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BIOHYDROGEN PRODUCTION FROM CULL PEACH MEDIUM BY HYPERTHERMOPHILIC BACTERIUM *Thermotoga neapolitana*

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BIOHYDROGEN PRODUCTION FROM CULL PEACH MEDIUM BY HYPERTHERMOPHILIC BACTERIUM *Thermotoga neapolitana*

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ABSTRACT

Thermotoga neapolitana is a marine hyperthermophilic bacterium that ferments various sugars to hydrogen and acetate. In this study, cull peaches were used as carbon source in a defined medium for biohydrogen production and produced 18 % to 25% hydrogen in the headspace. The hydrogen production varied from 6.4-7.7 mmol H_2/g peach (dry weight). The hydrogen concentration did not increase after 20 hours of incubation. The final pH decreased to 4.9 after 20 hours. Unautoclaved peach medium can be used for hydrogen production. The hydrogen production did not increase with the increase in peach concentration from 50 g/L to 100 g/L (wet weight). Yeast extract, as nitrogen source, was found important for hydrogen production. Soybean meal was found to be a good nitrogen source with cull peaches as carbon source for biohydrogen production.

The pH had profound effect on biohydrogen production by *Thermotoga neapolitana*. The optimum initial pH for hydrogen production using peach medium as was 8.0. The mass of hydrogen produced increased when pH was adjusted after 12 hours of incubation. The hydrogen production increased from 7.07 mmol H_2/g peach (dry weight) to 8.73 mmol H_2/g peach (dry weight), when pH was adjusted. The incubation time also increased from 20 hours to 40 hours for complete fermentation on pH adjusted medium. The amount of soluble COD utilized increases from 3.81 to 4.95 g COD/L, when pH was adjusted. The number of moles of carbon dioxide produced was same as that of hydrogen. The maximum rate of production of hydrogen observed in this study was 3.31 mmol H_2/L.h. The hydrogen production was same when pH was adjusted to two different pH of 6.5 and

7.5 respectively after 12 hours. The amount of hydrogen produced decreased when substrate concentration was increased from 50 g/L to 100 g/L (wet weight0 and pH was adjusted after 12 hours of incubation.

DEDICATION

I dedicated this work to my parents, my brother Arpit and to my dear sisters Abhilasha and Megha.

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I would like to thank my advisor, Dr. Caye M. Drapcho, for her assistance and support throughout this study. I am grateful to other committee members: Dr. Terry H. Walker and Dr. J. Michael Henson for their guidance in the completion of this dissertation.

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CHAPTER ONE

INTRODUCTION

1.1. Introduction

The limited number of known fossil fuel deposits and the threat to environment due to the use of these fossil fuels has made it essential to look for alternative and renewable sources of fuels. Hydrogen is one of the renewable energy sources. High conversion efficiency, recyclability and non polluting nature justify its prospects as the future fuel. Hydrogen has the energy density of 122 kJ/g which is 2.75 times greater than hydrocarbon fuels (Han and Shin, 2004). Hydrogen gas is a potential sustainable environmentally friendly fuel because it combusts to form only water and energy (Das, 2001; Lee, 1996; Sperling, 2004).

$$H_2 + 1/2O_2 \rightarrow H_2O + 282.119kJ \qquad (1)$$

As shown in (1), hydrogen does not contribute to the climate change and global warming (Levin et al., 2004).

The amount of hydrogen reported to be traded worldwide is 50 billion kilograms annually with a growth rate of nearly 10% per year for the time being (Winter, 2005). The amount of hydrogen produced in US is 9 billion kilograms annually. The hydrogen is mainly used for feedstock and intermediate chemical industries, such as for syntheses of ammonia or alcohols. The amount of hydrogen used as an energy carrier is very little. DOE estimated that 40 billion kilograms of hydrogen will be required to fuel about 100 million fuel-cell

powered cars, or to provide electricity to about 25 million homes if US would shift to a hydrogen-economy (DOE, 2002; DOE, 2004). Hydrogen gas can be produced by reformation of hydrogen rich compounds, electrolysis of water and gasification of coal. But these processes are energy intensive, costly and environmentally problematic. One of the most energy efficient and commercialized technologies for producing hydrogen available today is steam methane reforming and it produces about 95% of the hydrogen produced in United States (DOE, 2002). But this method catalyzes the reaction of steam with natural gas to produce hydrogen and carbon dioxide which is a greenhouse gas.

Hydrogen production using biological systems has gained much attention in the last decade. Biological production of hydrogen can be done by photo-biologically or through fermentation (Benneman, 2000; Hallenbeck, Benneman, 2002). Photo-biological hydrogen production utilizes solar energy, but the light conversion efficiencies and rate of hydrogen production remains low (Levin et al., 2004). Fermentation of organic wastes combines hydrogen production with waste treatment making it the most promising method of hydrogen production (Benemann, 1996). Hydrogen production by fermentation can use a large variety of renewable biomass including agriculture waste (Logan et al., 2002: Hussy et al., 2005), municipal waste (Wang et al., 2003), food processing waste (Van Ginkel et al., 2005). The rate of hydrogen production through fermentation usually is larger than 1 mmol/(L·h) whereas, the rate of hydrogen production through photolysis system is less than 1 mmol (L·h) (Levin, 2004). Hydrogen can be produced by wide variety of bacteria including mesophiles (Kotay and Das, 2007;

Shin et al., 2007), thermophiles, extreme thermophiles and hyperthermophiles (van Niel et al., 2003). These chemoorganotrophic microorganisms use organic substrates as energy source and hydrogen ion as electron acceptor to produce hydrogen. Hydrogen production by fermentation also depends on end products and metabolic pathways. The theoretical maximum yield for fermentation is 4 mol H_2/mol glucose when acetic acid is the byproduct (Thauer, 1976). 4 moles of hydrogen contains 33% of the combustion energy of glucose. The theoretical yield for fermentation is 2 mol H_2/mol glucose when butyric acid is the byproduct (Nandi and Sengupta, 1998). 2 moles of hydrogen contains 16.5% of the combustion energy of glucose. If ethanol and acetic acid are the end-products, then 2 mol H_2/mol glucose are produced (Hwang et al., 2004). If propionic acid is the end-product of fermentation, no hydrogen is produced (Ren et al., 2006). 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*).* Hyperthermophiles are a promising group for hydrogen production because they have higher hydrogen conversion efficiency and hydrogen production rates. Moreover, these microorganisms grow at the temperature of around 80°C. Almost no microorganism can grow at these temperatures and hence sterilization may be omitted thereby saving a lot of energy.

The genus *Thermotoga* lies in order Thermatogales under family Thermotogaceae. Most species of Thermotogales produce certain amount of hydrogen during their growth (Van Ooteghem et al., 2002; Van Ooteghem et al., 2004; Vrijie et al., 2002). *Thermotoga*

neapolitana was isolated around the Bay of Naples, Italy (Belkin et al., 1986). It 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). Yu (2008) reported that *T. neapolitana* accumulated 28%-30% hydrogen in the headspace, using glucose as carbon source after 20 hours of incubation. The end products of hyperthermophilic fermentation also have less variety. *Themotoga maritima* completely ferments 1 mole glucose as carbon and energy sources to 2 mole acetate, 2 mole CO_2 and 4 mole H_2 through Embden-Meyerhof pathway (Schroder et al., 1994; Schonheit and Schafer, 1995). *T.maritima* and *T.neapolitana* are closely related, based on 16s rRNA analysis (Huber and Hannig, 2006). Most of the glucose- fermenting anaerobes produce less than 2 mole of acetate and 4 mole of hydrogen from 1 mole of glucose because various byproducts such as lactate, ethanol, butyrate, propionate are also produced (Schonheit and Schafer, 1995).

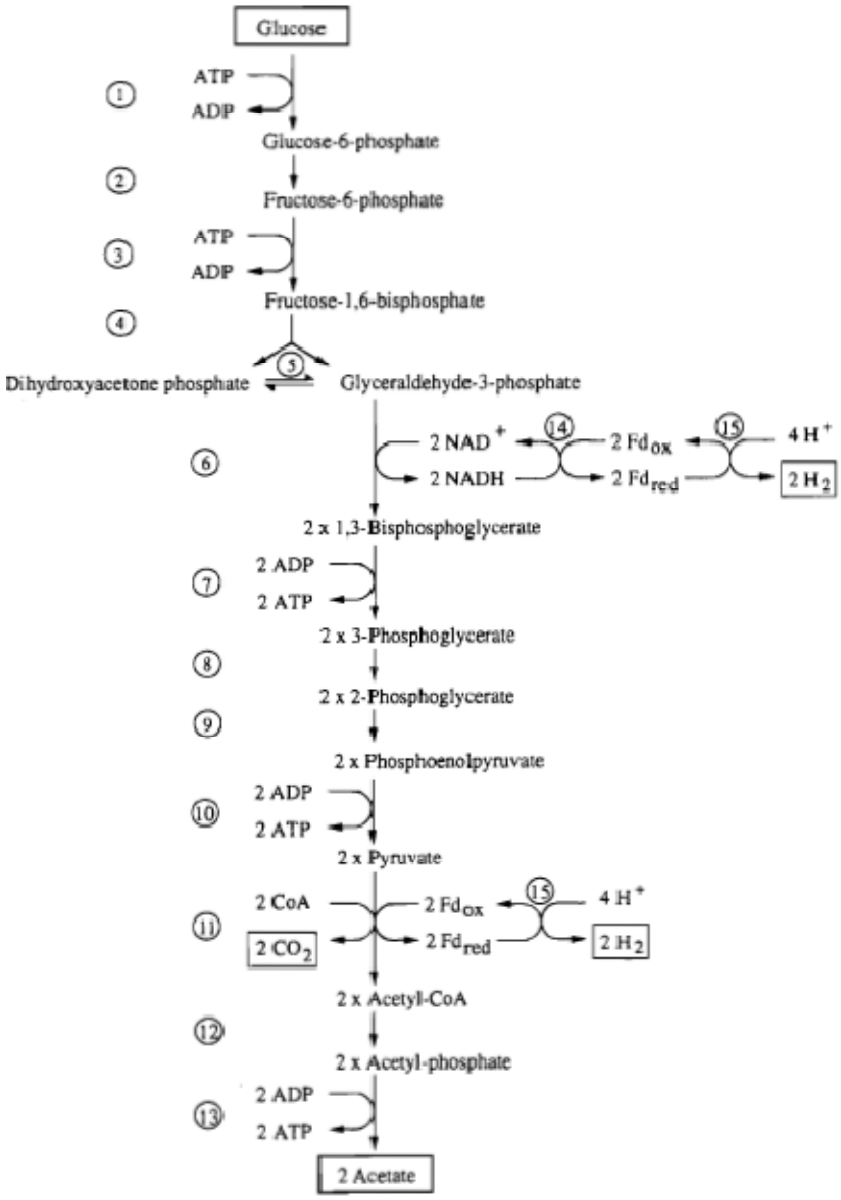

Fig 1.1.1. Metabolic pathway of glucose being fermented to 2 acetate, 2 CO_2 and 4 H_2 by *Thermotoga maritima (Schroder et al., 1994).*

Thermotoga neapolitana can use simple or complex carbohydrates or complex organic matter as carbon source or nitrogen source (Huber and Hannig, 2006: Zhu, 2007; Yu,

2008; Nguyen et al., 2008). Starch-rich and lignocellulosic wastes have been found to be good sources for hydrogen production. Yokoi used dried sweet potato starch residue for hydrogen production by the mixed culture of *C. butyricum* and *E. aerogenes* (Winter, 2005**)**. Ginkel studied hydrogen production from confectioners, apple and potato processor industrial effluents and also from domestic wastewater. The highest production yield was obtained as 0.21 L H_2/g COD from potato processing waste water (Winter, 2005). The maximum specific hydrogen production rate was 237mLH_2/g VSS d when 24 g/L edible corn starch was used as the substrate by *C. pasteurianum*. Molasses is another carbohydrate-rich substrate with sucrose as the main carbohydrate (Winter, 2005). The maximum and available rate of hydrogen production in continuous operation with *E. aerogenes* strain E.82005 was 36 and 20 mmol H_2/L h respectively. The available yield was 1.5 mol H_2/mol sugar expressed in terms of sucrose (Winter, 2005). Though, lignocellulosic biomass provides large pool of sugars which can be used, but the breakdown of cellulose into relatively simple sugars, including monosaccharides and disaccharides remains the bottleneck in its use. Vrije et al. (2002) reported *Thermotoga elfii* utilizes pretreated *Miscanthus* and produces a significant amount of hydrogen. Yu (2008) reported that *Thermotoga neapolitana* can utilize cellulose as the carbon source, though the hydrogen production was really less. *T.neapolitana* can also use a wide variety of nitrogen sources. Zhu (2007) reported that *T. neapolitana* can use agricultural feed stocks for hydrogen production. Energy from water containing biomass including sewage sludge and agricultural waste can be used for biofuels production mainly through microbial fermentation. Lactate and lactate-containing wastewater, cow-dung slurry,

vegetable starch, sugarcane juice and whey, bean product wastewater, tofu wastewater have been extensively used for hydrogen production (Nath & Das, 2004). One such agricultural waste produced in South Carolina is peaches. South Carolina is the second largest producer of peaches in the US. There are more than 200 million pounds of peaches harvested in the state in a normal year (SCDA, 2007). This large production is accompanied with large amount of rotten and spoiled peaches produced as peach waste (about 20 million pounds). These peaches have good amount of accessible sugars which might be used by the bacteria, without any pretreatment, for production of biohydrogen. Sucrose is the dominant sugar in peaches (Genard and Souty, 1996). Mateja et al. (2004) reported that total sugar content in peaches varied from 61.53 to 93.70 g/kg of the fruit. Therefore there is large variation in the sugar content in different varieties of peaches. This may affect the hydrogen production from peaches, based on variety. The amount of sucrose varied from 46.14 to 70.17 g/Kg; whereas the amount of glucose and fructose varied from 5.43 to 11.11 g/kg (Mateja et al., 2004). The amount of different sugars and organic acids in the peaches also changes at different times of the maturity and ripening of the fruit.

