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Anaerobic biohydrogen production using

different bacterial seed sources

by

Steven Wenetta Van Ginkel

A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Civil Engineering (Environmental Engineering)

Major Professor: Shihwu Sung

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This is to certify that the Master's thesis of

Steven Wenetta Van Ginkel

has met the thesis requirements of Iowa State University

Signatures have been redacted for privacy

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CHAPTER 1 INTRODUCTION

Biological hydrogen production seems to be a well studied process.   Nandi and Sengupta (1998) believe hydrogen production to be a well studied microbial process and hydrogen to be a clean alternative energy source to fossil fuels. However, they also believe there is not much demand for this process due to current inexpensive fossil fuels. Yet, gas prices have risen dramatically in the year 2000.

It is the belief of this author that there will be a day when some clean alternative energy source will be needed to replace fossil fuels. There is a wide range of figures estimating the rate of depletion of the world's fossil fuels. These reserves are finite. In essence, it really does not matter when our fossil fuels will run out. What matters is – will the world be ready for it?

Currently, there is little demand for alternative energy sources. However, as the cost benefit ratio of fossil fuel exploration increases, fossil fuels will become more expensive. This increased expense will increase the demand for alternative energy sources whether they will be more expensive or less expensive than fossil fuels at that particular time. A look at the 1970s oil shortage shows how expensive gasoline can be. At that time, President Carter of the U.S.A. developed an alternative energy program but it quickly lost interest as exploration tapped into new fossil fuel reserves. In spite of fossil fuel exploration, according to Maugh (1972), fossil fuel production will not keep up with demand and remaining reserves would be better used in the chemical industry and not for their energy content.

It is the opinion of this author that the demand for alternative energy sources will likely increase according to two scenarios. First, economically speaking, the alternative energy source must be cheaper than conventional fossil fuel reserves. Second, the pollution caused by fossil fuel combustion will become so destructive to the world as a whole that fossil fuel combustion will have to be reduced.

In the first scenario, the only driving force is technology. The world's universities and research institutes, whether it be for economic gain or education will try to develop alternative energy sources. This implementation will require research which

involves equipment, time, and manpower, all of which costs money. So, the quesiton is - who will pay for it? Many corporations will likely fund some grants for new research, but to provide financial resources to try to replace fossil fuel consumption with alternative energy consumption in this day will not happen unless some immediate financial gain and other benefits result from introducing the process to the public. It is the opinion of this author that, at least in the heartland of the U.S., fossil fuels are inexpensive and any pollution due to fossil fuel combustion has not caused enough recognizable deleterious effects to warrant the current use of a nonpolluting alternative energy source. In this regard, global warming has not been proven to the community at large.

In the second scenario, the most evident pollution concern is probably global warming due to the increased production of greenhouse gases. According to Maugh (1972), about 90% of energy end use is supplied by the combustion of fossil fuels to produce heat energy for transportation, industry, and homes. The other 10% is supplied by electricity. The combustion of fossil fuels puts a variety of chemicals into the air including carbon dioxide, carbon monoxide, sulfur dioxide, and nitrogen oxides. It is the belief of this author, at least in the United States, that the combustion of fossil fuels in power plants is one of the cleaner methods of fossil fuel combustion. At least this form of combustion is localized, so high expense/high technological pollution abatement equipment could easily be implemented. On the other hand, regulations on the combustion of fossil fuels by automobiles may be adequate when recognizing each individual automobile, but this idea is severely outweighed by the number of automobiles and the total number of miles driven.

No one can prove beyond any reasonable doubt that fossil fuel combustion causes or increases the greenhouse effect which may lead to global warming, fluctuations in El Nino and La Nina, hurricanes, flooding, tornadoes, etc. but it does seem that these natural disasters are increasing in number. One point is clearly evident though - the world is converting organic carbon (oil, natural gas, coal) into inorganic carbon (carbon dioxide) at an ever increasing rate. According to Dr. Eugene Takle at Iowa State University, "If we examine proxy data, we can get very good estimates of the carbon dioxide levels over

longer periods of time. At the time of the industrial revolution in the late 1700s, the amount of carbon dioxide in the earth's atmosphere was about 270 parts per million. The record showed that concentrations grew slowly until the 20th century but have grown very rapidly since then, particularly in the last 50 years ." Between the years 1973 and 1985, carbon dioxide levels have risen from 30 to 350 parts per million. At this rate, carbon dioxide levels will double by the year 2050. Some scientists agree that the debate of global warming is now a non-issue, rather, we should just be concerned how to adapt now that global warming is a reality (Takle, 2000).

Whether the plant life in the oceans, in the Amazon rain forest, or in the corn belt can assimilate this addition of carbon dioxide is a question that can not be proved at the moment. Some research suggests that typical Iowa crops will not benefit from global warming caused by increased levels of carbon dioxide (Rosenzweig and Hillel, 1995). However, 'proof' has never been an adversary to human technological development. The greenhouse effect does not have to be proven and the finiteness of the world's energy resources does not have to be proven either. In this regard, one way to decrease global warming is to replace traditional fossil fuel combustion with cleaner fuel combustion such as the combustion of hydrogen gas.

Currently, the production of hydrogen is 2.28 trillion standard cubic feet (scf) per year in the United States and the world produces 6 trillion scf (Maugh, 1972). According to the Institute of Gas Technology, in 1968, the U.S. would have had to produce 60 trillion scf of hydrogen to provide the energy supplied by natural gas at that time (Maugh, 1972). This would require 1 million megawatts of electricity at current electrolyzer efficiencies, currently three times the current U.S. electrical generating capacity. By the year 2000, it is estimated that the U.S. would have to produce 295 trillion scf of hydrogen to replace fossil fuels other than those used for generation of electricity. It is evident that hydrogen production falls short in supplying this high demand for energy. However, there are numerous technologies in place that could help bridge this gap (Maugh, 1972).

Currently renewable energy accounts for only 8% of the world's energy source while petroleum, natural gas, coal, and nuclear account for 39%, 24%, 23%, 7% respectively. Hydropower, biomass, geothermal, solar, and wind consist of 55%, 38%,

5%, 1%, and 0.5% of the renewable sources of energy (Takle, 2000). Solar energy is probably the best seemingly 'infinite' energy source and can be directly used to create electricity. The same situation exists for wind energy and hydropower although sites are limited for harvesting this energy. Some suggest the production of energy from wind to increase more rapidly than the increases in production of energy from other renewable energy sources (DOE, EIA). Nuclear energy was thought at one time to provide the majority of the world's energy demand, yet several accidents involving the release of radioactive waste limited its use. Fuels derived from plant materials – biomass energy, such as ethanol, have made a place in Iowa and they help Iowa's economy, yet the production of these fuels from plant materials competes with their use as food crops.

Bioenergy or energy derived from bacteria and algae has recently caused much attention. In anaerobic bioenergy production, methane or hydrogen is produced as a result of the degradation of a substrate by microorganisms. This substrate could be wastewater from a food processing plant, a slaughterhouse, or a municipal wastewater treatment plant. In this instance, the dual benefit of waste reduction and energy production is achieved in one step. Aerobic treatment methods could be used to treat this waste but the energy content in the waste would be eliminated and energy would be needed to supply aeration. Although no figures will be given here, there is undoubtedly huge amounts of wastewater from the sources listed above that could be used to generate methane or hydrogen to be used as fuel. In addition, it is apparent that these sources of wastewater are spread evenly throughout the globe. In other words, where there are people, there is wastewater. So, bioenergy could be generated anywhere. If the utilization of bioenergy is not sufficient in supplying the energy needs of the local community, then another method of energy production would have to be used either alone or in addition to bioenergy. Each particular community would have to decide which combination of methods would fit their needs best.

The focus of this research is biohydrogen production for the reasons stated above. Methane production is an additional alternative energy source and has its place in certain situations yet the combustion of methane yields carbon dioxide, a greenhouse gas. Methane is also a greenhouse gas. The combustion of hydrogen in air produces only

small amounts of nitrogen oxides (from nitrogen gas in the air), and the combustion of hydrogen with oxygen produces water. Hydrogen gas can also be used to directly produce electricity via a fuel cell in which no combustion is needed. Hydrogen gas is also used in the chemical industry for purifying petroleum products, for fertilizer production, and to start and warm gasoline fired automobiles (Ramachandran and Raghu, 1998). It has been mentioned that most of the pollution from the internal combustion engine arises from the first moments after starting a cold engine (Ramachandran and Raghu, 1998). In addition, the efficiency of a car burning hydrogen has been estimated to be 50% greater than that of a gasoline fired automobile (Maugh, 1972). According to Maugh (1972), hydrogen can be used to produce synthetic fuels such as methanol and Fischer-Tropsch gasoline from atmospheric carbon dioxide for as little as 30 to 45 cents per gallon.

