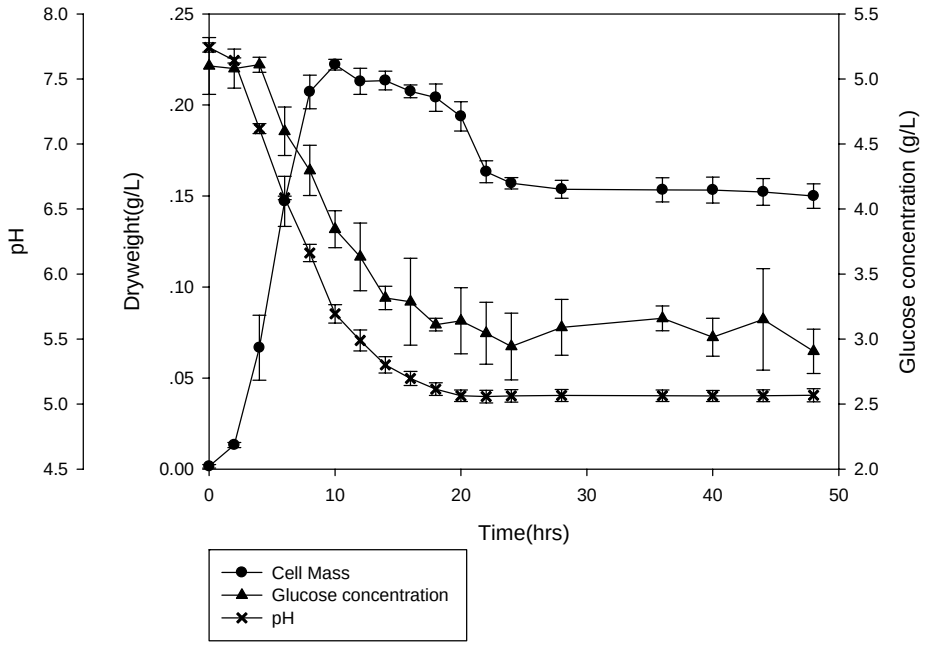

Figure 5.1. The pH value of medium, the dry weight of cell mass and the concentration of reduce sugar with incubation time of *Thermotoga neapolitana* with glucose as the sole carbon source.

In Figure 5.1, cell mass reached the maximum dry weight of 0.22 g/L after 10 hrs cultivation, then cell mass gradually decreased for 10 hrs. The pH was around 5.6 at 10 hrs, which might have inhibited the growth of the bacterium. After 20 hrs incubation, cell mass had a big decrease due to the rate of cell death being greater than the rate of cell growth. After 20 hrs, cell mass did not decline like the death phase of the typical bacterial growth phase. We used optical density to measure the cell mass. Optical density measure the total mass of cells rather than the mass of living cells.

Childers reported the maximum growth was reached in 24 hrs (Childers et al., 1992). While van Ooteghem reported the log phase of this bacterium began at 6 hrs, and maximum cell density was achieved after 14 hrs at 70 °C (Van Ooteghem et al., 2004). From our study, lag phase lasted for 2 hrs, and exponential phase lasted for 8 hrs from 2 to 10 hrs. The bacterium reached maximum cell mass at 10 hrs. After 10 hrs, cell growth entered stationary phase and lasted for 10 hrs. Death phase of cell growth began at 20 hrs. Temperature may be the reason for different results, since this study used optimal temperature of the bacterium (77 °C).

In Figure 5.1, the concentration of glucose kept constant for 4 hrs in the beginning of incubation. After that, the concentration of glucose rapidly decreased from 5.1 to 3.8 g/L in 10 hrs. The concentration of glucose slowly decreased from 3.8 to 3.1 g/L during the incubation period from 10 to 20 hrs. Glucose concentration remained constant after

20 hrs incubation, which again verified the conclusion made from cell mass measurement. This result was also consistent the changing pH over time. The pH drop was an important factor to inhibit the bacterium growth, since carbon substrate was not depleted in this experiment. The pH value kept stable after 20 hrs of incubation due to the rate of cell death being larger than that of cell growth. Alkali substrate released when only a few cells lyses after 20 hrs incubation, which react with organic acids produced by the bacterium, so pH is kept constant.