The hydrogen production by fermentation is affected by temperature, pH, partial pressure of hydrogen, partial pressure of carbon dioxide, organic acid concentration and inorganic elements. Extreme thermophilic anaerobic hydrogen fermentation can achieve more hydrogen production and higher hydrogen production rates than mesophilic anaerobic fermentation (van Groenestijn et al., 2002). The optimal growth temperature of

Thermotoga neapolitana is 77°C (Jannasch et al., 1988). The pH has profound effect on hydrogen fermentation. The organic acid from fermentation causes the pH to drop, and the accumulation of organic acids also inhibits the reaction of evolving hydrogen. High concentration of organic acids disturbs the pH gradient across the membrane, inhibiting all the metabolic functions of the cell (Jones and Woods, 1986). The undissociated or total acetate concentration can inhibit the hydrogen fermentation (Jones and Woods, 1986; Van Ginkel and Logan, 2005; Van Niel et al., 2003). Liu (2008) reported that acetate concentration at more than 50 mM started to inhibit the hydrogen fermentation. The acetate concentration also affected the duration of lag phase for the mixed culture (Liu, 2008). It has been reported that total acetate concentration is the main inhibitor of extremely thermophilic hydrogen fermentation by *Caldicellulosiruptor sacchrolyticus* ; undissociated acetate concentration does not have much effect on hydrogen production, at pH 6.5 and 7.2 (van Niel et al., 2003). pH also has the effect on the metabolism pathways of *T. neapolitana* (Nguyen et al., 2008). Jannasch et al. (1988) reported that pH range for growth of *T. neapolitana* is between 5.5 and 9. pH control is important for the hydrogen production because of the effect of pH on hydrogenase activity (Anna et al., 1991). Non-optimal pH may also prolong the lag phase in hydrogen fermentation (Cheng et al., 2002b; Liang 2003). Initial pH has been reported to have a significant effect on hydrogen fermentation using mixed microbial flora on sucrose solution (Lee et al., 2002). Liu (2008) reported an optimum initial pH of 7.0 for biohydrogen production from household solid waste, using an extremely thermophilic mixed culture. van Niel (2002) reported the unsuccessful attempt to increase the buffer strength of the medium for *T. elfii* as the

growth was completely inhibited by 50 mM phosphate. The decline in pH leads to the decline in growth and hydrogen production by the bacterium and hence pH maintenance is necessary for optimum hydrogen production. Strategies for pH control include optimum initial pH, addition of alkali to adjust the pH during exponential phase or the continuous maintenance at the optimum pH. Nguyen et al. (2008) reported significant effect of initial pH on growth and hydrogen production of both *T. maritima* and *T. neapolitana*. The hydrogen producing capability of hydrogen producing bacteria could increase with increase in pH in an appropriate range and will decrease with further increase in pH, at increasing levels (Wang, 2009). The range of initial pH has been reported between 6.5-7.5 for most of the extremely thermophilic hydrogen fermentations. van Niel (2002) maintained the pH of 7.0 and 7.4 at 70 °C throughout the experiment for pure cultures of *Caldicellulosiruptor saccharolyticus* and *Thermotoga elfii* respectively, for fermentation of glucose and sucrose. Schroder (1994) controlled the pH at 6.5 at 80°C using glucose as substrate for *Thermotoga maritima*. Kadar (2004) used paper hydrolysate for hydrogen production by pure culture of *Caldicellulosiruptor saccharolyticus* and maintained the pH at 7.2. Yokoyama (2007a) also reported the pH optimum of 7.0 for the extremely thermophilic mixed culture adapted from manure. Most of the data reported in literature is based on batch studies and without pH control and therefore, only the effect of initial pH has been accounted for in the reported studies. Most of the studies have used sucrose as the substrate. Wang (2009) recommends the investigation of the effect of pH on fermentative hydrogen production using organic waste as the substrate.

The hydrogen concentration in liquid phase is determined by the partial pressure of hydrogen in the headspace affects the hydrogen production (Hawkes et al., 2002). High hydrogen partial pressures cause the end product inhibition to inhibit the growth of hydrogen –evolving bacteria. 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). Different species have different hydrogen partial pressure limits, although the limit of hydrogen partial pressure for hydrogen-evolving bacteria growth can be increased at high temperatures. The hydrogen partial pressure limit increases to 2,000 Pa for *Pyrococcus furiosus* growing without S° at the temperature 98°C (Adams, 1990), and the limit of hydrogen partial pressure could be increased to 10,000-20,000 Pa for *Caldicellulosiruptor saccharolyticus* growing at the temperature 70°C (van Groenestijn et al., 2002; van Niel et al., 2003). Hydrogenase involves the hydrogen-evolving and hydrogen-consuming activity. The catalytic activity of hydrogenase is in favor of evolving hydrogen at high temperatures (Adams, 1990). Yu (2008) estimated a partial pressure limit of 38 kPa, the partial pressure of hydrogen at which further hydrogen production is inhibited. The removal of carbon dioxide can also improve the hydrogen production in fermentation (Tanisho et al., 1998). It has been reported that partial pressure of carbon dioxide has higher inhibition effect than partial pressure of hydrogen on hydrogen production by fermentation and hydrogen production was doubled when carbon dioxide was removed (Tanisho et al., 1998). Lee et al. (2001) reported that hydrogen production by fermentation increases

significantly by increasing iron concentration. The hydrogen production can therefore be increased by removing the limitation due to these factors.

1.2. Goals of This Study

The goals of the presented study are the following-

1. To study hydrogen production by the hyperthermophilic bacterium *Thermotoga neapolitana* using cull peaches as carbon source as compared to glucose.
2. To study the effect of nitrogen sources added to the peach medium on hydrogen production.
3. To determine the effect of pH on hydrogen production by *Thermotoga neapolitana* using cull peach medium.

1.3. CHAPTER TWO

This chapter presents the study on biohydrogen production by *Thermotoga neapolitana* using cull peaches as carbon source. It also includes the study on different nitrogen sources used in the medium. The data on hydrogen production with increased peach substrate concentration has also been presented.

1.4. CHAPTER THREE

The effect of pH on biohydrogen production has been studied in this chapter. It includes the effect of initial pH and the effect of pH adjustment on hydrogen production. The data includes the change in chemical oxygen demand (COD)

along with the amount of hydrogen and carbon dioxide produced. It also includes the effect of increased substrate concentration with pH adjustment.

1.5. CHAPTER FOUR

The chapter summarizes the conclusions of the study.

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CHAPTER TWO

Biohydrogen Production From Cull Peach Medium Using Hyperthermophilic Bacterium *Thermotoga neapolitana*

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Abstract

Thermotoga neapolitana is a marine hyperthermophilic bacterium that ferments various sugars to hydrogen and acetate. In this study, cull peaches were used as carbon source in a defined medium for biohydrogen production and produced 18 % to 25% hydrogen in the headspace. The hydrogen production varied from 6.4-7.7 mmol H_2/g peach (dry weight). The hydrogen concentration did not increase after 20 hours of incubation. The final pH decreased to 4.9 after 20 hours. Unautoclaved peach medium can be used for hydrogen production. The hydrogen production did not increase with the increase in peach concentration from 50 g/L to 100 g/L (wet weight). Yeast extract, as nitrogen source, was found important for hydrogen production. Hydrogen production was low

when ammonium chloride alone was used as the nitrogen source. Soybean meal was found to be a good nitrogen source with cull peaches as carbon source for biohydrogen production.

Keywords: Biohydrogen cull peaches unautoclaved yeast extract *Thermotoga neapolitana*

2.1. Introduction

One of the major challenges of the current century is the rising demand of fossil fuels. Fossil fuel resources are limited and non renewable. The estimated theoretical time of depletion for crude oil and nature gas reserves lies near 2060-2070 (Klass, 1998; Klass, 2003). Fossil fuels also contribute to environmental problems such as global warming, acid rain, and health problems (Levin et al., 2004). This makes it urgent to find environment friendly and renewable alternative sources of energy.

Hydrogen is one of the reliable answers to the foreseeable energy crisis and environmental pollution. High conversion efficiency and its non polluting nature justify its prospects as a future fuel. The mass of hydrogen reported to be traded worldwide is 50 billion kilograms annually with a growth rate of nearly 10% per year (Kargi, 2006; Winter, 2005). Hydrogen gas is a potential sustainable environmentally friendly fuel because it combusts to form only water and energy (Das, 2001; Lee, 1996; Sperling, 2004). Most of the hydrogen is produced from fossil fuels currently. Globally, hydrogen

is primarily produced via thermocatalytic reformation of natural gas (Benneman, 2000). Biological production of hydrogen presents much more sustainable and environmental friendly option.

Biological production of hydrogen can be done photo-biologically or through fermentation (Benneman, 2000; Hallenbeck, Benneman, 2002). Photo-biological hydrogen production utilizes solar energy, but the light conversion efficiencies and rate of hydrogen production remains low (Levin et al., 2004). Fermentation of organic wastes combines hydrogen production with waste treatment making it the most promising method of hydrogen production (Benemann, 1996). Hydrogen production by fermentation can be done in dark and it can use a large variety of renewable biomass including agriculture waste (Logan et al., 2002; Hussy et al., 2005), municipal waste (Wang et al., 2003), food processing waste (Van Ginkel et al., 2005).

Hydrogen can be produced by wide variety of bacteria including mesophiles, thermophiles, extreme thermophiles and hyperthermophiles (van Niel et al., 2003; Kotay and Das, 2007; Shin et al., 2007). These chemoorganotrophic microorganisms use organic substrates as energy source and hydrogen ion as electron acceptor to produce hydrogen. Hydrogen production by fermentation depends on the end products formed and metabolic pathways employed. The theoretical maximum yield for fermentation is 4 mol H_2/mol glucose when acetic acid is the byproduct (Thauer, 1976). Four moles of hydrogen contains 33% of the combustion energy of glucose. The theoretical yield for

fermentation is 2 mol H_2/mol glucose when butyric acid is the byproduct (Nandi and Sengupta, 1998). 2 moles of hydrogen contain 16.5% of the combustion energy of glucose. If ethanol and acetic acid are the end-products, then 2 mol H_2/mol glucose are produced (Hwang et al., 2004). If propionic acid is the end-product of fermentation, no hydrogen is produced (Ren et al., 2006).

Anaerobic bacteria capable of hydrogen production includes species of *Enterobacter* (Nath et al., 2006), *Bacillus* (Kotay and Das, 2007), *Clostridium* (Ferchichi et al., 2005; Zhang et al., 2006) and *Thermotoga* (Huber et al., 1986; Jannasch et al., 1988; Belkin et al., 1986). Hyperthermophiles are a promising group for hydrogen production because they have higher hydrogen conversion efficiency and hydrogen production rates. Moreover, these microorganisms grow at the temperature of around 80°C. Few microorganisms can grow at these temperatures and hence sterilization may be omitted thereby saving energy. The catalytic activity of hydrogenase is in favor of evolving hydrogen at high temperatures (Adams, 1990).

The genus *Thermotoga* lies in order Thermatogales under family Thermotogaceae. Most species of Thermotogales produce certain amount of hydrogen during their growth (Belkin et. al., 1986; Jannasch et al., 1988; Vrijie et al., 2002). *Thermotoga neapolitana* was isolated around the Bay of Naples, Italy (Belkin et al., 1986). It 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). Yu (2008) reported that *T. neapolitana* accumulated 28%-30% hydrogen, after 20 hours of incubation, using glucose as carbon source.

Themotoga maritima completely ferments 1 mole glucose as carbon and energy sources to 2 mole acetate, 2 mole CO_2 and 4 mole H_2 through Embden-Meyerhof pathway (Schroder et al., 1994; Schonheit and Schafer, 1995). *T.maritima* and *T.neapolitana* are closely related, based on 16s rRNA analysis (Huber and Hannig, 2006). Most of the glucose fermenting anaerobes produce less than 2 mole of acetate and 4 mole of hydrogen from 1 mole of glucose because various byproducts such as lactate, ethanol, butyrate are also produced (Schonheit and Schafer, 1995).