There are many abiotic ways to produce hydrogen. Currently, we produce 1 billion scf of hydrogen by steam reforming of natural gas (Padro, 1998). Other methods include electrolysis, endothermic chemical decomposition, photolysis, and biohydrogen production. Half of hydrogen produced today is produced by catalytic cracking of hydrocarbons (Maugh, 1972). This latter method would become impractical if the use of hydrogen is to replace hydrocarbon combustion in the future. Of the other methods, only electrolysis has been proved to work in practice on a large scale and fossil fuels are needed to produce the energy for electrolysis (Maugh, 1972). According to Maugh (1972), hydrogen is not really an alternative energy source if fossil fuels are required to produce it. In this regard, only photolysis (using energy from the sun) and biohydrogen production can be classified as alternative energy sources.

One form of biohydrogen production uses photosynthetic bacteria to split water into hydrogen and oxygen. In this method, high efficiencies of hydrogen production can be achieved. Some photosynthetic algae are able to increase the oxidative potential of electrons from water 0.3 volts higher than that of a hydrogen electrode. In turn, they use the reducing potential of these electrons to convert hydrogen ions to hydrogen gas with a hydrogenase enzyme (Maugh, 1972). Beneman (1996) prefers photosynthetic hydrogen production for its high efficiency, yet he says the 'dark' processes are simpler and can

produce hydrogen from a variety of carbohydrates and waste products. In addition, most of the 'dark' processes are anaerobic meaning little external energy is required.

The use of hydrogen as an energy source has its merits already. Currently, NASA uses hydrogen for rocket fuel and is the only consumer of hydrogen strictly for its energy content (Padro, 1998). According to Maugh (1972), combustion of hydrogen produces 325 BTUs per scf which is a third of that of natural gas, yet due to its lower viscosity hydrogen can be pumped at three times the rate of natural gas so one pipe can transmit nearly equal energy contents of either fuel. Hydrogen has 2.75 times the energy content per unit mass of any hydrocarbon, yet due to its low specific gravity (0.07) it requires a greater volume. In this regard, the storage of hydrogen gas is a practical problem. In addition, storing hydrogen in a liquid form is explosive. However, according to Ramachandran and Raghu (1998), metal hydrides are capable of reversibly absorbing hydrogen and they may provide an opportunity for the use of hydrogen as a fuel in automobiles. Metal hydrides enable the storage of hydrogen to be at higher densities and at lower pressures than in the liquid form. Currently, however, the cost of metal hydrides may be too high for automobile application.

The purpose of this thesis is to prove that anaerobic fermentation of synthetic wastes (sucrose) can be used to produce hydrogen in comparable amounts to other research. It seems other research focused only on a few isolated strains of bacteria. In a continuous reactor fermenting a waste to hydrogen, a mixed culture would have to be used since the wastewater contains a mixed culture of bacteria. So, an additional purpose of this thesis is to prove that a mixed culture of microorganisms could be used to produce hydrogen. This mixed culture was collected from the most abundant source of microorganisms – 'nature'. Since the bacteria is collected from nature, it is the opinion of this author that any community in the world could use the process described in this thesis to produce hydrogen. However, the diversity and the abundance of the bacterial culture will depend on where the microorganisms are collected. Since using a pure culture to produce hydrogen would not stay pure in a continuous reactor fermenting real wastes, a selective pressure must be induced to select for a dominant culture of hydrogen producing bacteria. This selective pressure is deemed heat shock treatment in this thesis

and is accomplished by boiling or baking the bacterial culture long enough to inactivate non-hydrogen producing bacteria.

In order to optimize this process, optimum growing conditions (hydrogen producing conditions) for the anaerobic microorganisms must be known. In this regard, this thesis explains how the optimum pH was found in this study using a series of batch experiments. It was also the goal to optimize the food to microorganisms ration. Yet, this was not accomplished because the biomass concentration was not accurately measured due to complications in the VSS measurement as described in this thesis.

Thesis Organization Section

This thesis consists of four chapters plus additional appendices. Chapter two is a literature review of current efforts to produce hydrogen. Chapter Three is a paper explaining the current efforts at Iowa State University to produce hydrogen. This paper explains the use of three variables to produce hydrogen – bacterial seed source, pH, and substrate concentration. Chapter Four is a paper confirming the results in chapter three using smaller sucrose concentrations and a pH buffer. Chapter five is the Conclusions section followed by the Literature Cited section. In appendix one, there is supporting data and graphs. In appendix two, future and ongoing research is discussed.

CHAPTER 2 LITERATURE REVIEW

Much research has been done using pure cultures of bacteria to produce hydrogen. *Escherichia coli* and *Citrobacter spp.* are known to produce hydrogen gas (Roychowdhury *et al.,* 1988). *Clostridium* can produce hydrogen gas (Nandi and Sengupta, 1998). *Enterobacter aerogenes* produces 1 mole of H_2 / mole of glucose consumed while some research focuses on immobilized pure cultures (Kumar *et al.,* 1995) and pure photosynthetic bacterial cultures (Nandi and Sengupta, 1988), other studies used continuous mixed cultures (Brosseau and Zajic, 1982b, Ueno *et al.,* 1996). It is the opinion of this author that the highest conversion rates from immobilized cultures are due to the high biomass concentrations used. Yet, since wastewater contains a high bacterial population, the purity of any wastewater treatment reactor would be lost and, therefore the treatment of wastewater would have to be a mixed culture.

A mixed culture would probably be more able to degrade the wide variety of substrates in a wastewater treatment facility. In a study by Sasikala *et al.* (1994), the authors concluded that hydrogen production was enhanced by using mixed cultures because they maintained the solution at a neutral pH. In the study by Roychowdhury *et al.* (1988) mentioned earlier, a mixed culture taken from sewage produced 0.7 moles H_2 / mole glucose, a rate comparable to that obtained with pure cultures.

The seeding of a reactor treating wastewater would need a large amount of bacteria to start the process. Therefore, this seed source should be present in large amounts. Natural soil and compost served as the seed source in the studies described in this thesis.

Many studies involving the production of hydrogen gas used inhibitors of hydrogen consumers especially the methanogens. To date, acetylene, 2-bromoethanesulfonate (BES), as well as aeration have been used to inhibit hydrogen uptake by the methanogens. According to Sparling *et al.* (1997), inhibition of methanogenesis is essential to efficient hydrogen production. Acetylene can be used as an inhibitor but this compound is known to inhibit the action of other anaerobes. An acetylene concentration of 5% inhibits 50% of the hydrogenase activity in some cultures

(Sparling *et al.*,1997). BES could be used but mutants resistant to this chemical are known to occur in cultures exposed to BES (Sparling *et al.*,1997). According to Roychowdhury *et al.* (1988), a mixed microbial flora from sewage and landfill sediments degrading sugars and natural sources rich in sugars produced hydrogen gas with a hydrogen gas concentration as high as 87%. The conversion from producing methane to producing hydrogen was rapid upon addition of pure sugars or sugar-rich preparations. In this instance, methanogenesis was inhibited by a shock load of high sugar content. When the sugar substrate was introduced, the pH was at neutrality and acidic conditions did not set in until several days later, so a low pH did not inhibit methanogenesis, but a high hydrogen concentration resulting from the rapid degradation of sugars may have (Roychowdhury *et al.*,1988). After acidic conditions set in, hydrogen gas production stopped, but after the addition of NaOH to bring the culture to neutrality, hydrogen gas production resumed. However in methanogenesis, most of the methane is generated from the cleavage of acetic acid with no hydrogen gas production. So, it would seem that the production of acetic acid was inhibited as well. Another explanation could be the high osmotic pressure induced by the high substrate concentration. According to Harper and Pohland (1985), hydrogen gas must be continually removed from the system to ensure the continued production of acetic acid. It has been estimated that a hydrogen partial pressure greater than 10^{-4} atm leads to the accumulation of propionic and butyric acids which inhibits hydrogen production. According to Grady *et al.* (1999), a high hydrogen partial pressure also inhibits acetic acid production and promotes higher molecular weight acid production. The production of butyric and acetic acids appear to coincide with hydrogen production, so a high hydrogen partial pressure would be inhibitive in this instance if these acids were not produced (Lay *et al.*, 1999).

Another method of inhibition of hydrogen uptake , described Lay *et al.* (1999), is heat shock. If a liquid medium containing bacteria is subjected to high temperatures, many bacteria species do not survive. In the cultures we are dealing with those bacteria that do survive are likely to be sporeformers. Once the liquid medium is subjected to natural bacterial growing conditions, the spores can germinate producing viable, growing

cells. According to Gottschalk *et al.* (1981), pasteurization at temperatures of 70 to 90'C for ten minutes is suitable for removing non-sporeforming bacteria.