Total Pressure

Total pressure readings at 25 ºC and hydrogen concentration are shown in Figure 5.2. The total pressures of gas in the headspace of medium measured at room temperature were decreasing during first 6 hrs of incubation. Then, it continuously increased for 6 hrs before becoming constant (Figure 5.2). A slight decrease then increase of total pressure occurred in all four replicate reactors.

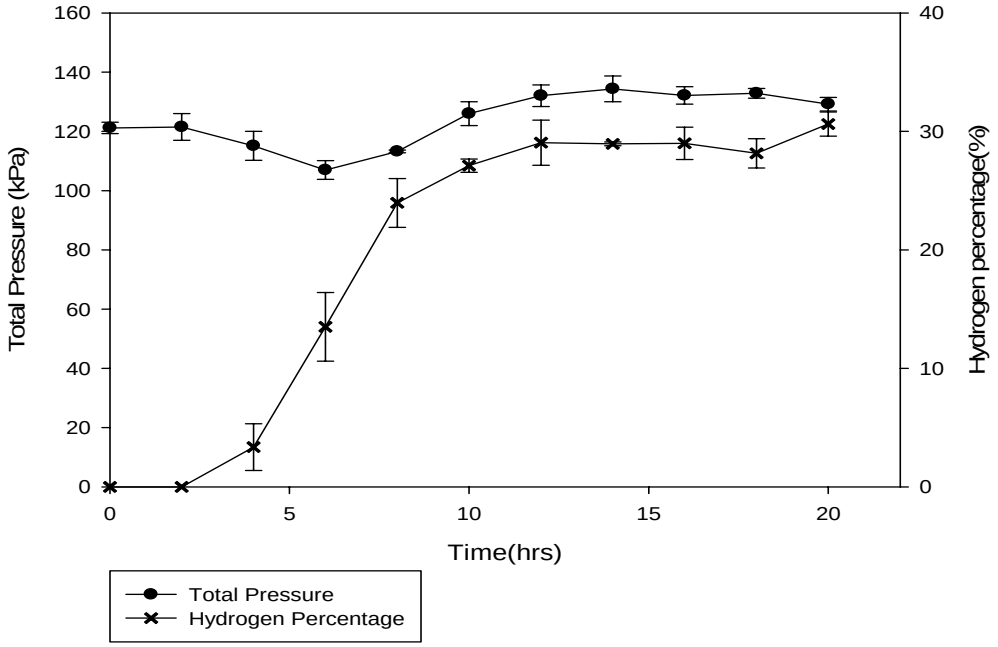

Figure 5.2. Total pressure of gas and percentage of hydrogen concentration produced by *Thermotoga neapolitana* with incubation time.

In Figure 5.3, hydrogen partial pressure produced by *Thermotoga neapolitana* in the headspace was 0 kPa in the beginning of the incubation. It increased to the maximum value of 38 kPa (measured at 25 °C or equivalent to 45 kPa at 77 °C) in 12 hrs incubation and then kept stable afterward, while the cell mass kept constant after 10 hrs incubation (Figure 5.2). The effect of hydrogen inhibition lagged behind cell mass, and the bacterium still released biogas in that time. After 12 hrs, bacterium did not produce hydrogen due to hydrogen inhibition and/or a decrease in pH.