Thermotoga neapolitana can use simple or complex as carbon source or nitrogen source (Belkin et al., 1986; Huber and Hannig, 2006: Zhu, 2007; Yu, 2008; Nguyen et al., 2008). Agricultural wastes constitute a large sugar deposit which can be used for hydrogen production. Agricultural biomass has accessible sugars and lingo-cellulosic components. Yu (2008) reported that hydrogen production by *T.neapolitana* was similar for glucose, sucrose, rice flour and xylan, used as the carbon source. Though, lingo-cellulosic biomass provides large pool of sugars which can be used, but the breakdown of cellulose into relatively simple sugars, including monosaccharides and disaccharides remains the bottleneck in its use. Vrije et al. (2002) reported *Thermotoga elfii* utilizes pretreated *Miscanthus* and produces a significant amount of hydrogen. Yu (2008) reported that

Thermotoga neapolitana can utilize cellulose as the carbon source to a limited extent. *T.neapolitana* can also use a wide variety of nitrogen sources. Yu reported that trypticase is important for hydrogen production and yeast extract may be replaced with soybean meal or canola meal, as alternative nitrogen source. Zhu (2007) reported that *T. neapolitana* can use agricultural feed stocks for hydrogen production. One such agricultural waste produced in South Carolina is peaches. South Carolina is the second largest producer of peaches in the US with more than 200 million pounds of peaches harvested in the state in a normal year (SCDA, 2007). Approximately 10% of these peaches (about 20 million pounds) are discarded annually due to bruising. These peaches have good amount of accessible sugars that might be used by the bacteria, without any pretreatment, for production of biohydrogen. Sucrose is the dominant sugar in peaches (Genard and Souty, 1996). Mateja et al. (2004) reported that total sugar content in peaches varies from 61.53 to 93.70 g/kg of the fruit (wet weight) due to varietal differences. The amount of sucrose varied from 46.14 to 70.17 g/Kg; whereas the amount of glucose and fructose varied from 5.43 to 11.11 g/kg (Mateja et al., 2004).The amount of different sugars and organic acids in the peaches also varied with fruit maturity and ripening. The objectives of this research is to study the hydrogen production by *Thermotoga neapolitana* using cull peaches as carbon source in a defined medium and compare it with glucose medium. Hydrogen production with and without autoclaving of the medium will also be studied

2.2. Materials and Methods

2.2.1. Organism

Thermotoga neapolitana was obtained from DSMZ (German Collection of Microorganisms and Cell Cultures).

2.2.2. Cultivation medium and conditions

Thermotoga neapolitana was maintained and cultivated on medium described by Van Ootegham (Van Ootegham et al., 2002): 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, 10.0 ml of vitamin solution (DSM medium 141), 10.0 ml of trace element solution (DSM medium 141), 0.121 g of trizma base per 1.0 L of distilled H_2O. Fresh peaches (Redhaven variety) were frozen at -70°C prior to use. Peaches were thawed and blended for 5 minutes prior to use. 50 g/L (wet weight) of depitted blended peaches was used as the carbon source in the medium. The initial pH of the medium was adjusted to 8.0 using 5N NaOH. 500ml serum bottles with 100 ml of the medium were used as the batch reactor for the experiments. All treatments were run in triplicates.

The bottles were sparged with nitrogen for 5 minutes and sealed. The medium was inoculated with 2ml inoculum using sterile syringe. The culture was incubated on an orbital shaker bed at 200 rpm and 77°C. The organism was preserved at 4°C.

2.2.3.1. Glucose and peach as carbon source- Medium was prepared with 5 g/L of glucose with other medium components as described above. The other set was added with 50 g/L (wet weight) of peaches. The initial pH was adjusted to 8.5 in both the reactors. Both sets were autoclaved at 121°C for twenty minutes. Both the sets were sparged with nitrogen and sealed. Reactors were incubated for 30 hours at 77°C and 200 rpm.

2.2.3.2. Autoclaved and unautoclaved peach medium- One set of reactors was set to pH of 8.5 and autoclaved at 121 °C for 20 minutes. The pH in these reactors dropped to 7.8 after autoclaving and was adjusted to 8.0 after autoclaving. The other set of reactors was not autoclaved (unautoclaved) and their initial pH was set to 8.0. Depitted blended peaches at 50 g/L (wet weight) were used as the carbon source. The nitrogen was sparged through these reactors and sealed. Unautoclaved medium has been used for the rest of the study.

2.2.3.3. Different concentrations of substrate- One set of reactors contained medium with 50 g/L and other set of reactors with 100 g/L of blended peaches, on

wet weight basis. The concentration of all the other components of the medium remained the same as described earlier.

2.2.3.4. Effect of nitrogen sources- The cultivation medium was prepared in the same manner, as described earlier, for all the reactors except for the nitrogen sources. The total amount of nitrogen added to the original medium with 2 g/L trypticase, 2 g/L yeast extract and 1 g/L ammonium chloride is 0.744 g/L. Three different sets of reactors were prepared, in which the amount of ammonium chloride was altered such that the total nitrogen added to the medium remained the same at 0.744 g/L. Depitted blended peaches at 50 g/L (wet weight) were used as the carbon source. Yeast extract contains 8-12% of nitrogen, therefore mean value of 10% of nitrogen in yeast extract was considered for calculations. Trypticase has 14.2% of nitrogen. Ammonium chloride contains 26.2% nitrogen. Four different reactor sets were prepared according to the table 2.2.3.4.1.

Table 2.2.3.4.1. Amount of different nitrogen sources added to different set of reactors.

Nitrogen source Ratio (Ammonium Chloride:Yeast Extract:Trypticase)	**Mass of Ammonium Chloride added (g/L)**	**Mass of Yeast Extract added (g/L)**	**Mass of Trypticase added (g/L)**	**Total nitrogen added (g/L)**
1:2:2	1	2	2	0.744
2.079:2:0	2.079	2	0	0.744
1.76:0:2	1.76	0	2	0.744
2.843;0:0	2.843	0	0	0.744

2.2.3.5. Soybean meal as nitrogen source- Soybean meal at 5 g/L was used as the nitrogen source instead of ammonium chloride, yeast extract and trypticase in the standard medium. Depitted blended peaches at 50 g/L (wet weight) were used as the carbon source.

2.2.4. Analysis Methods

2.2.4.1. Hydrogen concentration-The batch reactors were cooled to 25 °C by placing in water bath. Hydrogen gas in the headspace was sampled by collection with 1ml tuberculin syringe. 0.5 ml of gas was injected into gas chromatograph (SRI 8610C, SRI Instruments, Torrance, CA90503) with Thermal Conductivity Detector at 100°C and Silica Column (25°C). The pressure of Argon as carrier gas was 22 psi.

The volume of the headspace was 450 ml. According to ideal gas law, the hydrogen concentration was calculated through this equation,

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

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 (8.314 KPa.L/ (mol·K)), V_2 is the volume of medium (L). The Hydrogen partial pressure in 77°C was also calculated through ideal gas law

P1/T1 = P2/T2 (2)

2.2.4.2 Total headspace pressure-The pressure of the gas in the headspace of each reactor was measured with Traceable manometer (Fisher Scientific) after the reactor had cooled to 25 °C.

2.2.4.3 pH- pH was measured using a digital pH meter equipped with a gel electrode.

2.2.4.4 Dry weight of peach slurry- The dry weight of blended peaches was determined by placing 5 g of peach slurry (wet weight) in an aluminum weighing pan and dried at 105°C,until it reached a constant weight.

2.3 Results And Discussion

2.3.1 Biohydrogen production by *Thermotoga neapolitana* using peaches as carbon source-

The pH after autoclaving came down to 7.5 and 7.8 for glucose and peach medium respectively. Cull peaches were found to be a good carbon source for hydrogen production by the bacterium (table 2.3.1.1). The hydrogen concentration accumulated in headspace varied from 18% to 25%. The hydrogen production varied from 6.4 to 7.7 mmol H_2/g peach dry weight. The hydrogen production per liter of the medium varied from 38 mmol H_2/L to 54 mmol H_2/L, approximately. There was no difference found in the percentage concentration of hydrogen (α=0.05, p=0.564) produced in the head space after 30 hours of incubation, when glucose at 5 g/L and peaches at 50 g/L, on wet weight basis, were used as the carbon source.

Table 2.3.1.1. Hydrogen concentration for glucose and peach medium.

Carbon source	Mean Hydrogen concentration (%)	Absolute Pressure (kPa)	Mass of hydrogen in headspace (g)	Mean Hydrogen concentration (mmol /L medium)	Final pH
Glucose@ 5 g/L	23.21	122.06	0.01023	51.14	4.97
Peach @ 50 g/L, (wet weight)	22.99	122.46	0.01029	51.45	4.89

The major sugar present in Redhaven variety of peaches is sucrose. The other major sugars present are glucose and fructose respectively (Mateja et al., 2004). Yu (2008) reported that there was no difference in the amount of hydrogen produced when glucose and sucrose were used as the carbon source for *T.neapolitana* batch incubation. The amount of sucrose, fructose and glucose present in one kilogram of peaches are 51.67 g, 8.62 g and 7.08 g respectively (Mateja et al., 2004). The amount of different sugars and organic acids present in peaches also varies with different varieties and locations (Wu et al., 2003; Mateja et al., 2004). There was a distinct difference in the dry weight of the stored peaches (Redhaven variety) used during the course of study (table 2.3.1.2). The percentage solid content of peaches varied from

11.74% to 13.91%. The change in the dry weight, of peaches, explains the difference in the total number of moles and percentage concentration of hydrogen produced, for the standard peach medium, during the course of study. There was 4.8% of hydrogen accumulated in the headspace when standard medium without any peach or glucose was inoculated (table 2.3.1.3). This is because the bacterium used the available carbon from yeast extract and trypticase for its growth resulting in hydrogen production.

Table 2.3.1.2. Dry weight of peaches for all the experiments reported in the study.

Run (data only for reactor sets with peach concentration of 50 g/L, on wet weight basis)	**Percentage Solid in peach slurry (%)**	**Mean dry weight of peaches added/liter medium (g/L)**
Peach vs Glucose	13.34	6.73
Autoclaved vs Unautoclaved	13.91	6.97
Different peach concentration, without pH control	13.8	6.96
Different initial pH	13.69	6.95
pH adjusted to 6.5 pH not set to 6.5	13.67	6.97
pH set to 7.5 pH not adjusted to7.5	13.8	6.95
Different concentration with pH adjusted to 6.5	13.67	6.96
Soybean meal as nitrogen source	12.85	6.45
Different nitrogen sources	11.74	5.96
pH adjustment with COD measurement	12.22	6.16

Table 2.3.1.3. Hydrogen concentration for medium inoculated without any carbon source

Medium	**Mean hydrogen concentration (%)**	**Mean hydrogen concentration (mmol/L medium)**	**Mean total absolute pressure (KPa)**	**Final pH**
Medium without any peach or glucose added to it	4.8	10.15	116.29	6.80

2.3.2. Incubation time- Hydrogen concentration on peach medium, containing 50 g/L of blended peaches, increased until 20 hours of incubation. The partial pressure of hydrogen (fig.2.3.2.1) and headspace concentration did not increase after 20 hours (Fig. 2.3.2.2). Water vapor pressure at 25 °C is 5 KPa approximately, so it contributes little to total pressure measured in headspace. Yu (2008) reported the required incubation time for glucose and xylan to be 20 hours and 36 hours for sucrose. Our result is different from Yu's results as major sugar present in peaches is sucrose along with glucose and fructose. This may be explained as the bacteria utilizes glucose and fructose present in peaches first and the enzymes for sucrose metabolism might be produced simultaneously as sucrose is present in much greater amount. Also, by the time all of the glucose and fructose are used by the bacteria and it starts using sucrose, pH has already decreased considerably to limit the use of sucrose. The incubation time was set to 20 hours, until pH drops to 4.9, for rest of the study.

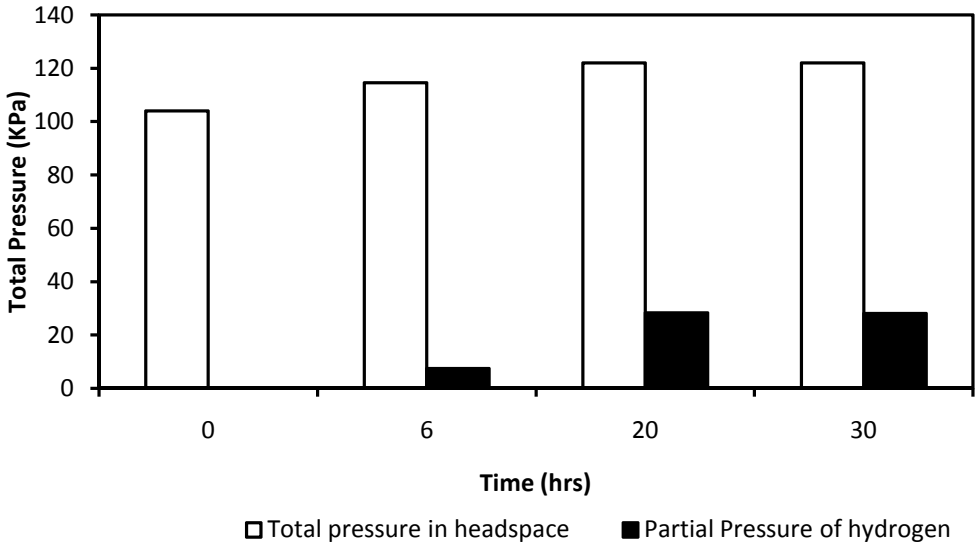

Fig.2.3.2.1. Partial pressure of hydrogen and total headspace pressure as function of time.