The purpose of this thesis is to discuss hydrogen gas production under anaerobic conditions using naturally occurring heterotrophic bacteria. There are many types of heterotrophic bacteria that are able to produce hydrogen. These bacteria are able to perform the anaerobic oxidation step in acidogenesis meaning they convert volatile and long chain fatty acids directly to hydrogen and acetic acid (Grady *et al.*, 1999). The most notable bacterial species producing hydrogen are in the genus *Clostridium*. Some members of this genus are well known as pathogens and as solvent or alcohol producers. The solvent producers are commonly used in industry as suggested by numerous publications on the subject (Dabrock *et al.,* 1992; Byung and Zeikus, 1985; Jones *et al.*, 1982). Some species, during their exponential growth phase, are capable of achieving highly efficient conversions of monosaccharides, polysaccharides, and other organic molecules into hydrogen gas. In the literature, it seems photosynthetic bacteria using light energy achieve high efficiencies of hydrogen production as well and many immobilized pure cultures of heterotrophic bacteria achieve high efficiencies of hydrogen production.

Despite the apparent inability of *Clostridium* species to generate hydrogen at the efficiency of photosynthetic organisms, there are some unique features of these organisms that lend themselves well to hydrogen production in a multitude of circumstances. The most important being their spore forming ability and their high prevalence in nature. *Clostridium* species can be obtained from almost any anaerobic condition including agricultural soil, compost, river sediment, and even termite intestines (Gottschalk, 1981).

The Clostridia are capable of producing hydrogen from a variety of substrates (Lay *et al.* 1999; Nandi and Sengupta, 1988). Unlike photosynthetic organisms, the Clostridia derive their energy from the fermentation of organic compounds rather than from light, so the process would be less energy intensive since no light would be used. In addition, no mixing would be needed since mixing coincides with the volatilization of the hydrogen gas. Furthermore, since organic substrates (i.e. wastewater) can be used,

fermentation would contribute to waste reduction in wastewater. As of yet, there seems to be no evidence of photosynthetic organisms degrading complex organic substrates. Clostridia has been shown to degrade sugary wastewater (Ueno *et al.*, 1996), starch (Taguchi *et al.*, 1991; Lay, 1996), municipal solid waste (Lay *et al.*, 1999), damaged wheat grains (Kalia *et al.*, 1994), and lignocellulosic waste (Sparling *et al.*, 1997).

Clostridia's spore forming ability is essential in inducing a selective pressure against hydrogen-consuming bacteria. Bacteria form spores under harsh environmental conditions. These conditions include high temperatures, extremes in pH, desiccation, low substrate availability – essentially almost any condition that does not induce or inhibits normal growth. Bacterial spores are the most resistant life form known and enable certain bacteria to survive extreme conditions under which they normally could not.

For the reasons mentioned, natural soils from an agricultural field and compost were collected and subjected to high temperatures to force the spore-forming bacteria into their spore form. It was the hope that all non-sporeforming bacteria (particularly methanogens) would die leaving just spores. Natural soil and ordinary compost were selected because of their widespread availability and because they were easy to obtain.

Other research in hydrogen production involves pure cultures or mixed cultures of two to three pure strains. These studies were carried out in a laboratory setting using simple substrates. Unfortunately, in a full-scale reactor treating real waste, bacteria are present in the wastewater that would contaminate any 'pure' culture. This is why 'natural' bacteria were obtained. They cost very little as well. Yet, the problem remains of inducing the formation of spores consistently in a full-scale reactor. Hence, this paper shall focus only on the start-up phase of a full-scale reactor by studying batch tests. Maintaining hydrogen-producing spore forming bacteria in a laboratory reactor will undoubtedly be one of the subjects of later research if it is to treat wastewater. This maintenance is termed selective pressure and it may be achieved by controlling the HRT or pH or by inducing shock loads of substrate or inhibitory chemicals such as acetylene or 2-BEA. Acidification may be an important pretreatment step in the hydrogen process to eliminate methanogens which desire a pH of 6.5 to 7.5.

CHAPTER 3 A COMPARISON OF ANAEROBIC BIOHYDROGEN PRODUCTION INOCULATED WITH VARIABLE SEED SOURCES

A paper to be submitted to the Water Research Journal

Authors: S. W. Van Ginkel, J.J. Lay, and Shihwu Sung

Abstract

Four different bacterial seed sources were collected from nature to determine if hydrogen production could be achieved using mixed cultures of bacteria. These sources were agricultural soils where both potatoes and soybeans were grown in as well as two different kinds of compost (designated compost one and compost two). The seed sources were baked for two hours to kill non-sporeforming, hydrogen-consuming bacteria. The study used 250 mL anaerobic batch serum bottle cultures incubated under mesophilic conditions. The bottles were filled to the 150 mL level with 30 grams of seed source, sucrose, and nutrients allowing a 100 mL headspace. A fractional factorial experimental design was used to explain the effects of sucrose concentration and pH on hydrogen production potential and rate. In the design, the sucrose concentration was varied from 2 to 6 grams per bottle (15 to 45 g COD / L) while the pH was varied from 4.5 to 6.5. Nine combinations of pH and sucrose concentration were used in the design. The average conversion efficiencies based on H_2–COD to sucrose–COD for potato, soybean, compost one, and compost two seed sources were 2%, 2%, 10%, and 4%, respectively. The highest hydrogen concentration in the biogas was 82% with an average of 55%. %. The highest values of hydrogen conversion rate using potato soil, soybean soil, compost one, and compost two as the seed sources were 12, 15, 18, and 19 mL / (g COD*L), respectively, while the highest hydrogen production rates were 572, 290, 672, and 592 mL / (L reactor volume*day), respectively. The average values of hydrogen conversion rate using potato soil, soybean soil, compost one, and compost two as the seed sources were 7, 9, 14, and 11 mL/ (g COD*L), respectively. The average hydrogen production rates were 512, 247, 568, and 514 mL / (L reactor volume*day), respectively. The average

lag phases or the time it took to reach the exponential hydrogen production rate were 30, 30, 10, and 10 hours, respectively. Overall, the use of compost as the seed source produced the highest amount of hydrogen and at the highest rate while using agricultural soil as the seed source produced a smaller amount of hydrogen and at a smaller rate. In conclusion, this research demonstrated that hydrogen gas can be produced using mixed bacterial cultures obtained from nature. The optimum pH and sucrose concentration was not conclusively determined in these experiments because the medium was not buffered with respect to pH so the pH became inhibitory at an unknown time. The pH would have to be maintained for more accurate results.

Introduction

Due to the combustion of fossil fuels, carbon dioxide and other pollutants enter the atmosphere. A balanced planet readily assimilates pollutants through its natural processes, but when the planet is overloaded with pollutants and the means by which it assimilates these pollutants is reduced, the planet falls away from the equilibrium. The main concern over the combustion of fossil fuels is the production of excess carbon dioxide, a greenhouse gas, released to the atmosphere. In fact, the greenhouse effect can be directly linked to fossil fuel combustion whether it be from a coal fired power plant or an automobile.

According to Dr. Eugene Takle at Iowa State University, "If we examine proxy data, we can get very good estimates of the carbon dioxide levels over longer periods of time. At the time of the industrial revolution in the late 1700s, the amount of carbon dioxide in the earth's atmosphere was about 270 parts per million. The record showed that concentrations grew slowly until the 20th century but have grown very rapidly since then, particularly in the last 50 years ." For example, annual mean carbon dioxide levels in the atmosphere as measured at Mauna Loa Observatory have risen from 325.5 ppm in 1970 to 338.5 in 1980 to 354.0 ppm in 1990 to 366.7 ppm in 1998. At this rate of increase atmospheric CO_2 levels will double the pre-industrial level (275 ppm) by about 2050 and triple (to 825 ppm) before 2090. Models estimate that a doubling of CO_2 could raise global mean temperatures by 1.5 to 3.5 degrees C above present levels.

Humans, in general, do not want to do without this energy source. So, there is a need for nonpolluting alternative energy sources. In this paper, that alternative energy source considered is hydrogen gas and the combustion product of hydrogen gas is a non-pollutant, water. In addition, hydrogen can be directly used to produce electricity through fuel cells without any combustion.

Furthermore, there is a need to eliminate biological and chemical wastes in an environmentally friendly manner. Much of this waste is carried in water and is a product of food processing. Some examples of this waste include waste from corn, soybean, and rice processing plants. This waste is high in energy content as measured as COD and is treated in conventional wastewater treatment plants. Aerobic wastewater treatment methods could be used to treat this waste, but the energy content would be eliminated and much energy to supply aeration would have to be added.

The need for an alternative energy source and the need for waste reduction can be accomplished using an anaerobic digestion process that produces hydrogen as a fuel. This is one purpose of this study. The hydrogen gas produced can be used in the chemical industry for purifying petroleum products, to directly produce electricity by the means of a fuel cell, and to start and warm gasoline fired automobiles (Ramachandran and Raghu, 1998). It has been mentioned that most of the pollution from an automobile engine arises from the first moments after starting a cold engine so starting and warming an automobile engine using hydrogen would cut down significantly on this pollution (Ramachandran and Raghu, 1998).