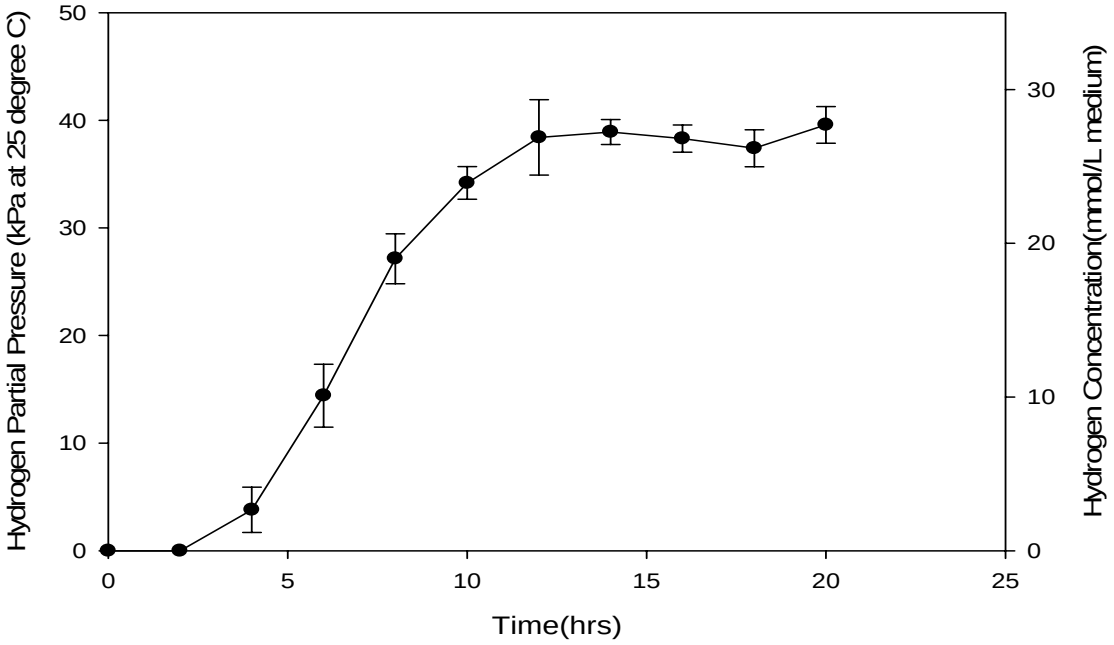

Figure 5.3. The partial pressure of hydrogen (measured at 25 °C) and hydrogen concentration (mmol H_2/L medium) produced by *Thermotoga neapolitana* with glucose as the sole carbon source with incubation time.

Hydrogen partial pressure is an important factor to inhibit hydrogen production (Claassen et al., 1999). The limit of hydrogen partial pressure was 2,000 Pa for *Pyrococcus furiosus* at 98 °C (Parameswaran et al., 1988), and 10,000 – 20,000 Pa for *Caldicellulosiruptor saccharolyticus* at 70 °C (van Niel et al., 2003). From our study, the limit of hydrogen partial pressure for *Thermotoga neapolitana* growth at 77 °C was 38 kPa when Han and Levenspiel equation (equation (6)) as described by van Neil et al was used (van Niel et al., 2003). In Figure 5.3, the hydrogen partial pressure passed over this limit after 10 hrs, and hydrogen partial pressure still increased after 10 hrs. However, the rate of hydrogen pressure production was decreased, and hydrogen partial pressure stopped increasing after 12 hrs. The reason for this is that hydrogen is the end-product of fermentation, and hydrogen partial pressure inhibits the evolving of hydrogen through negative feedback inhibition.

According to Henry's law, using Equation (3), when the Henry's law constant is 6.02 X10^{-9} M/Pa, the concentration of hydrogen dissolved in medium was 229 μM at the limit hydrogen partial pressure at 77 °C, and hydrogen concentration was 271 μM at hydrogen partial pressure of 45 kPa. However, according to Perry's data (Perry and Green, 1999), k_H increase with temperature grater than 70 °C. So, using the k_H of 7.29 X10^{-9} M/Pa, the dissolved hydrogen concentration was 277 μM at the limit hydrogen partial pressure of 38 kPa at 77 °C, and hydrogen concentration was 327 μM at hydrogen partial pressure of 45 kPa at 77 °C.

The trend of the concentration of hydrogen production by *Thermotoga neapolitana* with glucose as carbon source was similar to of the hydrogen partial pressure by the bacterium, as shown in Figures 5.2 and 5.3. The maximum hydrogen concentration by the bacterium was 26.64 mmol/L medium or 29% at 12 hrs incubation, after 12 hrs, the hydrogen concentration stayed stable. The maximum rate of hydrogen production by *Thermotoga neapolitana* was 4.48 mmol H_2/(L·h) at 77 °C between 6 and 8 hrs of incubation.

Cell Mass Changed with Different Concentration of Glucose

In Figure 5.4, the growth rate of *Thermotoga neapolitana* at the exponential phase was different when different concentrations of glucose. A higher concentration of glucose resulted in a faster growth of the bacterium. So the growth rate of the bacterium was

$$r_{15g/L\ glucose} > r_{10g/L\ glucose} > r_{7.5g/L\ glucose} > r_{5g/L\ glucose} > r_{2.5g/L\ glucose}.$$

But all of them had same maximum of cell mass in the end of exponential phase.