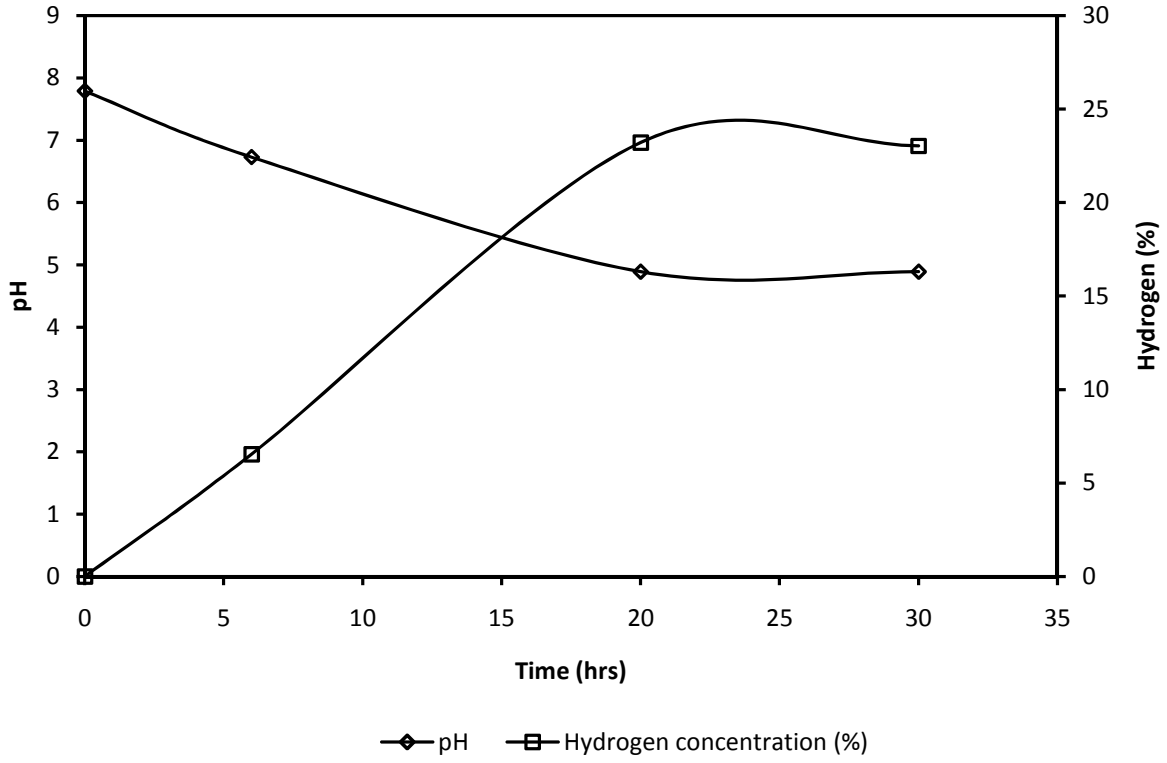

Fig. 2.3.2.2. pH and hydrogen concentration on autoclaved peach medium.

2.3.3. pH changed with time

The pH of the medium dropped from the initial pH of 8.5 to 7.8, at 25 °C, after sterilization and decreased to 4.88 after 20 hours of incubation (fig. 2.3.2.2). The pH does not change after 20 hours. Thermotogales form acetate, lactate, L-alanine, ethanol, carbon dioxide, and hydrogen gas as the products from fermentation when glucose is used as carbon source (Huber and Hannig, 2006; Ravot et al., 1995). The organic acids from fermentation cause the pH to drop. Jannasch et al (1988) reported that pH range for growth is between 5.5 and 9 (Jannasch et al., 1988). Van Ooteghem (2004) also reported that pH of medium dropped from 7.5 to 4.5 within 20hrs; but if adding itaconic acid, the

pH decreased from 7.5 to 5.9, and hydrogen production reached a maximum plateau value after 20hrs. The proton concentration affects the yield and rate of hydrogen production (Nath and Das, 2004; Mu et al., 2006), and the range of pH favorable to hydrogen production is narrow (Lay, 2000). So optimizing pH or blocking the formation of organic acids becomes necessary.

2.3.4. Unautoclaved medium vs. autoclaved medium- There was no difference found in the amount of hydrogen produced (α=0.05, p=0.7060) for non autoclaved medium as compared to the autoclaved medium (table 2.3.4.1). Therefore, non autoclaved peach medium was used for the rest of the study. This result is important because energy is saved by not autoclaving the medium.

Table 2.3.4.1. Hydrogen concentration for autoclaved and unautoclaved peach medium@50 g/L (wet weight).

Medium	**Mean Hydrogen concentration (mmol/L medium)**	**Mean Hydrogen Production (mmol/g dry weight peach)**	**Final pH**
Autoclaved Peach Medium @50 g /L (wet weight)	52.2	7.5	4.88
Unautoclaved Peach Medium @50 g/L (wet weight)	53.4	7.7	4.88

2.3.5. Effect of different concentrations of peaches- There was no difference found in the final amount of hydrogen produced (α=0.05, p= 0.8947) for two different peach concentrations, 50 g /L and 100 g /L, on wet weight basis, respectively (table 2.3.5.1). The amount of hydrogen produced per gram of peach for peach concentration of 100 g/L was almost half that of produced for peach concentration of 50 g/L. The final pH after 20 hours, for the batch reactors with peach at 100 g/L, dropped to 4.75. The final pH after 20 hours, for the batch reactors with peach at 50 g/L, dropped to 4.89. These results indicate inhibition due to pH or hydrogen partial pressure. Therefore, pH control is needed for determination of hydrogen production for greater peach concentrations.

Table 2.3.5.1. Hydrogen concentration for different peach concentrations.

Medium	**Mean Hydrogen concentration (mmol/L medium)**	**Mean Hydrogen Production (mmol/g dry weight peach)**	**Final pH**
Unautoclaved Peach Medium @50 g/L (wet weight)	52.4	7.5	4.89
Unautoclaved Peach Medium @100 g /L (wet weight)	52.5	3.8	4.75

2.3.6. Effect of nitrogen sources

Yeast extract was found to be important for hydrogen production; hydrogen production also increased with the use of trypticase (table 2.3.6.1). There was

very little amount of hydrogen produced when ammonium chloride alone was used as the nitrogen source. Yu (2008) reported that no hydrogen was produced when yeast extract alone was used as the nitrogen source and glucose was used the carbon source. Therefore our results are different from Yu's results. Yu also reported that trypticase may be added along with yeast extract as nitrogen source to obtain high hydrogen yield by *T. neapolitana*. Nguyen et al. (2008) reported that hydrogen production for *T. neapolitana* increased as yeast extract concentration in the medium increased from 0.5 g/L to 4.0 g/L. Van Niel et al. (2002) described yeast extract enriched medium to be important for hydrogen production by *T.elfii*. Apparently, certain micronutrients, other than amino acids, present in yeast extract are used by the bacteria for better hydrogen production (van Niel et al., 2002). The trypticase used in the medium contains amino acids in the form peptides which are the preferred form of amino acids used by certain lactate acid bacteria (van Niel et al., 2002). Tryptone also contains much more amount of proline as compared to yeast extract; otherwise yeast extract and trypticase are almost similar in composition (van Niel et al., 2002).

Table 2.3.6.1. Hydrogen concentration for different combinations of nitrogen sources in medium.

Nitrogen source Ratio (Ammonium Chloride:Yeast Extract:Trypticase)	**Mean Hydrogen concentration (mmol/L medium)**	**Mean Hydrogen production (mmol/g dry weight peach)**
1:2:2	38	6.37
2.079:2:0	30	5.08
1.76:0:2	17.6	2.95
2.843:0:0	6.4	1.07

2.3.7. Soybean meal as nitrogen source- The percentage hydrogen in headspace decreased by 27%, as compared to standard medium containing ammonium chloride, yeast extract, trypticase at 1 g/L, 2 g/L, 2g/L respectively (table 2.3.7.1), when soybean meal was used as the sole nitrogen source. Yu (2008) reported that soybean meal to be a good nitrogen source for hydrogen production but the medium used in his study also contained ammonium chloride at 1g/L, whereas soybean meal at 5g/L is the only nitrogen source present in the medium in our study.

Table 2.3.7.1. Hydrogen concentration when soybean meal was used as the nitrogen source.

Nitrogen source in the medium	**Mean Hydrogen concentration (mmol/L medium)**	**Mean Hydrogen Production (mmol/g dry weight peach)**	**Final pH**
Soybean meal@5g/L	32.04	4.9	5.05
Ammonium chloride@1g/L, Yeast extract @ 2g/L, Trypticase @ 2 g/L	45.19	7.0	4.89

2.4. Conclusions

Peaches are a good carbon source for hydrogen production using *Thermotoga neapolitana*. Little pretreatment is required for the peaches to use as carbon source. Unautoclaved medium can be used. The incubation time of 20 hours is enough for hydrogen production, without pH control. The increase in substrate concentration does not lead to rise in hydrogen production; though the pH inhibition or hydrogen partial pressure inhibition can be the reason for this result. Therefore, further studies where the pH is controlled at optimum and hydrogen partial pressure is maintained below the inhibitory limits are necessary. Yeast extract is important for hydrogen production on peach medium; though a little amount of yeast extract might be used by the bacteria for hydrogen as a carbon

source because very small amount of hydrogen was produced when medium was inoculated without any carbon source. Further studies with medium containing optimum salt concentration are necessary to bring down the medium cost.

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CHAPTER THREE

Effect of pH on Biohydrogen Production From Cull Peaches by Hyperthermophilic Bacterium *Thermotoga neapolitana*

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Abstract

The pH had profound effect on biohydrogen production by *Thermotoga neapolitana*. The optimum initial pH for hydrogen production using peach medium as was 8.0. The mass of hydrogen produced increased when pH was adjusted after 12 hours of incubation. The hydrogen production increased from 7.07 mmol H_2/g peach (dry weight) to 8.73 mmol H_2/g peach (dry weight), when pH was adjusted. The incubation time also increased from 20 hours to 40 hours for complete fermentation on pH adjusted medium. The amount of soluble COD utilized increases from 3.81 to 4.95 g COD/L, when pH was adjusted. The number of moles of carbon dioxide produced was same as that of hydrogen. The maximum rate of production of hydrogen observed in this study was 3.31 mmol H_2/L.h.

The hydrogen production was same when pH was adjusted to two different pH of 6.5 and 7.5 respectively after 12 hours. The amount of hydrogen produced decreased when substrate concentration was increased from 50 g/L to 100 g/L, on wet weight basis

Keywords: Biohydrogen cull peaches initial pH pH-adjustment *Thermotoga neapolitana*

3.1. Introduction

Thermotogales can use simple or complex carbohydrates or organic matter as carbon or nitrogen sources. Thermotogales form acetate, lactate, L-alanine, ethanol, carbon dioxide, and hydrogen gas as the products from fermentation using glucose as carbon and energy source (Ravot et al., 1995; Huber and Hannig, 2006). On the basis of 16s rRNA gene sequence analysis, *Thermotoga maritima* and *Thermotoga neapolitana* are closely related (Huber and Hannig, 2006). *Themotoga maritima* completely ferments 1 mole glucose to 2 mole acetate, 2 mole CO_2 and 4 mole H_2 (Schroder et al., 1994). *Thermotoga martima* degrades glucose through simultaneous operation of both conventional Embden-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 and *Thermotoga maritima* is the most archaea-like bacteria (Nelson et al., 1999).

Most species of Thermotogales produce 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). 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). 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). *Thermotoga neapolitana* can utilize a wide variety of carbon sources including glucose, sucrose, xylose, xylan, cellulose, cellobiose, starch, corn starch, and beet pulp pellet (Yu, 2008). In the carbon studies, glucose, sucrose, rice flour, and xylan produced similar levels of hydrogen (Yu, 2008).

Some hyperthermophiles use sulfur compounds like elemental sulfur, polysulfides and cystiene as alternative electron acceptors (Adams; 1990, Childers; 1997). *Thermotoga spp.* cannot use sulfate as the electron acceptor which is a common electron acceptor for facultative organisms (Childers, 1997). Huber et al. (1986) reported that, when *T.maritima* was grown in absence of sulfur, the growth was completely inhibited at 240

KPa (at culture temperature); whereas the growth continued even at partial pressure of 240 KPa, though the hydrogen production reduced by 40%, when sulfur was added to the medium. The presence of sulfur in the medium reduce hydrogen production when hydrogen is not at inhibitory levels, but it does not stimulate growth (Schroder, 1994). *T. maritima* has also been reported to reduce Fe (III) to Fe (II) with hydrogen as electron donor, when hydrogen levels become inhibitory (Vargas, 1998).

The pH of the culture medium influences growth and hydrogen production. The organic acid causes the pH to drop, and the accumulation of organic acids also inhibits the reaction of evolving hydrogen. High concentration of organic acids disturbs the pH gradient across the membrane, inhibiting all the metabolic functions of the cell (Jones and Woods, 1986). The undissociated or the total acetate concentration can inhibit the fermentation (Jones and Woods, 1986; Van Ginkel and Logan, 2005; van Niel et al, 2003). Liu (2008) reported that acetate concentration at more than 50 mM started to inhibit hydrogen fermentation. Acetate concentration also affected the duration of lag phase for the mixed culture (Liu, 2008). It has been reported that total acetate concentration is the main inhibitor of extremely thermophilic hydrogen fermentation; undissociated acetate concentration does not have much effect on hydrogen production, at pH 6.5 to 7.2, by *Caldicellulosiruptor sacchrolyticus* (van Niel et al 2003). pH also has the effect on the metabolism pathways of *T. neapolitana* (Nguyen et al., 2008). Nguyen et al. reported that the optimal initial pH for hydrogen production by *T. neapolitana* was from 6.5 to 7.5 and the hydrogen production decreased as pH was increased from 8 to 9.

Jannasch et al. (1988) reported that pH range for growth of *T. neapolitana* is between 5.5 and 9. pH control is important for hydrogen production because of the effect of pH on hydrogenase activity (Anna et al., 1991). Non-optimal pH may also prolong the lag phase in hydrogen fermentation (Cheng et al., 2002b; Liang, 2003).