There are many ways to produce hydrogen gas. Electrolysis involves splitting water by an electric current into hydrogen and oxygen. Chemical methods include cracking hydrocarbons into hydrogen leaving lower molecular weight hydrocarbons behind. Yet, these methods do not accomplish the combined role of waste reduction/energy production and furthermore, these methods require large inputs of energy as electricity derived from fossil fuel combustion as well. This kind of hydrogen of production does not fall under the alternative energy category since conventional polluting energy sources were used to produce them (Maugh, 1972).

In addition, some forms of cyanobacteria and other photosynthetic bacteria as well as some forms of algae are able to produce hydrogen while photosynthesizing, yet this is a complicated process and can be complicated by highly turbid water and requires high UV light intensities for optimum performance (Benemann, 1996). Yet, because of the high light intensities used, hydrogen production by photosynthetic methods yield comparatively high hydrogen production rates. This method could serve the dual purpose of waste reduction, if the substrate is a waste, although no evidence of this has been published. These studies usually use some simple sugar to produce the hydrogen gas (Sasikala *et al*, 1994; Xu *et al.*, 1995).

Much research has been done using pure cultures of bacteria to produce hydrogen gas (Brosseau and Zajic, 1982a,b; Kalia *et al.*, 1994; Karube *et al.*, 1976). Some research focuses on immobilized pure cultures (Kumar *et al.*, 1995; Xu *et al.*, 1995) while others use a chemostat - completely mixed regime (Brosseau and Zajic, 1982; Ueno *et al.*, 1996). It is the opinion of this author that the comparatively high conversion rates from immobilized cultures are probably due to the high biomass concentration supported by this method. However, Brosseau and Zajic (1982) claim chemostats to have a higher hydrogen production potential because immobilized cultures can become diffusion limited. Yet, whether a chemostat or immobilized bacteria are used, since wastewater is contaminated by bacteria, the purity of any wastewater treatment reactor would be lost and therefore the treatment of wastewater would likely have to be a mixed culture. According to a study by Sasikala *et al.* (1994), the highest hydrogen production rates came from mixed cultures because, collectively, the culture was able to maintain the pH around neutrality.

The seeding of a wastewater treatment reactor would need a large amount of bacteria to start the process. Therefore, this seed source should be present in large amounts. Natural agricultural soil, as determined in this study, served as this seed source. In addition, it would be advantageous to have a high hydrogen-producing bacterial population in this seed source. Luckily, *Clostridium* species – the bacteria possibly responsible for the hydrogen production in this study, are prevalent in any environment.

According to Gottschalk *et al.* (1981), 'The ability to form spores that resist dryness, heat, and aerobic conditions makes the clostridia ubiquitous.'

The genus *Clostridium* has been well studied for its potential to generate hydrogen gas (Brosseau and Zajic, 1982a,b; Lay *et al.*, 1999; Ueno *et al.*, 1992). There are many species of the genus *Clostridium* including saccharolytic and proteolytic species (Minton and Clarke, 1989). According to *Bergey's Manual of Systemic Bacteriology* (Cato *et al.*, 1986), there are 83 species. However, since this publication, 25 additional species have been described (Gottschalk *et al.* 1981). Some species of *Clostridium* are pathogens and others produce solvents or alcohols. The latter use is common in industry as suggested by numerous publications on the subject (Dabrock *et al.,* 1992; Byung and Zeikus, 1985; Jones *et al.*, 1982). Some species during their exponential growth phase, are capable of achieving highly efficient conversions of monosaccharides, polysaccharides, and other organic molecules into hydrogen gas. Another important aspect of *Clostridium* is its ability to form spores. As mentioned previously, a heat shock treatment can be used to select for a 'pure' culture of *Clostridium* and other sporeformers such as *Bacillus*. According to Gottschalk *et al.* (1981), several species of *Clostridium* produce heat-resistant spores when cells are subjected to temperatures of 100°C or more for several hours. Some species of *Clostridium* are more heat-resistant than others, so it may be advantageous to use as low of heat as possible for pasteurization to initiate the production of clostridial spores so as not to eliminate less heat tolerant hydrogen-producing Clostridia. Incubation for 10 min at temperatures of 70, 80, or 90°C is sufficient to eliminate mesophilic non-sporeformers (Gottschalk *et al.*, 1981).

The Clostridia are obligate anaerobic heterotrophs that ferment and do not contain a cytochrome system (Nandi and Sengupta, 1998). Clostridia are fermentative bacteria transferring electrons from one organic compound to another rather than having a terminal electron acceptor. This genus produces hydrogen using the activities of pyruvate-ferredoxin-oxidoreductase and hydrogenase enzymes. *Clostridium* species have an optimal pH range of 6.5 to 7.0 for growth which is similar to that of methanogens (Grady *et al.,* 1999; Minton and Clarke, 1989).

In typical anaerobic processes, hydrogen is produced in a step called anaerobic oxidation where hydrogen is produced from the degradation of volatile and long chain fatty acids. During the exponential growth phase of *Clostridium*, hydrogen gas is mainly produced, yet when the population reaches the stationary phase of growth, solvents or alcohols are mainly produced. This shift occurs when the pH drops to 4.5 (Byung and Zeikus, 1985). Apparently, the build up of volatile fatty acids and hydrogen during the exponential growth phase inhibits additional hydrogen production. Accordingly, it is important to remove excess hydrogen from the system and maintain the pH at neutrality to keep the system producing hydrogen.

According to Harper and Pohland (1986), hydrogen must be continually removed from the system to insure the continued production of acetic acid which is the main precursor to methane. According to Brosseau and Zajic (1982 a,b), pathways in the degradation of glucose could yield one mole of acetic acid and four moles of hydrogen or one mole of butyric acid and two moles of hydrogen. So, if hydrogen needs to be produced continuously at an optimum rate, acetic acid needs to be produced continuously as well. It has been estimated that a hydrogen partial pressure greater than 10^{-4} atm leads to the accumulation of propionic and butyric acids instead of acetic acid which inhibits further hydrogen production. In hydrogen production with the inhibition of methanogenesis, the conversion of acetic acid into carbon dioxide and methane is blocked, so acetic acid would build up in the system potentially lowering the pH. This pathway of methane production is deemed aceticlastic methane production (Grady *et al.*, 1991). This accounts for 2/3 of methane produced in anaerobic processes. The other 1/3 of methane produced is from H_2-oxidizing methanogens which reduce carbon dioxide. If both of these pathways are blocked by inhibiting methanogens, the pH may become inhibitory, H_2 builds up in the system and higher molecular weight acids such as butyric and propionic acid are produced instead of acetic acid (Grady *et al.*, 1999). However, in a study by Lay *et al.*, (1998) on hydrogen production, most of the hydrogen production coincides with the degradation of butyric acid.

If methane formers are inhibited, hydrogen must be removed by other means to allow continuous hydrogen production. One way to help achieve this goal is using a

continuously stirred reactor to help the hydrogen gas leave the system. In this way, hydrogen is removed and some loss of COD can be accomplished although this loss of COD is quite small compared to methanogenesis. In this regard, this study does not focus on COD reduction. In a real wastewater treatment facility, the optimum alternative energy production and waste reduction scheme would be to produce hydrogen gas followed by methane production.

As stated earlier, many studies involving the production of hydrogen gas rely on the inhibition of hydrogen consumers - especially methanogens (Lay *et al.*, 1999; Roychowdhury *et al.*, 1988; Sparling *et al.*, 1997; Ueno *et al.*, 1996). To date, acetylene, 2-bromoethanesulfonate (BES), as well as aeration have been used to inhibit methanogens. According to Sparling *et al.*, (1997), the inhibition of methanogens is essential to efficient hydrogen production. Acetylene can be used as an inhibitor but this has been known to inhibit the hydrogenase enzyme of other anaerobes. It has been mentioned that a 5% acetylene concentration inhibits 50% of the hydrogenase activity in some cultures. BES could be used yet there are mutants resistant to this chemical (Sparling *et al.*, 1997).

Another method of inhibition is heat shock. If a liquid medium containing bacteria was subjected to high temperatures, many bacteria species could not survive. Those that do survive are likely to be sporeformers. Once the liquid medium is returned to natural bacterial growing conditions or inoculated into fresh growth medium, the spores can germinate producing viable, growing cells (Gottschalk *et al.*, 1981).

The Clostridia's spore forming ability is essential in inducing a selective pressure. Bacteria form spores under harsh environmental conditions. These conditions include high temperatures, extremes in pH, desication, low substrate availability – essentially almost any condition that does not induce or inhibits normal growth. Bacterial spores are the most resistant life form known and enable bacteria to survive extreme detrimental conditions.