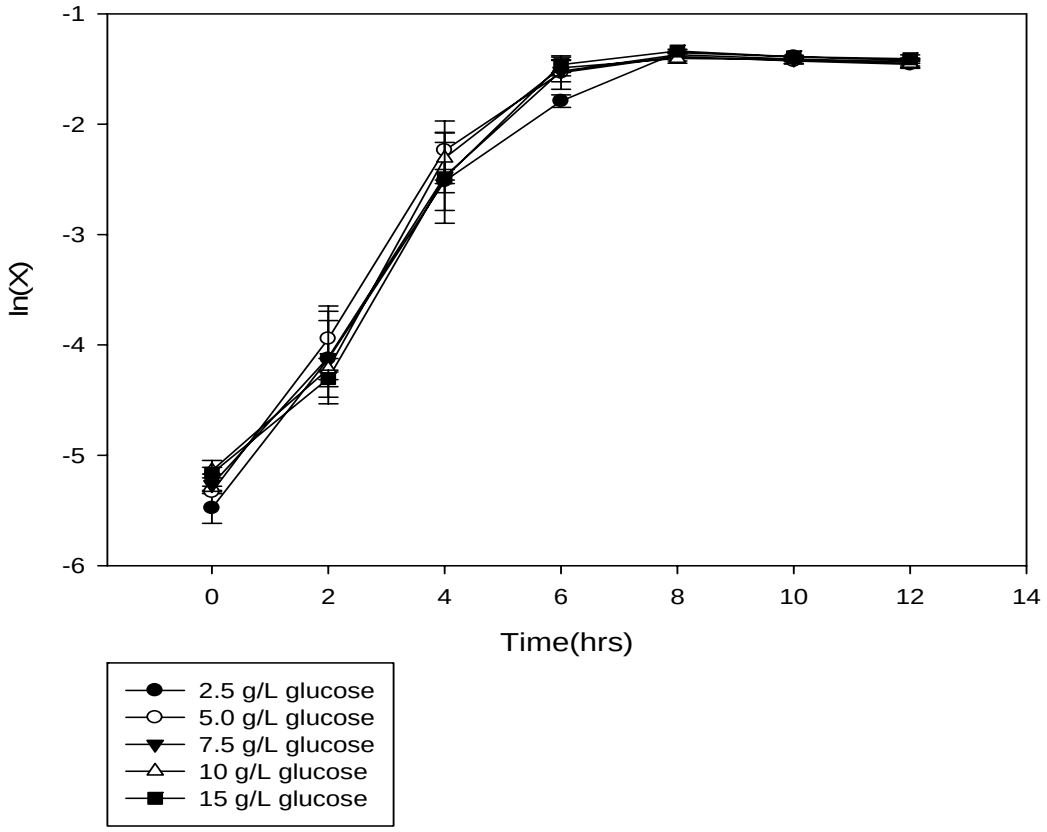

Fig 5.4. The log of dry weight of cell mass of *Thermotoga neapolitana* with incubation time for different concentration of glucose.

Kinetic Parameters

Biomass yield and product yield were calculated as 0.25 g/g glucose or 44.59 g/mol glucose for the observed biomass yield from substrate, 0.028 g H_2/glucose or 2.50 mol H_2 /mol glucose for the observed hydrogen yield from substrate, and 0.114 g H_2/g dry weight for the observed hydrogen yield from biomass.

$$\frac{1}{\mu} = \frac{K_S}{\mu_{\max}} * \frac{1}{S} + \frac{1}{\mu_{\max}} \qquad (7)$$

Here, μ is specific growth rate of bacterium, $\mu_{\max}$ is maximum specific growth rate, K_s is the substrate constant, and S is the concentration of growth-limiting substrate.

The Monod model is a classic model to describe bacterial growth with one substrate as growth-limiting substrate. According to the data we acquired, the parameters were calculated according to the Lineweaver-Burk plot (Equation (7)) from the Monod model with intercept equals to $\frac{1}{\mu_{\max}}$, and slope equals to $\frac{K_S}{\mu_{\max}}$ (Figure 5.5).