Initial pH has been reported to have a significant effect on hydrogen fermentation using mixed microbial flora on sucrose solution (Lee et al., 2002). Liu (2008) reported an optimum initial pH of 7.0 for biohydrogen production from household solid waste, using an extremely thermophilic mixed culture. van Niel (2002) reported the unsuccessful attempt to increase the buffer strength of the medium for *T. elfii* as the growth was completely inhibited by 50 mM phosphate. The decline in pH leads to the decline in growth and hydrogen production by the bacteria and hence pH maintenance is necessary for optimum hydrogen production. Some of the steps can include optimum initial pH, addition of alkali to adjust the pH during exponential phase or the continuous maintenance at the optimum pH. Nguyen et al. (2008) reported significant effect of initial pH on growth and hydrogen production of both *T. maritima* and *T. neapolitana*. The range of initial pH has been reported between 6.5-7.5 for most of the extremely thermophilic hydrogen fermentations. van Niel (2002) maintained the pH of 7.0 and 7.4 at 70 °C throughout the experiment for pure cultures of *Caldicellulosiruptor saccharolyticus* and *Thermotoga elfii* respectively, for fermentation of glucose and sucrose. Schroder et al. (1994) controlled the pH at 6.5 at 80°C using glucose as substrate for *Thermotoga maritima*. Kadar (2004) used paper hydrolysate for hydrogen production

by pure culture of *Caldicellulosiruptor saccharolyticus* and maintained the pH at 7.2. Yokoyama (2007a) also reported the pH optimum of 7.0 for an extremely thermophilic mixed culture adapted from manure. Most of the data reported in literature is based on batch studies and without pH control and therefore, only the effect of initial pH has been accounted for in the reported studies. Most of the studies have used sucrose as the substrate and Wang (2009) recommends the investigation of the effect of pH on fermentative hydrogen production using organic waste as the substrate. The objective of this study is to study the effect of initial pH and pH adjustment during the batch incubation on hydrogen production by *T. neapolitana* using peach medium.

3.2. Materials And Methods

3.2.1. Organism

Thermotoga neapolitana was obtained from DSMZ (German Collection of Microorganisms and Cell Cultures).

3.2.2. Cultivation medium and conditions

Thermotoga neapolitana was maintained and cultivated on medium described by Van Ootegham (Van Ootegham et al., 2002): 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, 10.0 ml of

vitamin solution (DSM medium 141), 10.0 ml of trace element solution (DSM medium 141), 0.121 g of trizma base per 1.0 L of distilled H_2O. Fresh peaches (Redhaven variety) were frozen at -70 °C prior to use. Peaches were thawed and blended for 5 minutes prior to use. 50 g/L of depitted blended peaches was used as the carbon source in the medium. The initial pH of the medium was adjusted to 8.0 using 5N NaOH. 500ml serum bottles with 100 ml of the medium were used as the batch reactor for the experiments. All treatments were run in triplicates.

The bottles were sparged with nitrogen for 5 minutes and sealed. The medium was inoculated with 2ml inoculum using sterile syringe. The culture was incubated on an orbital shaker bed at 200 rpm and 77 °C. The organism was preserved at 4 °C.

3.2.3.1. Initial pH- Three sets of reactors were prepared. The initial pH at 25 °C was set to 7.5, 8.0 and 8.5 using 5N NaOH in the three set of reactors respectively. Depitted blended peaches at 50 g/L (wet weight) were used as the carbon source.

3.2.3.2. pH adjustment- Two sets of fifteen reactors were prepared. The initial pH was adjusted to 8 with 5 N sodium hydroxide in all the reactors. The pH was adjusted to 7.5, after 12 hours of incubation, in one set of reactors. The pH was not adjusted in the other set of reactors. Depitted blended peaches at 50 g/L (wet

weight) were used as the carbon source. Three reactors each from the two set of reactors were taken out at 6, 12, 20, 40 and 50 hours respectively for analysis.

3.2.3.3. pH adjustment to two different pH levels- Two set of reactors were prepared. The initial pH was adjusted to 8 with 5 N sodium hydroxide in all the reactors. Depitted blended peaches at 50 g/L (wet weight) were used as the carbon source.The pH was adjusted to 6.5 and 7.5 respectively, after 12 hours of incubation, in the two set of reactors.

3.2.3.4. Different substrate concentrations with pH adjustment- Two sets of reactors containing peach slurry at 50 g/L and 100 g/L, on wet weight basis, were prepared. The concentration of rest of the medium components remained the same as described earlier. The initial pH was adjusted to 8 with 5 N sodium hydroxide in all the reactors. The pH was adjusted to 6.5 in these reactors after 12 hours.

3.2.4. Analysis methods

3.2.4.1. Hydrogen concentration-After incubation, the batch reactors were cooled to 25 °C by placing in water bath. Hydrogen gas in the headspace was sampled by collection with 1ml tuberculin syringe. 0.5 ml of the gas was injected into gas chromatograph (SRI 8610C, SRI Instruments, Torrance, CA90503) with Thermal

Conductivity Detector at 100°C and Silica Column (25°C). The pressure of Argon as carrier gas was 22 psi.

The volume of the headspace was 450 ml. According to ideal gas law, the hydrogen concentration was calculated through this equation,

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

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 ideal gas law

P1/T1 = P2/T2. (2)

3.2.4.2. Total headspace pressure-The pressure of the gas in the headspace of each reactor was measured with Traceable manometer (Fisher Scientific) after the reactor had cooled to 25 °C.

3.2.4.3. Carbon dioxide concentration- Carbon dioxide in the headspace was measured by injecting 0.5 ml of the headspace gas into gas chromatograph (Agilent 7890A) with Carbon Plot column at 35C And TCD at 150C. Helium at 30 ml/min was used as the carrier gas. The number of moles of carbon dioxide was calculated in the same way as described for hydrogen.

3.2.4.4. Chemical Oxygen Demand- Chemical oxygen demand (COD) was measured using accu-TEST Chemical Oxygen Demand Systems. Closed reflux method was used for measuring COD. The samples were diluted 10 times with distilled water, to bring the salt concentration below 2 g/L (the maximum salt concentration required for the method is 2 g/L). The samples were filtered through 0.45 µ filter to measure the soluble COD. Unfiltered samples were used to measure total COD.

3.2.4.5. pH- pH was measured using a digital pH meter equipped with a gel electrode.

3.2.4.6. Dry weight of peach slurry- The dry weight of blended peaches was determined by placing 5 g of peach slurry (wet weight) in an aluminum weighing pan and dried at 105°C, until it reached a constant weight

3.3. Results and discussion

3.3.1. Effect of initial pH- The initial pH of 8.0, at 25°C, was found to be the best for hydrogen production on peach medium (fig 3.3.1.1). The batch reactors with initial pH of 8.0 produced 52.6 mmol H_2/Liter of medium as compared to 47.3 mmol H_2/liter of medium for reactors with initial pH of 8.5, the next best initial pH for hydrogen production. Hydrogen production was increased as initial pH was increased from 7.5 to 8.0 and decreased when pH was increased to 8.5. Nguyen et al. (2008) also reported an increase in hydrogen production as pH was increase from 5.5 to 6.0 - 7.0 and a decrease

in hydrogen production as pH was increased from 8.0 to 9.0. An optimal pH of 6.5-7.5 has been reported for growth and hydrogen production by *Thermotoga neapolitana*, on *T. maritime* basal medium (Nguyen et al., 2008). The initial pH has also been reported to have an effect on other metabolic pathways of the bacteria (Nguyen et al., 2008). Zhu (Zhu, 2007) reported an optimum initial pH of 8.5, for growth and hydrogen production on agricultural residues. Therefore, our results match very well with these previous reports on *Thermotoga neapolitana*.

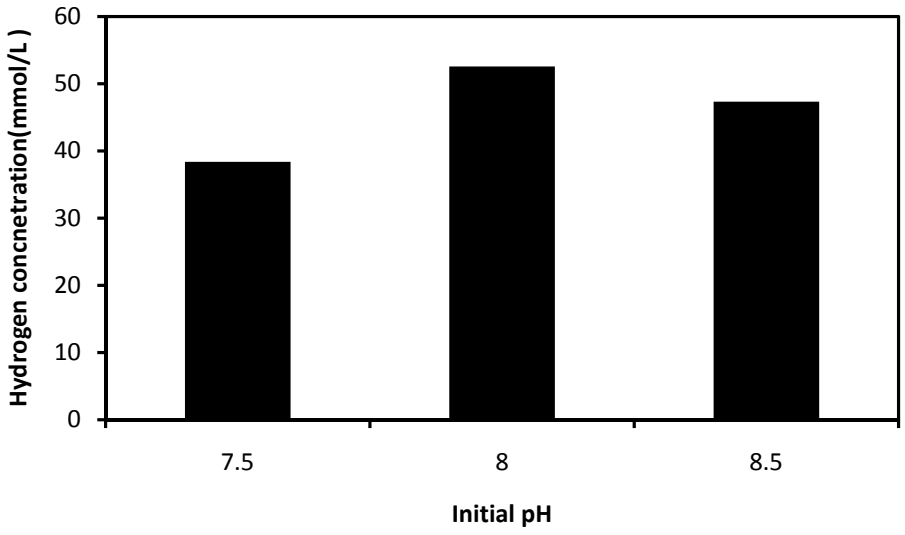


Fig.3.3.1.1. Hydrogen concentration at different initial pH of the peach medium.

Table 3.3.1.1 Hydrogen concentration for different initial pH of the peach medium.

Initial pH (at 25 ℃)	Percentage of hydrogen concentration (%)	Absolute Pressure (kPa)	Mass of hydrogen in headspace (g)	Mean Hydrogen concentration (mmol /L medium)	Mean Hydrogen Production (mmol/g dry weight peach)
7.5	18.37	114.99	0.00768	38.38	5.52
8.0	24.23	119.46	0.01052	52.58	7.57
8.5	22.08	117.99	0.00946	47.31	6.81

3.3.2. Effect of pH adjustment

The hydrogen production increased from 7.07 mmol H_2/g peach (dry weight) to 8.73 mmol H_2/g peach (dry weight), when pH was adjusted to 7.5 after 12 hours of incubation (table 3.3.2.1). Zhu (2007) reported increased hydrogen production by *Thermotoga*

neapolitana when pH was adjusted in early stationary phase. Our results are well in tandem to these reported results. The hydrogen concentration, in head space, increased till 40 hours and remains stable after that, as compared to the incubation time of 20 hours, after which there was no increase in hydrogen concentration, in batch reactors without any pH adjustment (fig. 3.3.2.1.). Also, the cumulative hydrogen concentration was a little less at 20 hours in batch reactors with adjusted pH. This can be explained as the metabolism might have shifted to the production of more reduced products when pH was suddenly adjusted. Sudden changes in environmental conditions including pH and temperature lead to lactate production (Demeril and Yenigun, 2004; Han and Shin, 2004; Liu et al. 2008a; Temudo et al., 2007). Liu (2008) reported the shift from acetate to butyrate pathway, leading to decrease in hydrogen production, for a mixed culture with the decrease in pH. The final pH dropped to 4.88, in all the reactors (fig.3.3.2.2.).

Table 3.3.2.1. Hydrogen and carbon dioxide concentration when pH was adjusted to 7.5 after 12 hours of incubation.

Medium	**Mean Hydrogen concentra -tion (mmol/L medium)**	**Mean Hydrogen Production (mmol/g dry weight peach)**	**Mean Carbon dioxide concentra -tion (mmol/L medium)**	**Mean Carbon dioxide production (mmol/ g dry weight peach)**	**Final pH**
pH not adjusted to 7.5 after 12 hours of incubation	39.31	6.38	43.74	7.07	4.88
pH adjusted to 7.5 after 12 hours of incubation	53.83	8.74	53.54	8.73	4.88

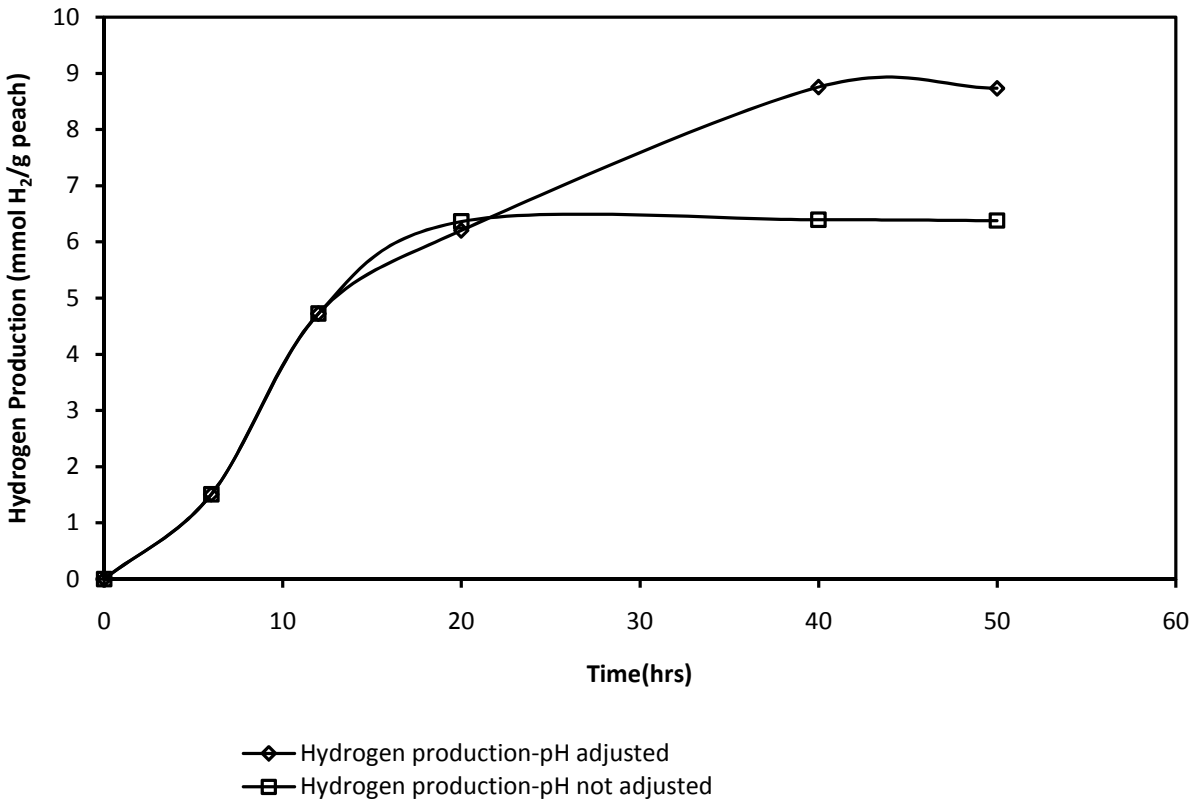


Fig.3.3.2.1. Hydrogen production as function of time.