For the reasons mentioned, natural soil from an agricultural field and compost were obtained and subjected to high temperatures to force the bacteria into their spore form. It was the hope that all non-sporeforming bacteria (particularly methanogens)

would perish leaving only spores. Natural soil and ordinary compost were selected as seed sources because of its widespread availability and because it is easy to obtain. Some studies have used mixed cultures to produced hydrogen gas (Ueno *et al.*, 1995; Roychowdhury *et al.*, 1988). However, it seems most research in hydrogen production involves pure cultures or mixed cultures of two to three pure strains (Sparling *et al.*, 1997; Brosseau and Zajic, 1982a; Kumar *et al.*, 1995; Taguchi *et al.*, 1992; Kalia *et al.*, 1994). These studies were carried out in a laboratory setting using simple substrates. Unfortunately, in a full-scale reactor treating real waste, bacteria are present in the wastewater that would contaminate any 'pure' culture. This is why 'natural' bacteria were obtained. They cost very little as well. Yet, the problem remains of inducing the formation of spores consistently in a full-scale reactor. Hence, this paper shall focus only on the start-up phase of a full-scale reactor by studying batch tests. Maintaining hydrogen-producing spore forming bacteria in a laboratory reactor will undoubtedly be one of the subjects of later research if it is to treat wastewater. This maintenance may be achieved by controlling the HRT or pH or by inducing shock loads of substrate or inhibitory chemicals such as acetylene or 2-BEA.

Materials and methods

Seed source

Four types of bacterial seed sources were obtained in this study. Two different types of soil were obtained from the Iowa State University Student Farms in Ames, Iowa. The first type was soil in which potatoes were grown the previous season and the second type was soil where soybeans were grown the previous season. The soil was collected in February and March. The ground was not frozen and the soil was obtained from a depth of roughly two to eight inches. The other two types of seed source were compost obtained from the Iowa State University compost facility in Ames, Iowa. The first type was obtained from the upper portion of the compost pile and was high in organic content due mainly from leaves and grass from the campus. The second type of compost was

obtained from the same compost pile as the first, but more towards the bottom. It was high in sand content. Both types of compost were very warm to the touch and strong in smell.

Heat shock

These four bacterial seed sources were spread out on flat aluminum pans and baked in a 104'C oven for two hours. This dry mix was then crushed into a fine material (less than 4 mm in diameter) using a mortar and pestle for easy handling.

Fractional factorial design

In order to describe the effects of initial substrate concentration (sucrose) and initial pH and their interaction, eleven 250 mL batch serum bottles were subjected to a fractional factorial design as shown in Table 1. The sucrose was varied from two to six grams per bottle with a central value of four grams while the pH adjustment varied from 4.5 to 6.5 with a central value of 5.5. Each 'X' in Table 1 designates one batch serum bottle while 'X^3' designates three batch serum bottles.

Table 1. Experimental Design

		pH				
		4.5	5	5.5	6	6.5
	2			X		
	3		X		X	
Sucrose	4	X		X^3		X
g/150mL	5		X		X	
	6			X		

In order to fit the factorial design, the initial pH and sucrose values were evenly distributed by the means of coded units. The levels of sucrose and pH in coded units were

$$x_i = (X_i - X_i^*)/\Delta X_i$$

where x_i is the coded value of the ith test variable, X_i is the uncoded value of the ith test variable, X_i^* is the uncoded value of the ith test variable at the center point and ΔX_i is the step change value as described by Lay *et al.,* (1998), Box *et al.,* (1978). In his research, the coded values were (-2, -1, 0, 1, 2) corresponding to sucrose values of (2, 3, 4, 5, 6) in grams / 150 mL bottle. The step change value ΔX_i was one.

Five levels of initial pH and five levels of sucrose were used. Twenty-five combinations of these factors could have been used, yet this study used nine *who* combinations encircling the central point of (4, 5.5). See Table 1. The center point was replicated three times to achieve an accurate value at this point. This center point was chosen based on earlier research performed by Lay *et al.,* (1999).

Procedure

Thirty grams of the dry bacterial seed source mix was added to each of the eleven batch serum bottles along with the prescribed sucrose amount and a half a mL of nutrient stock solution. Each liter of nutrient stock solution, modified from Lay *et al.* (1999), contained 200 g of NH_4HCO_3, 100g of KH_2PO_4, 10g $MgSO_4$-$7H_2O$, 1.0 g of NaCl, 1.0 g of $Na_2MoO_4\bullet2H_2O$, 1.0 g of $CaCl_2\bullet2H_2O$, 1.5g of $MnSO_4\bullet7H_2O$ and 0.278g of $FeCl_2$. The bottles were then filled to the 150 mL mark using nanopure water. The pH was then adjusted to the prescribed amount using either 1 M HCL or 1 M KOH and then immediately capped with a rubber septum stopper. The bottles were then flushed with nitrogen gas for fifteen seconds to achieve an anaerobic condition inside the bottle. The stoppers were immediately replaced and tied down with plastic fasteners.

The batch of eleven bottles were then placed in a incubator/shaker at 37°C and at a horizontal rotational speed of 180 rpm. Subsequently, usually after twenty-four hours,

the biogas was sampled using 2 to 50 mL syringes according to the Owen approach (Owen *et al.*, 1979).

The percentage of hydrogen gas present in the biogas was determined by comparing the sample biogas to a pure standard of hydrogen gas. A 0.5 mL Gastight™ syringe was used to inject the sample and standard into a GOW-MAC Series 350 gas chromatograph (GC) equipped with a thermal conductivity detector. The column was a 8 foot by 1/4 inch stainless steel (SS) 350A Molesieve 13X 80/100. The operational temperatures of the injection port, the oven and the detector were 100, 50, and 100°C, respectively. Nitrogen was used as the carrier gas at a flowrate of 60 mL / min.

Nitrogen , carbon dioxide, and methane were detected by comparing the sample biogas with a standard of pure nitrogen, carbon dioxide, and methane using a GOW-MAC Series 350 GC equipped with a thermal conductivity conductor. The column was a 8 foot by 1/4 inch SS 350B Hayesep DB 80/100. The operational temperatures of the injection port, the oven and the detector were 150, 50, and 100°C, respectively. Helium was used as the carrier gas at a flowrate of 60 mL / min.

For each bottle, hydrogen production was calculated by multiplying the amount of biogas produced by the respective hydrogen gas concentration at that sampling time. These values were summed after each sampling time to create a cumulative hydrogen production curve. In the Appendix is given one entire series of spreadsheets for one batch experiment. Eight cumulative hydrogen production curves using potato as the seed source is shown in Figure 1. The symbols on the figure represent actual experimental data points from a batch at several pH and sucrose concentration values while the lines were fitted to the experimental data using a sigmoidal function described in the next section.

Data analysis

Once the cumulative hydrogen production curves were obtained over the course of the entire experiment, a curve was modeled to the data using a modified Gompertz equation (Lay *et al.*,1999).