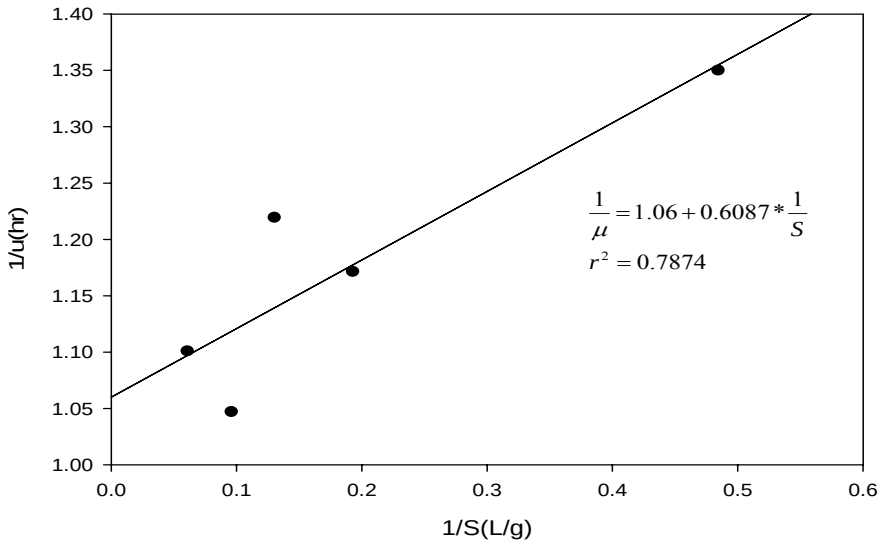

Figure 5.5. Lineweaver-Burk Plot for specific growth rate vs. substrate concentration of *Thermotoga neapolitana*.

The maximum specific growth rate (μ_{max}) of *Thermotoga neapolitana* growth with glucose as carbon source was 0.94 hr^{-1} at 77 °C. The substrate constant (K_S) was 0.57 g/L. When glucose concentration was 5.0 g/L, the doubling time was 0.84 hr or 49 mins.

The observed biomass yield for *Thermotoga maritima* was 45 g /mol glucose when glucose is converted to acetate, CO_2 and H_2 as fermentation products (Schroder et al., 1994). Therefore, the observed biomass yield from glucose in this experiment was parallel to the results for *Thermotoga maritima*. Jannasch et al also reported the doubling time for *Thermotoga neapolitana* was 45 mins in agreement to our data (Jannasch et al., 1988). van Niel et al reported the maximum hydrogen production rate by *Thermotoga elfii* was 2.7-4.5 mmol H_2/(L·h) (van Niel et al., 2002), but van Ooteghem showed the

maximum hydrogen production rate by *Thermotoga neapolitana* at 70 °C was 0.597 mmol H_2/(L·h), so 4.48 mmol H_2/(L·h) of the maximum rate of hydrogen production by *Thermotoga neapolitana* matched the result from van Niel et al.

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2\ CH_3COOH + 4H_2 + 2CO_2 \quad (8)$$

The observed hydrogen yield from substrate was 2.50 mol H_2 /mol glucose in this study. But the theoretical maximum yield for dark fermentation is 4 mol H2/mol glucose from the equation (8) (Thauer, 1976). The equations (8) used so far to describe biohydrogen production through dark-fermentation did not include cell mass, and hydrogen is a growth-associated product. Therefore, it is important to include cell mass in the equation to precisely describe the process. Schroder et al also reported 1 mol of glucose consumed by *Thermotoga maritima* form 2 mol acetate, 2 mol CO_2, and 4 mol H_2 (Schroder et al., 1994). van Ooteghem et al also reported the ratio of H_2/CO_2 produced by *Thermotoga neapolitana* is 2:1 (Van Ooteghem et al., 2004). The elemental composition of the cell mass of *Themotoga neapolitana* was assumed to be $CH_{1.8}O_{0.5}N_{0.2}$ (molecular weight is 24.6 g/mol) (Doran, 1995). When those assumptions were combined with the kinetic coefficients from this study, a revised equation was used as follows:

$$C_6H_{12}O_6 + 0.5H_2O + 0.34NH_3 \rightarrow 1.72CH_{1.8}O_{0.5}N_{0.2} + 1.25CH_3COOH + 2.50H_2 + 1.25CO_2 \quad (9)$$

According to this new equation, the calculations of elemental mass weight for both side of equation for carbon, hydrogen, and oxygen were closely matched (9% different for carbon, 4% different for hydrogen, and 10% different for oxygen).