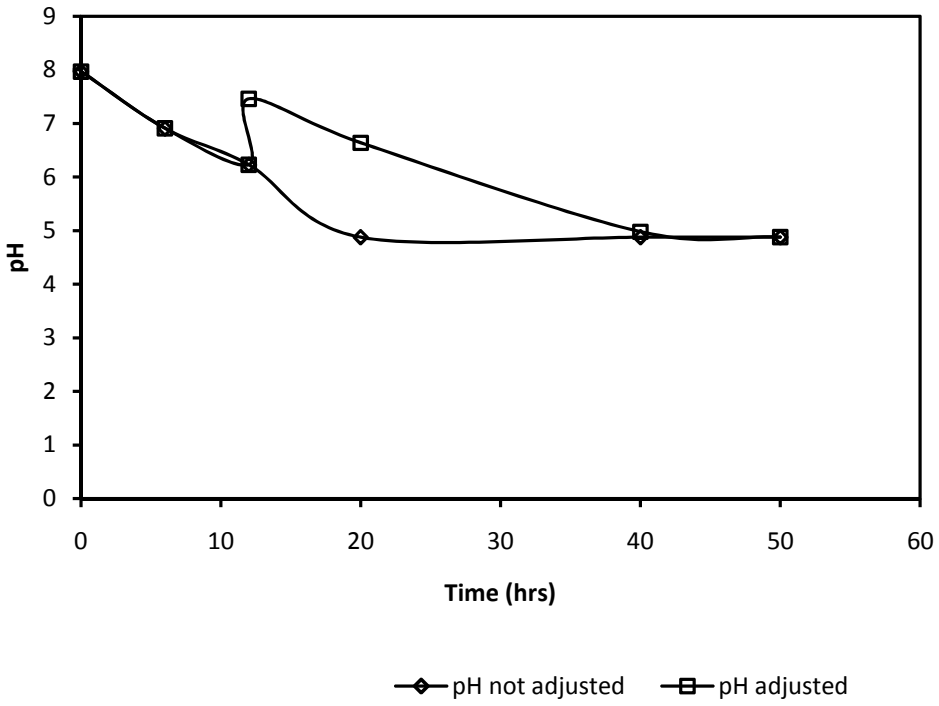


Fig.3.3.2.2. pH as function of time.

The amount of soluble COD utilized also increased from 3.81 g/L COD to 4.95 g/L COD, when pH was adjusted (fig. 3.3.2.3.). The hydrogen yield in terms of soluble COD utilized also increased from 10.3 mmol H_2/g COD to 10.87 mmol H_2/g COD. Yu (2008) reported 2.57 mol H_2/mol glucose which is equivalent to 13.34 mmol H_2/g COD (considering 1 g glucose = 1.07 g COD/L). The pH adjustment, at early stationary stage, has been reported to increase the conversion efficiency of *T. neapolitana* strains from 2.2 mol H_2/mol glucose to 3.6 mol H_2/ mol glucose (Zhu, 2007). The amount of hydrogen produced using corn husk with pH control methods has been reported to be more than the amount of hydrogen produced using glucose without pH control methods (Zhu, 2007).The bacteria

utilized the nutrients from the medium and formed the biomass and therefore, the total COD remained the same throughout the experiment. The soluble COD reported include the contributions from the reduced products and acetate as well. Therefore, actual soluble substrate utilized would still be higher.

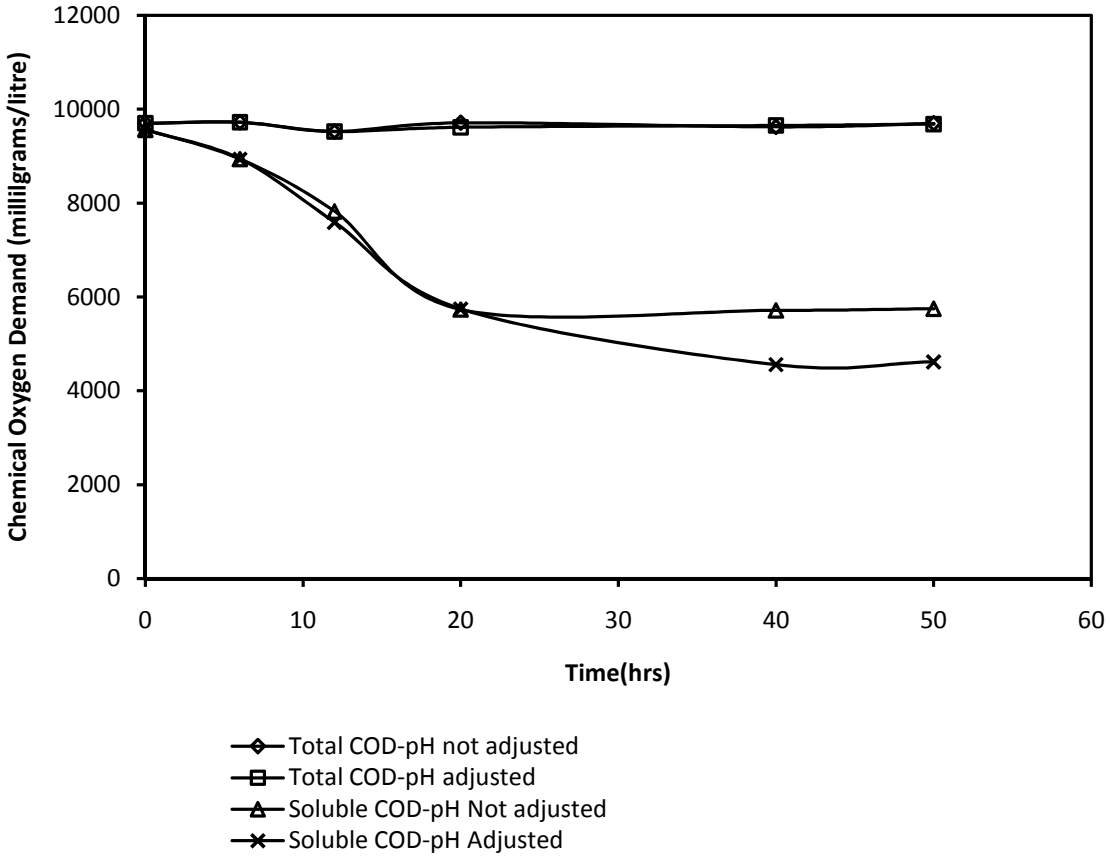


Fig. 3.3.2.3. Total and soluble Chemical Oxygen Demand (COD) as function of time.

Moles of carbon dioxide present in the headspace remained almost equal to the number of moles of hydrogen at each point of time (fig.3.3.2.4.). This result is different from the metabolism reported in literature for *T. maritima*, according to which 2 moles of carbon dioxide are produced for every 4 moles of hydrogen produced (Schroder et al., 1994).

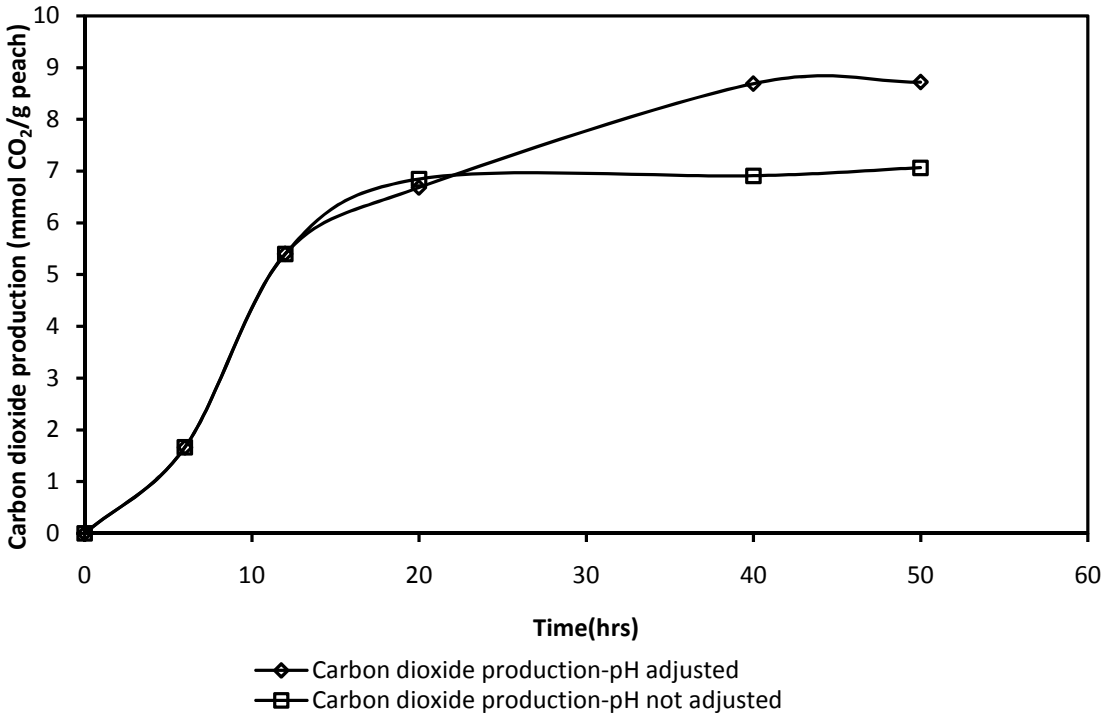
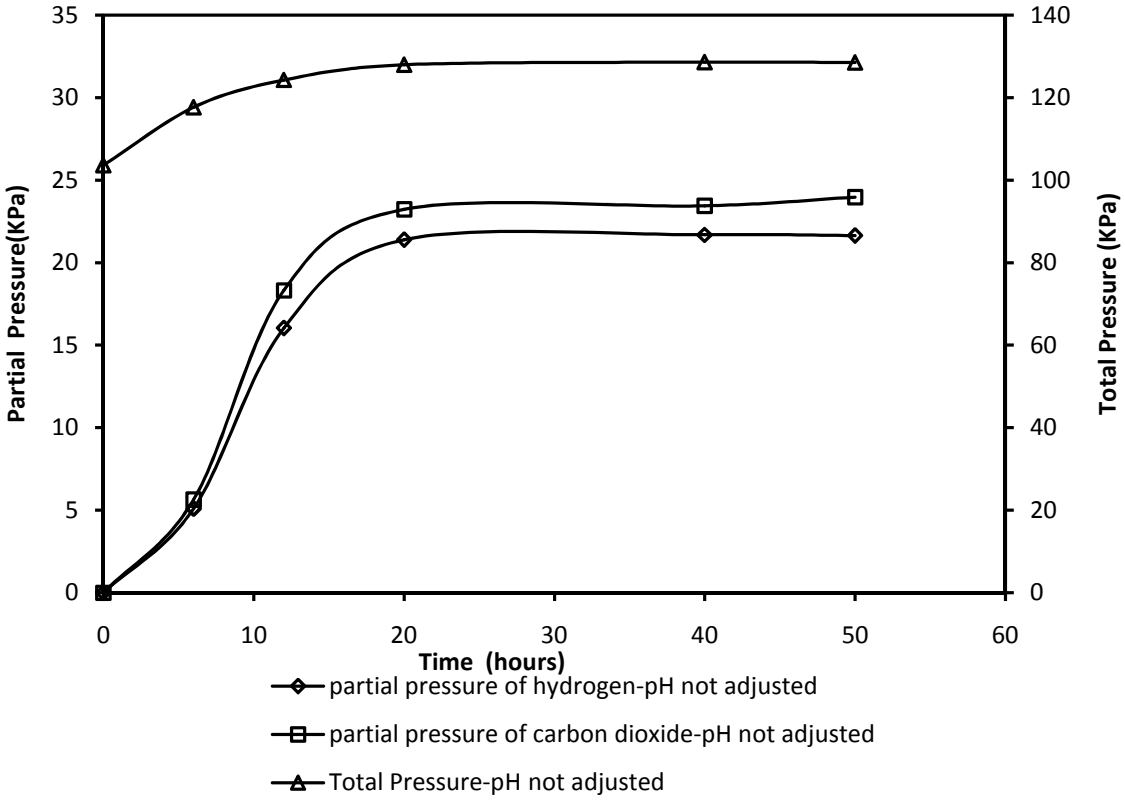


Fig.3.3.2.4. Carbon dioxide production as function of time.

The total pressure in the headspace rose from 128.6 KPa in batch reactors without pH adjustment, to 133.7 KPa in batch reactors with pH adjustment; whereas the partial pressure of hydrogen rose from 21.6 KPa in batch reactors without pH adjustment to 29.6 KPa in batch reactors with pH adjustment (fig. 3.3.2.5a. and fig. 3.3.2.5b.). The partial pressure of carbon dioxide also rose from 24 KPa to 29.6 KPa in batch reactors with pH adjustment. This strengthens our result that hydrogen yield that is the hydrogen produced per gram of COD increased with adjustment of pH, as the metabolism could have shifted towards hydrogen production instead of production of reduced products when pH was adjusted.