$$H = P * \exp\{-\exp[(R_m * e / P)(\lambda - t) + 1]\}$$
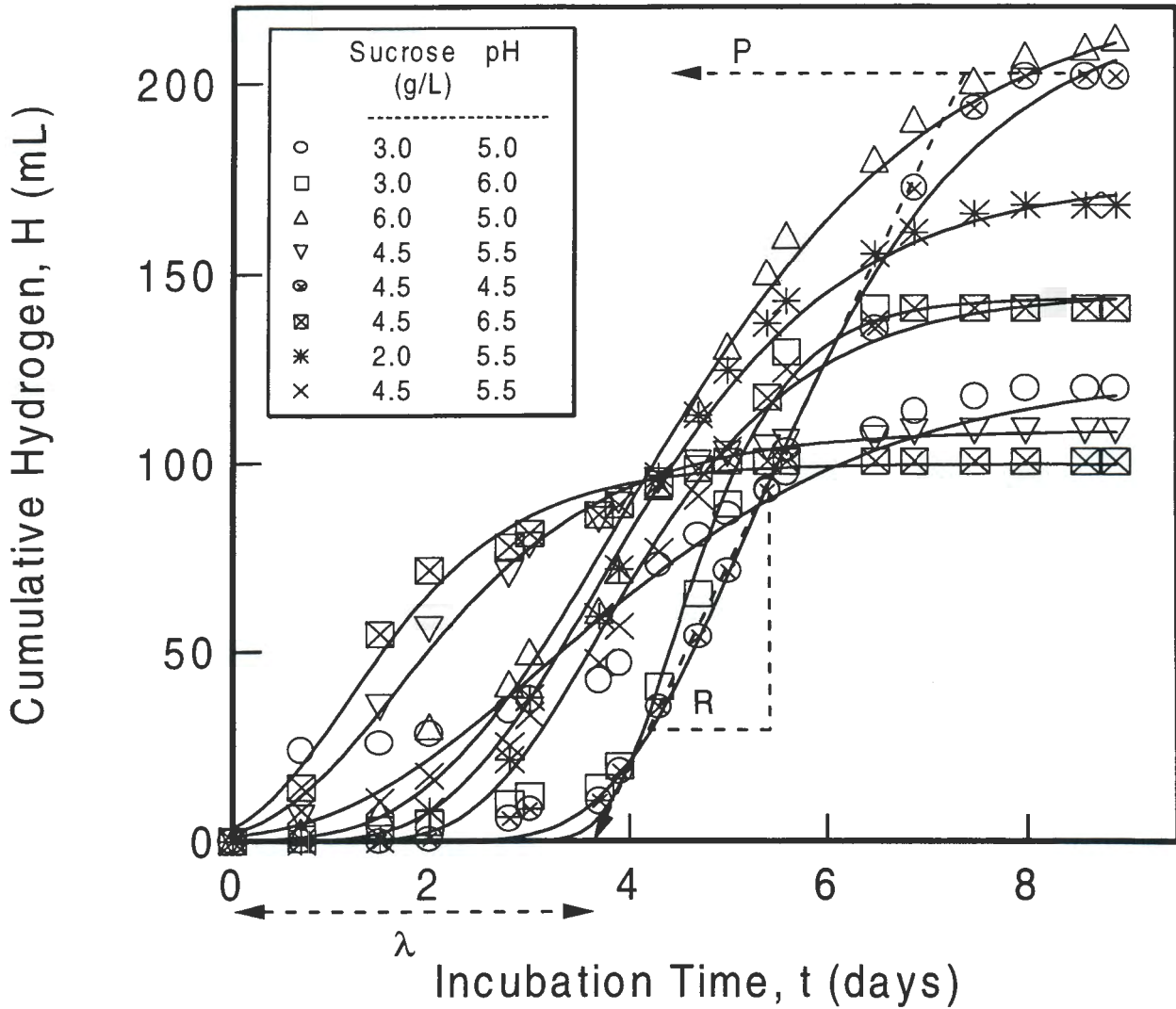
Figure 1. P, R, and λ for a cumulative hydrogen production curve.

The curve was best fitted by minimizing the ratio of the sum of square error to the correlation coefficient (SSE / R^2) using the 'Solver' function in the 'Tools' menu in Microsoft Excel 1995. The curve was initially fit by eye and was subjected to the constraints of hydrogen production potential, P (or total amount of hydrogen produced in mL), the hydrogen production rate, R (mL hydrogen produced / hour), and the lag phase, λ (hr) or the time to exponential hydrogen production. In summary, P, R, and λ were obtained for each of the eleven bottles for each seed source experiment. These parameters

are shown in Figure 1. The experiments utilizing potato soil were replicated nine times while the soybean soil, compost one, and compost two experiments were replicated only once. To confirm that there was no hydrogen production resulting from any degradation of the soil matter, two blank experiments were run without any sucrose added.

Results

Feasibility of natural bacterial seed sources converting sucrose to H_2.

To confirm that there was no background hydrogen production resulting from degradation of the soil matter, two blank experiments were run without sucrose added. As Figure 2 shows, no hydrogen production occurred from the blank serum bottle reactor. The curve designated '2 g Sucrose delayed' was delayed by adding the two grams of sucrose 48 hours after the start of the experiment. The curves show that the only difference between the curves with 2 grams of sucrose added is the lag phase. These two experiments confirm the lack of hydrogen production from the soil matter itself. In addition, chiefly by accident, a whole batch was delayed by 24 hours because no nutrient stock solution was added. By hour 48, the entire batch behaved as normal – the only difference being the lag phase was extended an additional 24 hours (data not shown).

Tables 2 to 5 show the values of P and R using the Gompertz equation for the potato, soybean, compost one, and compost two seed sources, respectively. Confidence intervals are given for the potato seed source treatments where applicable. In addition, for the potato seed source treatments, additional P and R values are given for additional bottles conducted at an initial pH and sucrose concentration as shown in Table 2 using boiling as the heat shock method.

The average conversion efficiencies of sucrose-hydrogen to hydrogen gas for potato, soybean, compost one, and compost two seed sources were 3.4, 6.9, 10.6, and 8.4%, respectively. The average conversion ratios of sucrose to hydrogen over all experiments varied from 0.4 to 1.2 moles of H_2 / mole sucrose with an average value of 0.8 moles of H_2 / mole sucrose. The highest hydrogen concentration in the biogas was 82% with an average of 55%. The highest values of hydrogen production using potato
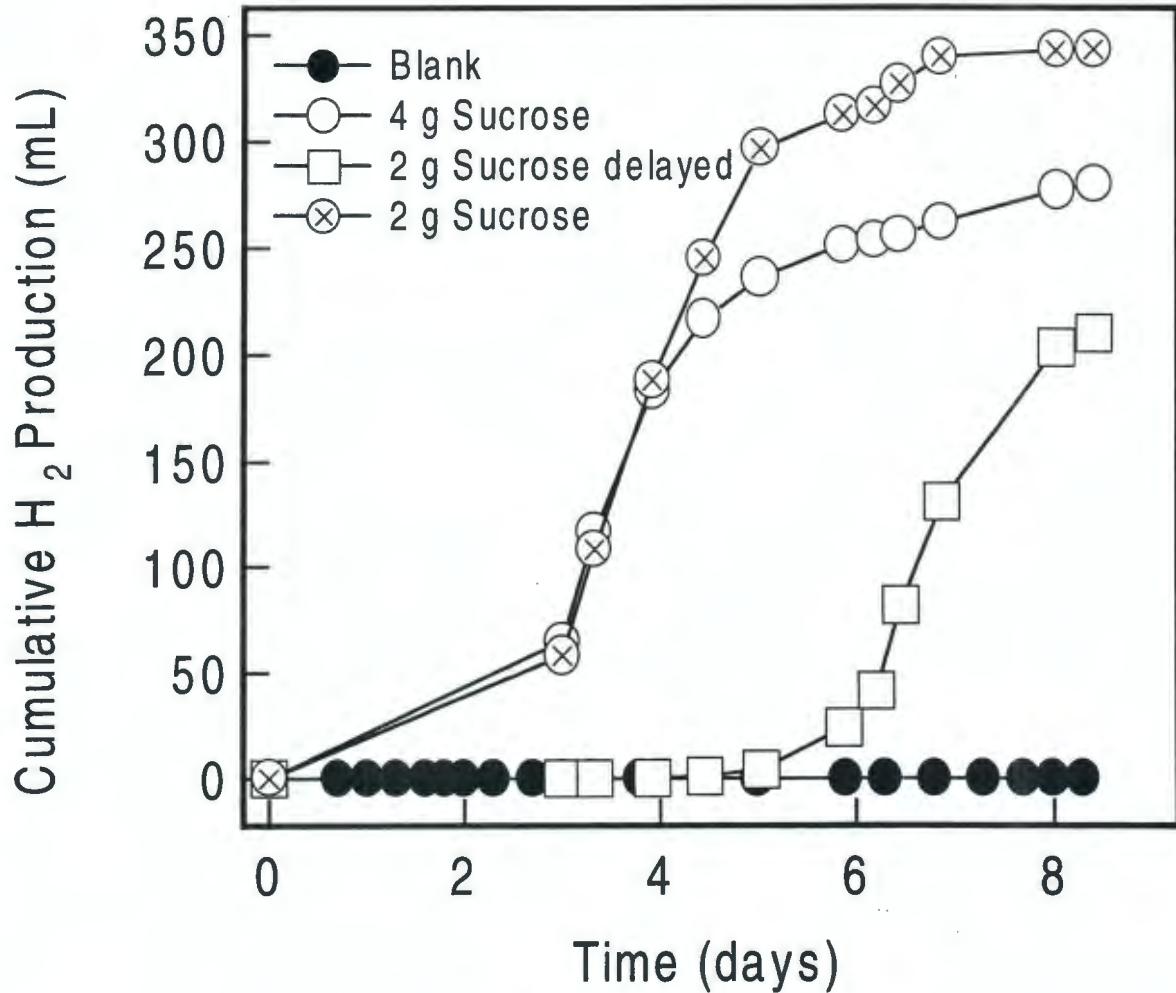
Figure 2. Zero background hydrogen production.

Table 2. Confidence intervals for hydrogen production potential (mL) and rate (mL/hr), respectively, using potato soil as the seed source. The rate lasted 37 hours in each case. The lag ranged from 15 to 25 hours.