CONCLUSION

Thermotoga neapolitana, a hypertheromophilic bacterium, ferments glucose to produce acetate, carbon dioxide, and hydrogen as major fermentation products. The growth curve of this bacterium was typical bacterial growth curve. When glucose was the carbon source and energy source, the exponential phase was between 2 and 10 hrs of incubation, the maximum cell mass concentration was reached after 10 hrs of incubation, the stationary phase lasted for 10 hrs, and the death phase began at 20 hrs.

pH and hydrogen partial pressure are two important factors that affect the hydrogen production and bacterial growth. The pH of broth was decreased due to acetate and/or other organic acid accumulated as end-products of the bacterium growth. When pH decreased to 5.0, it appears to inhibit hydrogen production. The maximum hydrogen partial pressure in this study was 45 kPa at 77 °C, which is larger than the limit of 38 kPa according to our estimation based on the growth inhibition equation. Therefore, hydrogen

partial pressure may inhibit the hydrogen production in this batch fermentation. More experiments will be required in the future to study hydrogen production inhibitors.

The maximum specific growth rate (μ_{max}) of *Thermotoga neapolitana* growth with glucose as carbon source was 0.94 hr^{-1} at 77 °C. The substrate constant (K_S) was 0.57 g/L, the observed biomass yield from substrate was 0.25 g/g glucose or 44.59 g/mol glucose, the observed hydrogen yield from substrate was 0.028 g H_2/glucose or 2.50 mol H_2 /mol glucose, and the observed hydrogen yield from biomass was 0.114 g/g dry weight. When glucose concentration was 5.0 g/L, the doubling time was 0.84 hr or 49 mins at 77 °C.

The equation,

$C_6H_{12}O_6 + 0.5H_2O + 0.34NH3 \rightarrow 1.72CH_{1.8}O_{0.5}N_{0.2} + 1.25CH_3COOH + 2.50H_2+1.25CO_2$, may be use to described the hydrogen production by *Thermotoga neapolitana* with glucose as the growth-limiting substrate.

Acknowledgments

This research was supported by funding of Department of Agricultural and Biological Engineering, Clemson University. Yu was partially supported by the Stackhouse fellowship of the College of Agricultural, Forest, and Life Science, Clemson University.

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CHAPTER VI

CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS

The ultimate goal of this study was to produce hydrogen by Thermotoga neapolitana utilizing agricultural feedstocks. To reach this goal, difference carbon sources and nitrogen sources need to be screened, and physiology and fermentation characteristics of this hypertheromophilic bacterium should be known.

Carbon sources affected the incubation time of Thermotoga neapolitana to produce hydrogen. For most monosaccharides or oligosaccharides, 20 hrs incubation could be selected as good choice. But for polysaccharides or agriculture waste, 36 hrs incubation was necessary. The exactly incubation time for each carbon sources needs to be decided through experiments.

Thermotoga neapolitana can utilize different carbon sources to produce hydrogen. Ten carbon sources, glucose, sucrose, xylan, rice flour, cellobiose, corn starch, starch, beet pulp pellet, and cellulose, were screened. Among those carbon sources, sucrose, rice flour, and xylan were nearly as good as glucose as a carbon source for the bacterium to produce hydrogen.

Trypticase was a vital part of the nitrogen source. Trypticase combined with alternative nitrogen sources can efficiently increases the yield of hydrogen produced by *Thermotoga neapolitana*. Without the combination of nitrogen sources and trypticase, bacterium might not produce high yield of hydrogen. Soybean meal, and canola meal were promising nitrogen sources to replace yeast extract.

A completely randomized design experiment to screen three selected carbon sources, glucose, sucrose, and rice flour, with three selected nitrogen sources, yeast extract, soybean meal, and canola meals, were completed. From this experiment, sucrose and rice flour were promising carbon sources to replace glucose as carbon source, soybean meals was promising nitrogen source to replace yeast extract as nitrogen source.

Uniform design was used to attempt optimization of the fermentation medium with rice flour as carbon source, and soybean meal and trypticase as dual nitrogen sources. The optimized medium was 9 g/L rice flour, 4.5 g/L soybean meal, and 4.5 g/L trypticase. The hydrogen concentration for this optimized medium was 0.07083 ±0.006198 g/L. The increased hydrogen concentration from control medium to optimized medium was 21.6%.