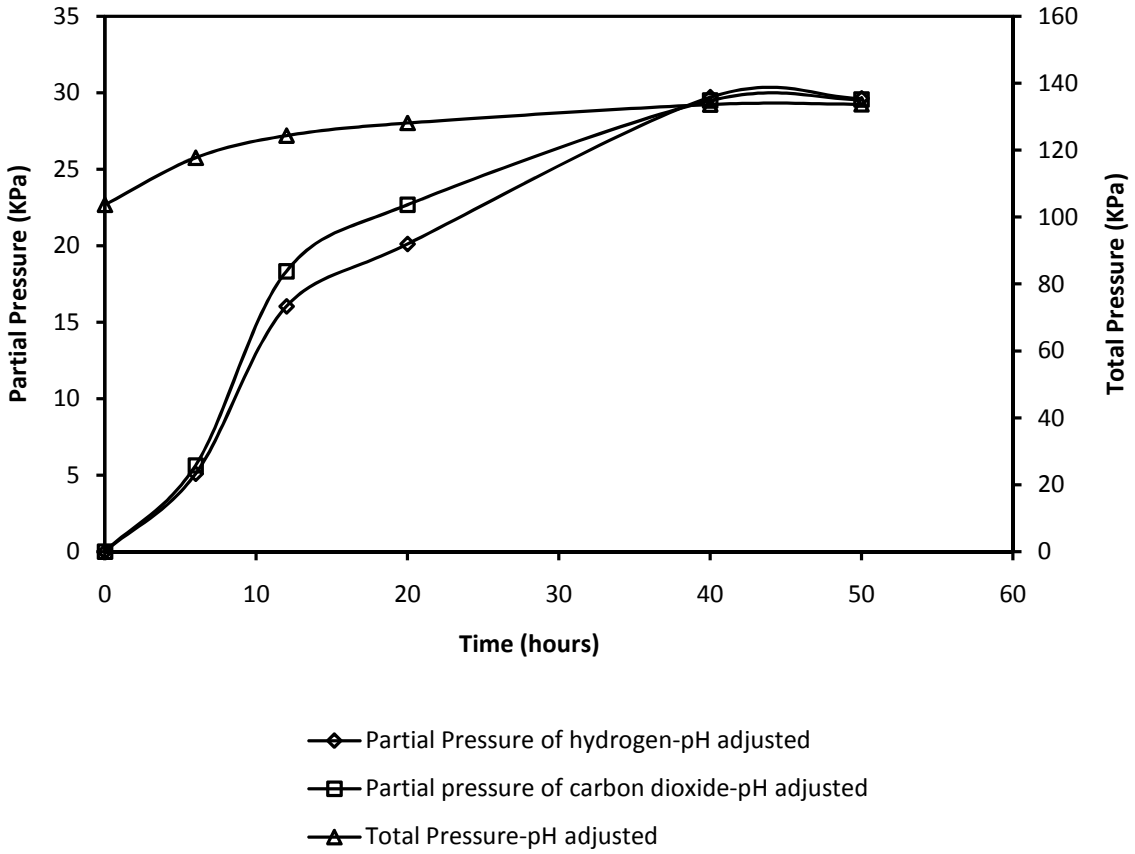


Fig. 3.3.2.5(a, b). The total pressure and partial pressure of hydrogen and carbon dioxide as function of time.

The maximum rate of hydrogen production observed was 3.31 mmol H_2/L.h and was same for reactors with and without pH adjustment (fig. 3.3.2.6.). Yu (2008) reported the maximum rate of hydrogen production on glucose by *Thermotoga neapolitana* to be 4.8 mmol H_2/L.h. Nguyen et al. (2008) reported the maximum hydrogen production rate by *T. neapolitana* on glucose media to be 8.7 mmol H_2/L.h. The maximum hydrogen production rate for *Thermotoga elfii* reported by van Niel et al. was 2.7- 4.5 mmol H_2/L.h (van Niel et al., 2002). Therefore, our

results are similar to reported by van Niel. This rate was observed for incubation time of 6-12 hours. Though, the hydrogen production rate for reactors without pH adjustment dropped to almost negligible after 20 hours; the production rate for reactors with pH adjustment was 0.786 mmol H_2/L.h for 20 hours to 40 hours period of incubation time.Yu (2008) also reported that the exponential phase for *T. neapolitana* was from 2 hours to 10 hours of incubation whereas hydrogen was produced till 12 hours. As hydrogen is a growth associated product, therefore our results of maximum hydrogen production rate lying between 6 to 12 hours of incubation corresponds to observations reported by Yu. The hydrogen production rate was maintained at relatively low rate, in reactors with pH adjustment, because the limitation due to partial pressure of hydrogen is also inhibiting the production. But the hydrogen production on peach medium continues till 20 hours, which is different from Yu's observation on glucose medium. This is because major sugar in peaches is sucrose (Mateja et al., 2004) along with glucose and fructose. Yu reported fermentation time of 30 hours for sucrose. The bacteria should have utilized glucose first followed by sucrose and xylose.

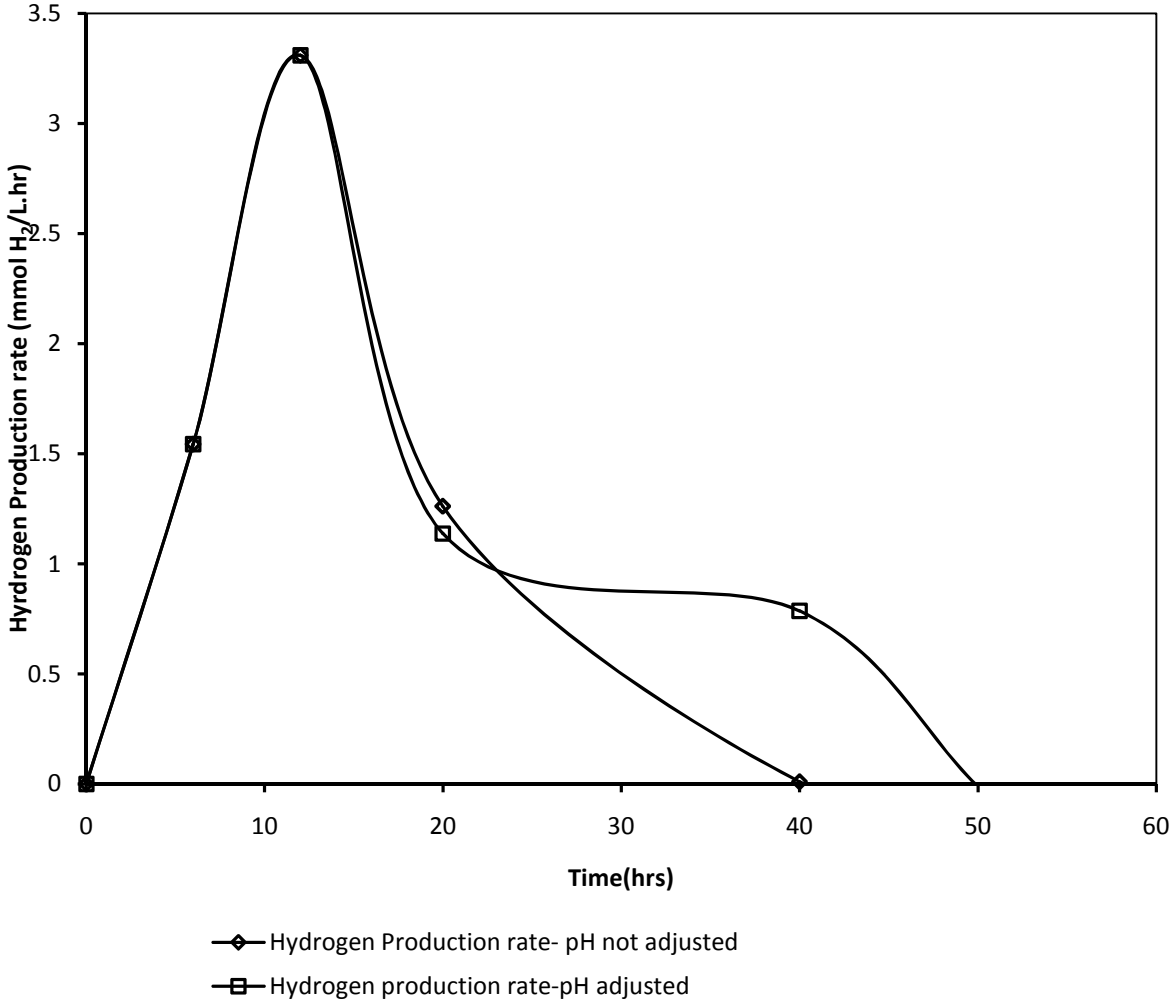


Fig.3.3.2.6. Hydrogen production rate (mmol hydrogen/hour.literof medium)

3.3.3. Effect of pH adjustment to two different pH levels after 12 hours of incubation-There was no difference found in the hydrogen concentration when pH was adjusted to 6.5 or 7.5 after 12 hours (α=0.05, p=0.2084) (table 3.3.3.1.). The reason might be the inhibition on hydrogen production due to partial pressure of hydrogen. Hydrogen

partial pressure is an important factor to inhibit hydrogen production (Claassen et al., 1999). The limit of hydrogen partial pressure increases to 2,000 Pa for *Pyrococcus furiosus* at 98°C (Adams, 1990), and the limit of hydrogen partial pressure increase to 10,000 – 20,000 Pa for *Caldicellulosiruptor saccharolyticus* at 70°C (van Niel et al., 2003). Yu (2008) reported the limit of hydrogen partial pressure for *Thermotoga neapolitana* growth at 77°C was 38 KPa, based on growth inhibition equation. The final partial pressure of hydrogen, when pH was adjusted to 6.5 and 7.5, was 37.1 KPa and 37.5 KPa respectively. These values are almost equal to the partial pressure limit reported by Yu.

Table 3.3.3.1. Hydrogen concentration when pH was adjusted to two levels after 12 hours of incubation.

Medium	Mean Hydrogen concentration (%)	Absolute Total Pressure (KPa)	Mass of hydrogen in headspace (g)	Mean Hydrogen concentration (mmol /L medium)	Mean Hydrogen Production (mmol/g dry weight peach)
pH adjusted to 6.5after 12 hours	25.94	142.96	0.013472	67.36	9.68
pH adjusted to 7.5 after 12 hours	26.20	142.96	0.01361	68.04	9.78

3.3.4. Effect of substrate concentration with pH adjustment-There was a difference in the hydrogen concentration produced at two different substrate concentrations (α=0.05, p=0.0259), when pH was adjusted (table 3.3.4.1). The amount of hydrogen produced decreased when the peach concentration was raised from 50 g/L to100 g/L (wet weight).Yu (2008) reported that the rate of growth for *T. neapolitana* increased with the increase in glucose concentration, though the maximum cell mass produced at the end of

exponential phase remained the same. Nguyen et al. (2008) reported the increase in glucose consumed and acetic acid produced at higher concentrations of glucose.Hydrogen production and growth was reported to increase as glucose concentration increase from 5 g/L to 7.5 g/L, but it decreased as the glucose concentration was further increased (Nguyen et al., 2008). Therefore, the substrate inhibiton at higher concentrations of peach might be the reason for decrease in hydrogen production.

The major sugar in peaches is sucrose. The amount of sucrose in peaches is 5.2 g/100 g peach as compared to 6.8 g total sugars/100 g peach (Mateja et al., 2004). Thus the actual sugar concentration increased from 3.4 to 6.8 g/L approximately, when we increase the peach substrate concentration from 50 g/L to 100 g/L, on wet weight basis. The presence of sucrose being the major sugar in our studies instead of glucose might explain the different inhibitory sugar concentration in our results as compared to reported by Nguyen et al. (2008).

Table 3.3.4.1. Hydrogen concentration for two peach concentration when pH was adjusted after 12 hours of incubation.

Medium	Mean hydrogen concentration (%)	Absolute Pressure (KPa)	The mass of hydrogen in headspace (g)	Mean Hydrogen concentration (mmol /L medium)	Mean Hydrogen production (mmol/g dry weight peach)
Peach medium @50g/L (wet weight) with pH adjustment	25.56	143.13	0.01314	65.72	9.45
Peach slurry @100g/L (wet weight) with pH adjustment	24.27	142.13	0.01239	61.97	8.91

3.4. Conclusion

pH affects the hydrogen production by *Thermotoga neapolitana* using cull peaches. Initial pH has profound effect on the amount of hydrogen produced. It increases as the initial pH rose from 7.0 to 8.0 and decreases as the initial pH increases further. pH control is neceassary for hydrogen production, otherwise the metabolism shifts to the production fo more reduced products like lactate. Hydrogen partial pressure may also be

inhibiting the hydrogen prodution and therefore the amount of hydrogen produced was same when pH was adjusted to two different pH of 6.5 and 7.5 respectively. Hydrogen production by *T. neapolitana* may also be inhibited at higher substrate concentraions.