		Sucrose (g / 150 mL bottle)				
		2	3	4	5	6
pH	4.5	100±21, 2.4±0.2		183±50, 3.0±0.6	200±10, 2.9±0.5	
	5.0	95±26, 2.4±0.6	188±69, 3.3±1.2		179±59, 3.2±0.9	178, 1.7
	5.5	183±49, 3.2±0.8		198±55, 3.3±1.1		189±39, 3±0.8
	6.0	82±21, 1.7±0.5	209±64, 3.6±1.1		177±59, 3.1±0.9	
	6.5	165±21, 1.3±0.8		192±50, 3.2±0.7		

Table 3. Values of hydrogen production potential (mL), 1st rate (mL/hr), and 2nd rate (mL/hr), respectively, using soybean soil as the seed source. The first rate lasted 62 hours while the second rate lasted 80 hours. The lag was 25 hours.

		Sucrose(g/ 150 mL bottle)				
		2	3	4	5	6
pH	4.5			195, 1.4, 0.9		
	5.0		200, 1.6, 2.1		213, 1.4, 1.1	
	5.5	222, 1.4, 1.7		255, 1.6, 1.5		318, 1.6, 2.1
	6.0		256, 1.7, 1.4		278, 1.8, 1.5	
	6.5			317, 1.7, 2.1		

Table 4. Values of hydrogen production potential (mL) and rate (mL/hr), respectively, using compost one as the seed source. The rate lasted 53 hours. The lag was 7 hours.

		Sucrose(g/ 150 mL bottle)				
		2	3	4	5	6
pH	4.5			440, 3.2		
	5.0		334, 4.2		312, 3.7	
	5.5	267, 3.4		510, 4.0		356, 3.7
	6.0		355, 2.9		336, 3.3	
	6.5			507, 3.14		

Table 5. Values of hydrogen production potential (mL) and rate (mL/hr), respectively, using compost two as the seed source. The rate lasted 46 hours. The lag was 14 hours.

		Sucrose(g/ 150 mL bottle)				
		2	3	4	5	6
pH	4.5			296, 3.4		
	5.0		287, 3.7		304, 3.3	
	5.5	284, 3.2		303, 3.1		286, 3.4
	6.0		401, 3.3		301, 3.4	
	6.5			296, 2.5		

soil, soybean soil, compost one, and compost two as the seed sources were 13, 12, 15, and 16 mL/(g COD*L), respectively, while the highest hydrogen production rates were 464, 240, 768, and 640 mL / (L reactor volume*day), respectively. The average hydrogen production rate were 384, 208, 592, and 528 mL / (L reactor volume*day). The average lag phases or the time it took to reach the exponential hydrogen production rate were 30, 30, 10, and 10 hours, respectively.

In the analysis of the hydrogen production rate, the Gompertz equation values were higher than rates determined by a simple linear regression equation ($y = mx + b$). The hydrogen production rates were therefore determined using this equation where 'x' was set at a standard of 24 hours or more to make an equal comparison among seed sources. Despite the inconsistency of these two model equations, the Gompertz equation is quick and useful in helping to determine the differences among the different pH levels and sucrose concentrations so it was used to make this comparison. Furthermore, it seems that the Gompertz model values can be correlated to the simple linear regression values so some correction factor could be used to equate these two models. See the Appendix (Figures A-1 to A-8) for examples of the high hydrogen production rates and characteristic curve shapes of each seed source.

Discussion

Comparison of seed sources

where dox it come from?

The highest average hydrogen production potential (372 mL) occurred using compost one as the bacterial seed source. Compost one was greater than compost two (223 mL) in producing hydrogen, soybean was third (203 mL), and potato soil (126 mL) came in a distant fourth. The high hydrogen production values of the compost could be entirely due to the bacterial population present in the compost and the fermentation of sucrose, but it could also be due to the high organic content in the compost. *blank → no H_2 production?* Compost one had the highest organic content followed by compost two while soybean and potato soil had comparable organic content as measured by VSS (data not shown). The average conversion efficiencies based on H_2–COD to sucrose–COD for potato, soybean, compost one, and compost two seed sources were 2, 2, 10, and 4%, respectively. The large

difference in conversion efficiencies between compost one (10%) and potato and soybean soil (2%) could be due to the production of hydrogen from the organic matter, particularly cellulose. Compost two (4%) follows the same trend since it seemed to have less organic matter than compost one.

The potato soil treatment rate value was averaged over three batches while the other treatments show values from one batch. The higher rates of the two composts could also be explained by the short lag phase (10 hours) compared to that of agricultural soil (30 hours) which may suggest a higher biomass concentration in the seed source. When the composts were collected, they were rather warm and wet and they were high in cellulose content (leaves and grass). This type of environment is much more conducive for high biomass concentrations (and hydrogen production) than the agricultural soil which was dry, rather inorganic, and left open to the elements.

According to Gottschalk *et al.* (1981), there exists many species of cellulytic clostridia. The degradation of organic matter may have occurred in the compost one treatment since the cumulative hydrogen production curves show two characteristic growth phases (see Appendix Figure A - 7). The first growth phase would involve the degradation of sucrose – an easily digested substrate. This first growth phase is quite similar to the growth phase using potato soil as a seed source in that they both lasted until roughly the 100 hour mark. The second growth phase of the compost could be due to cellulose degradation once the culture developed the enzyme capability to degrade the cellulose. However, some experiments conducted with the soybean soil (which was not high in cellulose) shows similar curves (See Appendix Figure A – 6).

In addition, these high potentials could be due to the high buffering capacities of the compost. During the initial pH adjustment, a comparatively large amount of acid had to be injected to the bottles to lower the pH. Although this amount was not quantified, the amount was much greater than that for the soybean and potato seed sources which had similar apparent buffering capacities. This is likely since these two soils came from adjacent fields (soil sampling points were 50 feet apart). The only differences between these soils is the particular type of crop grown in the field the previous year and that the

potatoes were organically grown while the soybeans were grown using traditional farming methods (herbicides and fertilizers).

This high buffering capacity would keep the media in the optimum range for hydrogen production. According to Minton and Clarke (1989), the optimum pH for *Clostridium* is from 6.5 to 7.0. Since the high end of the pH range used in this experiment was 6.5, the production of volatile acids from the degradation of sucrose would quickly lower the pH out of the optimum range of 6.5 to 7.0 if no buffering capacity were available.

The buffering capacity can also explain the duration of the experiments. Both the compost one and compost two treatments lasted about a month while the potato and soybean soil treatments only lasted about a week and a half. Apparently, the pH was maintained at some value during this month to allow for further hydrogen production above and beyond that of the potato and soybean soil treatments.

It was the initial intent of this research to find the optimum pH and sucrose concentration for hydrogen production. However, in this particular study, these values were not found. As stated earlier, an inhibition due to a drop in pH could be the limiting condition to hydrogen production. In this case, once the pH became inhibitory, sucrose degradation would decrease and finish. This undegraded sucrose became evident in a volatile solids determination where bottles with a higher initial sucrose concentration always had a higher final volatile solids content (see Appendix, Figure A-9).

Conclusions

In conclusion, biohydrogen production using seed sources obtained from nature was achieved. Although no species were identified as the hydrogen producers, each seed source was able to produce hydrogen in appreciable amounts and rates compared to other studies. Heat shock served well to select for hydrogen-producing bacteria. It was the original intent of this research to model the initial rate of hydrogen production at an initial pH level and initial sucrose concentration and eventually use the best combination of pH and sucrose concentration in a lab-scale reactor. However, the initial sucrose concentration was much too high so the substrate concentration was never

limiting. Furthermore, on completion of biogas production, the final pH was always between the values of 3.8 to 4.0 which means that the pH never held at its initial value in the experimental design (4.5, 5.0, 5.5, 6.0, or 6.5) and the media became inhibitory for the hydrogen-producing bacteria. At this low pH, *Clostridium* has been observed to switch from hydrogen production to solvent production (Byung and Zeikus, 1985). In other words, no significant differences were found in the hydrogen production rates at each pH level since they may have fell too quickly to discover such differences. Only a range of pH levels could be considered optimum. This range is believed to be between 5.0 and 6.0. On average, it was found that the two kinds of compost produced the most hydrogen and at a higher rate. The high potential and rate of the compost could be due to the high buffering capacity and high biomass concentration of the compost as described earlier. Subsequent batch experiments should be focused on low initial sucrose concentrations and the maintenance of pH to safeguard against a drop in pH. In this regard, buffered batch serum bottles were used with enough buffering capacity to last the duration of the experiment as explained in the next paper of this thesis. Based on the results of this research, compost seems to be the best seed source for hydrogen production, yet the actual species of bacteria responsible for the hydrogen production have not been identified. These species responsible may occur in both compost and agricultural soil or they may not. Since, the biomass concentration was not determined in this study, the specific hydrogen production rate (mL H_2/ g bacteria * hr) can't be determined. In other words, the higher rate of hydrogen production from the compost could entirely be due to the higher initial biomass concentration as explained by the short lag phase. A continuous reactor should have been run using each seed source to determine the optimum pH value. In theory, after a continuous reactor reaches a steady state, only one bacterial species will dominate at a given set of operating conditions. Thus, if the best hydrogen producing species in nature is present in both compost and agricultural soil, a continuous reactor using any of these seed sources will produce the same rate at a given set of operating conditions after reaching a steady state. Furthermore, the pH could easily be maintained in a continuous reactor as compared to a batch reactor.