The growth curve of *Thermotoga neapolitana* was a typical bacterial growth curve. When glucose was carbon source and energy source, the exponential phase was between 2 and

10 hrs of incubation, the maximum cell mass concentration was reached after 10 hrs of incubation, the stationary phase lasted for 10 hrs, and the death phase began at 20 hrs.

pH and hydrogen partial pressure are two important factors that affect the hydrogen production and bacterial growth. The pH of broth was decreased due to acetate and/or other organic acid accumulated as end-products of the bacterium growth. When pH decreased to 5.0, it appears to inhibit hydrogen production. The maximum hydrogen partial pressure in this study was 45 kPa, which is larger than the limit of 38 kPa according to our estimation based on growth inhibition equation. Therefore, hydrogen partial pressure may inhibit the hydrogen production in this batch fermentation. More experiments will be required in the future to study hydrogen production inhibitors.

The maximum specific growth rate (μ_{max}) of *Thermotoga neapolitana* growth with glucose as carbon source was 0.94 hr^{-1}. The substrate constant (K_S) was 0.57 g/L, the observed biomass yield from substrate was 0.25 g/g glucose or 44.59 g/mol glucose, the observed hydrogen yield from substrate was 0.028 g H_2/glucose or 2.50 mol H_2 /mol glucose, and the observed hydrogen yield from biomass was 0.114 g/g dry weight. When glucose concentration was 5.0 g/L, the doubling time was 0.84 hr or 49 mins. The equation, $C_6H_{12}O_6 + 0.5H_2O + 0.34NH3 \rightarrow 1.72CH_{1.8}O_{0.5}N_{0.2} + 1.25CH_3COOH + 2.50H_2+1.25CO_2$, may be use to described the hydrogen production by *Thermotoga neapolitana* with glucose as the growth-limiting substrate.

RECOMMENDATIONS

Thermotoga neapolitana has very strong ability to utilize different carbon or nitrogen sources, so a wide range of agricultural feedstocks, agricultural waste, municipal waste, and manufacture waste need to be considered. Since *Thermotoga neapolitana* has low ability to utilize cellulose material from this study and other studies. Vrije et al reported *Thermotoga elfii* utilizes pretreated *Miscanthus* and produces a significant amount of hydrogen (Vrijie et al., 2002), so proper pretreatment will be necessary for cellulose material used by *Thermotoga neapolitana*.

pH is an important inhibitor for hydrogen production by Thermotoga neapolitana. Adding chemicals or mixed culture with acid-consumed bacteria may be the way to solve the problem of pH drop. Hydrogen partial pressure is another important inhibitor for hydrogen production by Thermotoga neapolitana. So reducing hydrogen partial pressure in the headspace needs be more considered than other.

Continuous cultivation of Thermotoga neapolitana need be studied in the future, because biohydrogen need be continuously produced by this bacterium during whole incubation. There are several problems need be considered during this operation, such as, insulation, high-temperature cultivation, anaerobic cultivation, pH drop, hydrogen partial pressure, etc.

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APPENDIX A

THE COMPOSITION OF CARBON SOURCES

Rice Flour

Protein	6.0-9.0%
Fat	0.4-1.0%
Crude Fiber	0.3-1.0%
Ash	0.6-0.8%
Moisture	8.5-13.0%

http://www.labudde.com/LaBudde_Group_Ingredients.htm

Beet Pulp Pellets

Crude Protein	7.0%
Crude Fat	0.5-0.6%
Crude Fiber	15.0- 20.0%
Ash	4.0-5.0%

Moisture	8.0-12.0%

http://www.labudde.com/LaBudde_Group_Ingredients.htm

Corn Starch

Carbohydrate	87.5-89.5%
Moisture	10.0-13.0%
Protein	<0.1%
Fat	0.2%
Ash	0.2%
Crude Fiber	0.1%

http://www.labudde.com/LaBudde_Group_Ingredients.htm

Xylan (Oats Spelts)

Xylose	70.2%
Glucose	9.1%
Arabinose	6.2%

From Tokyo Kasei Company.