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CHAPTER FOUR

CONCLUSIONS AND SUGGESTIONS

4.1. CONCLUSIONS

Blended, depitted peach, used as carbon source in a defined medium, was studied for biohydrogen production by the hyperthermophilic bacteria, *Thermotoga neapolitana*. *T. neapolitana* produced 18%-25% of hydrogen concentration in headspace. No pretreatment was needed for fermentation.The fermentation of peaches completes in 20 hours, without pH control as hydrogen concentration doesnot increase after that. The final pH of the medium drops to 4.9 and is a major inhibitor of hydrogen production. There is no need for autoclaving the medium for hydrogen production by *Thermotoga neapolitana* as no difference in hydrogen production was observed for the autoclaved vs non autoclaved medium. Yeast extract is important, asnitrogen source, for hydrogen production; whereas addition of trypticase also increase the amount of hydrogen produced. Soybean meal as nitrogen source along with peaches as carbon source produce a good amount of hydrogen for fermentation.

pH has a profound effect on hydrogen production by *T. neapolitana*. The amount of hydrogen produced varies significantly with the initial pH. An initial pH of 8.0 is best for hydrogen production on peach medium. pH adjustment, to increase the pH, after 12 hours leads to increase in amount of hydrogen produced. The amount

of hydrogen increases from 7.07 mmol H_2/g peach to 8.73 mmol H_2/g peach, when pH is adjusted. Though there is no difference in the amount of hydrogen produced when pH is adjusted to 6.5 and 7.5 respectively. The maximum partial pressure of hydrogen obtained in this study is around 38 KPa approximately after pH adjustment. This is almost equal to the partial pressure limit, before it starts inhibiting hydrogen production, stated in other studies. Therefore, partial pressure of hydrogen may also be inhibiting the hydrogen production by the bacteria. There is a substrate inhibition on hydrogen production as it decrease when peach concentration was increased from 50 g/L to 100 g/L, on wet weight basis, with pH adjustment after 12 hours.

The dry weight of peaches has profound effect on the total amount of hydrogen produced in headspace and it changes with peach variety and storage conditions. The hydrogen yield increased from a mean value of 2.57 mol H_2/ mol six carbon sugar to 3.26 Mol H_2/ mol six carbon sugar when pH was adjusted.

4.2. Suggestions

pH and partial pressure of hydrogen are the major inhibitors for hydrogen production by *Thermotoga neapolitana*. Continuous control of pH by adding the suitable alkali (like sodium hydroxide) using a proper pH control method, should completely alleviate the role of pH in metabolic shift to reduced products. This needs to be studied first, to properly study the role of partial pressure. The study

on effect of headspace to volume ratio may be one way to reduce this inhibition; though the continuous evacuation of gases from headspace holds the key.

Resazurin has been used as the oxygen indicator in most of the studies. But the color produced by resazurin depends on pH and temperature as well along with oxygen. Therefore, the use of redox probe is recommended to measure the exact reduction potential needed to assure growth every time.

The medium used for cultivation of *Thermotoga neapolitana* consists of salt at 10 g/L. This adds a lot to the cost of the medium and needs to be reduced. Studies to find the optimum salt concentration in terms of the cost involved and hydrogen produced needs to be done.

Most of the studies reported include the batch reactor studies on hydrogen production, and continuous reactor operation for biohydrogen production needs to studies. A proper heat exchanger design to reduce the energy cost for the continuous reactor operation is recommended.

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APPENDIX A

RESULTS

A.1.Hydrogen yield

The following tables compiles the result obtained throughout the study in terms of dry weight of peach added and the yield obtained in terms of total sugar added.

Table A.1.1. Dry weight of peach slurry.

Run (data only for reactor sets with peach concentration of 50 g/L, on wet weight basis.	**Percentage Solid (%)**	**Dry weight added/lit-er medium (g/L)**	**Approxima-te total sugar added (g/L)**	**Hydrogen Concentration (Mmol H_2/L medium)**	**Hydrogen Yield (Mol H_2/ mol six carbon sugar)**
Peach vs Glucose	13.34	6.73	3.45	51.45	2.69
Autoclaved vs Unautoclaved	13.91	6.97	3.57	53.35	2.59
Different peach concentration, without pH control	13.8	6.96	3.57	52.38	2.64
Different initial pH	13.69	6.95	3.56	52.58	2.66
pH adjusted to 6.5 pH not set to 6.5	13.67	6.97	3.57	67.36 57.33	3.39 2.89
pH set to 7.5 pH not adjusted to7.5	13.8	6.95	3.56	68.04 53.58	3.44 2.71

Run (data only for reactor sets with peach concentration of 50 g/L, on wet weight basis.	**Perce-ntage Solid (%)**	**Dry weight added/lit-er medium (g/L)**	**Approxima-te total sugar added (g/L)**	**Hydrogen Concentration (Mmol H_2/L medium)**	**Hydrogen Yield (Mol H_2/ mol six carbon sugar)**
Different concentration with pH adjusted to 6.5	13.67	6.96	3.57	52.74	2.66
Soybean meal as nitrogen source	12.85	6.45	3.31	45.19	2.46
Different nitrogen sources	11.74	5.96	3.05	37.95	2.23
pH adjustment with COD measurement pH not adjusted pH adjusted to 7.5	12.22	6.16	3.16	39.31 53.83	2.24 3.07

The average of the percentage solid presented for different runs in the above table is 13.27%. Assuming that standard solids percentage for Redheaven variety of peaches used in the study is 13.27%, total solids present in 100 g of peach is 13.27 g. The total sugar content in standard Redhaven variety of peach is 6.8 g/100 g peach (Mateja et al., 2004). The following parameters are calculated –

1. Amount of sugar added per liter medium= (6.8/13.27)*(Dry weight added per Liter medium)

2. Moles of hydrogen produced per gram of sugar present = (moles of hydrogen produce per liter of medium)/(total sugar added per Liter of medium)

3. Moles of hydrogen produced per mole of six carbon sugar = (Moles of hydrogen produced per gram of sugar)*180

Table A.1.1. shows that the hydrogen yield varied from 2.23 Mol H_2/ mol six carbon sugars to 2.89 Mol H_2/ mol six carbon sugar, when pH was not controlled. Whereas the hydrogen yield increased to 3.07 Mol H_2/ mol six carbon sugar to 3.44 Mol H_2/ mol six carbon sugars when pH was adjusted after 12 hours. Zhu (2007) reported that hydrogen yield for glucose as carbon source increased from 2.2 mol H_2/ mol glucose to 3.6, when pH was adjusted in early stationary phase. Yu (2008) reported hydrogen yield of 2.54 Mol H_2/ mol glucose. Our results match these results.

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Zhu, Hongbin, 2007. Hydrogen Production and Utilization of Agricultural Residues by *Thermotoga species*. MS dissertation, University of Water LOO.

APPENDIX B

MATERIAL AND METHODS

B.1. Material

The standard procedure described by Yu (2008) for cultivating *Thermotoga neapolitana* was modified for cultivating it using cull peaches as carbon source.

B.1.1. Peaches- The first batch was obtained as frozen depitted peaches of Redhaven variety stored in a zip-lock bags. These peaches were preserved at -70°C. The second batch of peaches of Red Heaven variety was handpicked at Clemson University Musser Farms. These peaches were also stored at -70°C.

B.1.2 Preparation of peach slurry- Frozen peach was taken out from -70°C freezer and kept at room temperature, in a china dish, for two hours to allow it thaw. The peach was then cut into small pieces using a knife. The cut peach was then blended for two minutes using hand held blender, until uniform slurry was obtained. One peach weighed between 230 g to 250 g and therefore one fruit was enough for single run. Note that it is good to blend at least whole of the fruit every time to obtain uniform slurry.

B.2. Medium Preparation

1. The following medium was used to prepare the batch reactors of *Thermotoga neapolitana*-NH_4Cl, 1 g; K_2HPO_4, 0.3 g; KH_2PO_4, 0.3 g; $MgCl_2.6H_2O$, 0.2 g; KCl, 0.1 g; $CaCl_2.2H_2O$, 0.1 g; NaCl, 10 g; Cysteine $HCl.H_2O$, 1.1 g; Trypticase,

2 g; Yeast Extract, 2 g; Vitamin solution (DSM medium141), 10 ml; Trace element solution (DSM medium 141), 10 ml; Triazma base, 0.121 g; H_2O, 1 Liter.

2. Pour 100 ml of the medium in 500 ml serum bottles to allow 450 ml of actual head space.
3. Weigh 5 g of the peach slurry to a 50 ml of conical flask. Transfer it to the serum bottle to obtain 50 g/L of the peach concentration on wet weight basis.
4. When multiple serum bottles are prepared, peach slurry should be weighed separately for each of the serum bottle; otherwise equal amount of peach slurry might not be transferred to all the serum bottles.
5. 5 N NaOH was used to adjust the pH.

B.3. Sparging

7. Open the valve for nitrogen cylinder and keep the outlet pressure at 1 psi.
8. Release nitrogen for 1-2 minutes to remove air from the tube.
9. Place one tube needle into liquid and one tube needle in headspace, and sparge for 5 minutes.
10. Apply a flange type rubber stopper and seal it with an aluminum cap using crimper.

B.4. Inoculation

11. Sparge nitrogen into an empty serum bottle to create a nitrogen bottle.
12. Seal this bottle with a flange type rubber stopper and an aluminum cap.

13. Use syringes to continuously sparge nitrogen into the nitrogen bottle
14. Fill a 3 ml syringe with nitrogen from the nitrogen bottle.
15. Inject the nitrogen from the syringe in step 14 into the serum bottle containing seed culture and take out the seed culture using the same syringe.
16. Syringe 2 ml of the seed culture into fresh medium bottles prepared in sparged phase.
17. Put the inoculated bottle in shaker for incubation at 77°C and 200 rpm.

B.5. Adjusting the pH

1. Start the experiment with three extra reactors incubated initially.
2. Take out these three reactors at 12 hours and cool them down to 25°C by putting them in water bath at 25°C.
3. Decrimp these reactors. Measure the pH in these reactors
4. Adjust the pH to the required value, using 5N NaOH. Note the quantity of 5N sodium hydroxide required.
5. Take excess amount of 5N NaOH in serum bottle. Sparge this bottle with nitrogen for 5 minutes and seal the bottle as described earlier.
6. Using a 3 ml syringe take out the equal amount of 5N NaOH as noted in step 4 and it to the respective reactors in which pH has to be adjusted.
7. Take the samples from these reactors and measure their pH at 25°C to assure that the pH has actually been adjusted to the required value.

B.6. Seed Culture- The medium was prepared as described earlier with 50 g/L of peaches, on wet weight basis. It was inoculated with one glycerol stock of Thermotoga neapolitana, stored at -70°C. The bottle was incubated for three days at 77°C and preserved at 4°C. Subsequent seed cultures were prepared from this bottle by inoculating them with 2 ml of the inoculums and incubating for 20 hours. The seed culture was stored at room temperature and used for further experiments. The seed culture kept at room temperature loses their viability after two weeks and hence, needs to be prepared every two weeks.

B.7. Resazurin- The color due to resazurin in the medium was observed to be dependent on pH and temperature as well, along with the presence of oxygen in the medium. When resazurin was added to the peach medium, it imparted deep purple color to it. The color slowly shifts to dark to light pink as nitrogen is sparged. The color of the medium remained light pink when serum bottles are sealed, inoculated and put in the orbital shaker. The medium regained its original color (and not pink) after about 20-30 minutes it is placed in the orbital shaker at 77°C.

B.8. Effect of temperature on pH of peach medium-The pH of the medium was found to be different at room temperature (25°C) and 77°C for peach medium (table B.8.1). The pH of the medium was higher at lower temperature. The difference in pH of the medium at the two temperatures, 25°C and 77°C, was lower for low pH (fig.B.8.1).

Table B.8.1. The pH of the peach medium was different at 25°C and 77°C.

pH of the medium at 25 ℃ (pH 25)	**pH of the medium at 77℃ (pH77)**	**δpH (pH25-pH77)**
8.0	7.0	1.0
7.7	7.15	0.55
6.6	6.35	0.25
4.75	4.56	0.187

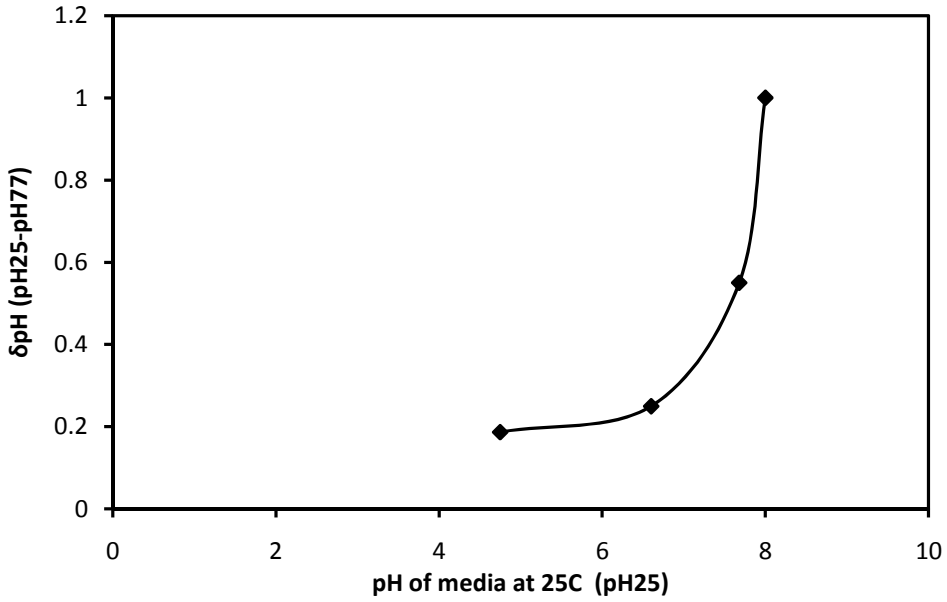

Fig. B.8.1. The difference in pH of peach medium at two temperatures.

Reference:

Yu, X., Drapcho, C.M., 2008. Biohydrogen production by the hyperthermophilic bacterium *Thermotoga neapolitana*. PhD dissertation, Clemson University.