APPENDIX B

THE COMPOSITIONS OF NITROGEN SOURCES

Yeast Extract

Typical Analysis

Total Solids	95%
Organic Solids	84-85%
Total Nitrogen	10-11%
Protein (N x 6.25)	62-68%
Ash	11-12%
Chloride (as NaCl)	2-3%
pH (aqueous soln.)	5-5.5%

Typical Amino Acid Profile (as % of Protein)

Arginine	4.1%	Cystine	1.4%
Histidine	1.9%	Methionine	1.1%
Lysine	7.2%	Threonine	3.9%
Tyrosine	1.9%	Leucine	5.7%
Tryptophan	0.7%	Isoleucine	4.4%

Phenylalanine	3.5%	Valine	5.2%

http://www.mpbio.com/product_info.php?cPath=491_1_12&products_id=194027&depth=nested&keywords=yeast%20extract

Trypticase™ peptone

Nitrogen Content/Physical Characteristics

Total Nitrogen (TN)	14.2 %
Amino Nitrogen (AN)	5.2 %
AN/TN	0.37 %
Ash	5.7 %
Loss on Drying	4.0 %
NaCl	0.1 %
pH (2% Solution)	7.2

Elemental Analysis

Calcium	295 (µg/g)
Magnesium	110 (µg/g)
Potassium	588 (µg/g)
Sodium	26600 (µg/g)

Chloride	0.09 %
Sulfate	0.18 %
Phosphate	2.54 %

Amino Acid Analysis

	Free	Total		Free	Total
Arginine	2.3 %	4.8 %	Cystine	0.3 %	*
Histidine	0.6 %	4.8 %	Methionine	1.1 %	2.5 %
Lysine	3.3 %	10.6 %	Threonine	0.6 %	2.4 %
Tyrosine	0.4 %	1.6 %	Leucine	5.3 %	10.4 %
Tryptophan	0.8 %	*	Isoleucine	1.1 %	8.3 %
Phenylalanine	2.7 %	7.1 %	Valine	1.5 %	9.1 %
Aspartic Acid	0.2 %	7.7 %	Glutamic Acid	1.1 %	13.2 %
Proline	0.2 %	10.9 %	Glycine	0.1 %	6.3 %
Serine	0.8 %	2.5 %	Alanine	0.9 %	5.7 %

http://www.bd.com/ds/technicalCenter/typicalAnalysis/typ-trypticase_peptone.pdf

Soybean Meal

Dry matter	89 %
Crude Protein	48.0 %
Fat	1.0 %
Crude Fiber	3.0 %
Neutral Detergent Fiber	7.1 %
Acid Detergent Fiber	5.3 %
Calcium	0.2 %
Phosphorus	0.65 %
Total Digestible Nutrients	78.0 %

http://www.labudde.com/LaBudde_Group_Ingredients.htm

Canola Meal

Typical Analysis

Dry matter	91.5 %
Crude Protein	36.0 %
Ether Extract	3.5 %
Crude Fiber	11.7 %

Ash	6.8 %

Typical Amino Acid Analysis

Crude Protein	36.0 %
Methionine	0.74 %
Cystine	0.91 %
TSAA	1.65 %
Lysine	2.10 %
Tryptophan	0.46 %
Threonine	1.61 %
Arginine	2.12 %
Isoleucine	1.38 %

http://www.labudde.com/LaBudde_Group_Ingredients.htm

Linseed meal

Dry matter	94 %
Crude Protein	41.0 %
Fat	4.5 %
Crude Fiber	12.5 %

Neutral Detergent Fiber	26.3 %
Acid Detergent Fiber	18.8 %
Calcium	0.15 %
Phosphorus	1.10 %
Total Digestible Nutrients	70.0 %

http://www.labudde.com/LaBudde_Group_Ingredients.htm

Fish meal

Dry matter	92 %
Crude Protein	62.0 %
Fat	9.8 %
Crude Fiber	1.0 %
Neutral Detergent Fiber	0.0 %
Acid Detergent Fiber	0.0 %
Calcium	5.0 %
Phosphorus	3.0 %
Total Digestible Nutrients	71.0 %

http://www.labudde.com/LaBudde_Group_Ingredients.htm

Cottonseed Meal

Dry matter	94 %
Crude Protein	41.0 %
Fat	4.5 %
Crude Fiber	12.5 %
Neutral Detergent Fiber	26.3 %
Acid Detergent Fiber	18.8 %
Calcium	0.15 %
Phosphorus	1.1 %
Total Digestible Nutrients	72.0 %

http://www.labudde.com/LaBudde_Group_Ingredients.htm