CHAPTER 4 BIOHYDROGEN PRODUCTION OPTIMIZATION USING NATURAL INOCULUM

Authors: S. W. Van Ginkel and Shihwu Sung

Abstract

Three buffered anaerobic batch cultures were inoculated with compost as the seed source for hydrogen gas production. The experiments were arranged in a fractional factorial design with the aim of discovering an optimal pH and sucrose concentration for hydrogen production potential and hydrogen production rate. The cultures were buffered with a 0.066 M KH_2PO_4 solution and the final pH was adjusted to its final value using 0.94 M Na_2CO_3 and 1 M HCl. The pH ranged from 4.5 to 7.5 in the cultures. Three concentration ranges of sucrose were used. In the high range, the sucrose concentration ranged from 1.5 to 7.5 g COD / L. In the medium range, the sucrose concentration ranged from 1 to 4 g COD / L. In the low range, the sucrose concentration ranged from 0.5 to 1.5 g COD / L. The high sucrose concentration range produced the most amount of hydrogen (33 mL / g COD * L) and had the highest hydrogen production rate (1790 mL / (L reactor volume * day)). The optimum pH range for hydrogen production potential and rate occurred between 5.0 and 6.0. The highest values of hydrogen production potential and hydrogen production rate occurred at a pH of 5.5. The highest conversion efficiency based on H_2–COD to sucrose–COD was 15% at the high sucrose concentration range. The average conversion ratios for the high, medium, and low sucrose concentrations were 1.6, 0.9, and 0.5 moles of H_2 produced per mole sucrose, respectively. In comparison to previous experiments using unbuffered media, buffering the medium clearly results in higher hydrogen production potential and hydrogen production rate.

Introduction

Three buffered sets of batch mixed bacterial cultures were set up as a result of suspected pH inhibition in unbuffered cultures described in the previous chapter. In these cultures, a high enough sucrose concentration was used for it all not to be consumed by the end of the experiment, so the true potential of the hydrogen-producing bacteria was not measurable. The remaining sucrose was determined by volatile solids tests. Those cultures with an initially high sucrose concentration always had a higher final volatile suspended solids concentration which means sucrose was probably one of the volatile solids detected (See Appendix, Figure A-9). A drop in pH likely inhibited further sucrose utilization. The pH of each bottle of several experiments was tested after biogas production stopped. The final pH was always in the range of 3.8 to 4.0 which means the pH became too acidic for the neutrality-preferring Clostridia - the suspected hydrogen producer in this study (Minton and Clarke, 1989). The optimum pH range for the entire genus *Clostridium* as stated by Minton and Clarke (1989) is 6.5 to 7.0. However, based on the experiences of the experiments mentioned in the previous chapter, the suspected optimum pH range for clostridial hydrogen producers in this study is in the range of 5.0 to 6.0. In the previous chapter, a high pH (6.5) coincided with high hydrogen production. A pH of 6.5 is not considered the optimum value. The pH probably quickly decreased from 6.5, through the optimum between 5.0 and 6.0, and then became inhibitory under 4.5. An initial pH of 6.5 just means the culture had more time or more pH units to go through the optimum (5.0 to 6.0) before the medium became inhibitory. Therefore, it is the goal of this study to test this hypothesis.

In the previous study, the initial pH value was one of the factors used to compare the hydrogen production potential and hydrogen production rate inoculating with variable seed sources. Initially, when the bacteria culture of each seed source were present in nature, they were either in their spore form or as vegetative cells. Their natural media was at a certain pH and the culture adjusted to this value by metabolizing any available substrate that they could at that particular pH. The bacteria that adjusted the best became dominant. In the lab, when the pH was adjusted to a new value, any metabolic history of the bacteria was erased if the pH deviated much from the natural value. In addition, since

heat shock (baking) was conducted to remove non-sporeformers, the majority of the bacteria were forced into their spore form and an entirely new set of growing conditions was established. In this regard, setting a new pH for the batch cultures would determine the new metabolic activity of the culture. Some bacteria will be able to grow well at this new pH while others would not. Subsequently, a dominant culture would be established and the bacteria would start producing degradation products, such as hydrogen, from the new substrate. Since the only two variables were pH and sucrose concentration, differences in hydrogen production would be determined by these two factors. Every other factor remained the same for the entire batch. However, the media was left unbuffered and the pH dropped at an undetermined time thereby inhibiting hydrogen production.

In order to get accurate estimates of the hydrogen production potential, P and hydrogen production rate, R three sets of buffered batch cultures were used. In these experiments, a much lower sucrose concentration was used. Since in hydrogen production, sucrose is the precursor to volatile acid production, a lesser amount of sucrose would produce less acid which would cause a smaller decrease in pH. It was the goal that the buffer used would maintain the desired pH long enough for cells to utilize all of the sucrose and to maintain the hydrogen production rate.

Materials and methods

Compost was obtained from the Iowa State University compost facility in Ames, Iowa. The compost was heat shocked (baked) at 104°C for two hours to kill hydrogen-consuming bacteria, primarily methanogens. After heat shock, the compost was sieved using a 1.19mm sieve and 30 grams of the compost was placed into each of fifteen batch serum bottles. A buffer solution was prepared by dissolving 18 grams of KH_2PO_4 in two liters with a resulting molarity of 0.066 M. The pH was adjusted to a prescribed value using 1 M HCl or 0.94 M Na_2CO_3.

Sucrose and pH variation

The fifteen batch serum bottles were setup as a fractional factorial experimental design as described by Box *et al.*, (1978). Subsequently, contour plots (Box *et al.*, 1978) were constructed to evaluate the main effects and the interaction between sucrose (x_1) and pH (x_2) on hydrogen production potential (P) and hydrogen production rate (R). The three buffered sets of cultures were designated the high range, the medium range, and the low range with respect to sucrose concentration.

In the first set of cultures - the high range, the added sucrose varied from 0.2 to 1.0 grams per bottle while the initial pH varied from 5.5 to 7.5 as described in Table 1. Each 'X' represents one batch serum bottle. In contrast to the unbuffered cultures of the previous study, the pH range was shifted one unit towards a more alkaline pH.

In the second set of cultures - the medium range, the added sucrose varied from 0.13 to 0.53 grams per bottle while the initial pH varied from 5.0 to 7.0 as described in Table 2. The center value was replicated twice.

In the third set of cultures - the low range, the sucrose concentration varied from 0.066 to 0.2 grams per bottle while the initial pH varied from 4.5 to 6.5 as shown in Table 3. The center value was replicated twice.

Table 1. Experimental Design – the *High Range*.

		pH				
		5.5	6	6.5	7	7.5
Sucrose g/150 mL	0.2	28		10		1
	0.4	60	69		16	6
	0.6	138		95		29
	0.8		184		83	
	1.0	242		113		24

Table 2. Experimental Design – the *Medium Range*.

		pH				
		5.0	5.5	6.0	6.5	7.0
Sucrose g/150 mL	0.13	7		4		3
	0.23		17		28	
	0.33	6		24		10
	0.43		45		28	
	0.53	61		61		34

Table 3. Experimental Design – the *Low Range*.

		pH				
		4.5	5	5.5	6	6.5
Sucrose g/150 mL	0.066	2		2		1
	0.1		5		3	
	0.133	6		6		2
	0.166		6		5	
	0.2	6		10		10

Experimental apparatus and procedure

The experiments were conducted at random as described by Steele *et al.* (1997). Using a random design, the batch serum bottles were numbered one through fifteen. Then, using a random number table, the numbers one through fifteen were randomly picked and consecutively assigned to a sucrose / pH combination. The experiments were conducted in 250 mL serum bottles filled to 150 mL with 30 grams of sieved compost with varying amounts of sucrose and pH adjustment and 0.5 mL of nutrient stock solution. Each liter of the nutrient stock solution, modified from Lay *et al.* (1999), contained 200 g of NH_4HCO_3, 100g of KH_2PO_4, 10g $MgSO_4$-$7H_2O$, 1.0 g of NaCl, 1.0 g of Na_2MoO_4-$2H_2O$, 1.0 g of $CaCl_2$-$2H_2O$, 1.5g of $MnSO_4$-$7H_2O$ and 0.278g of $FeCl_2$. After addition of sucrose and nutrients, the bottles were flushed for 15 seconds with nitrogen gas. The bottles were then incubated in a shaker chamber at 37°C at a rotational speed of 180 rpm. The volumes of the biogas collected were measured using 2 to 50 mL syringes as described by Owen *et al.* (1979).

Analytical methods

The hydrogen gas percentage was measured by comparing the sample biogas with a standard of pure hydrogen using a GOW-MAC Series 350 gas chromatograph (GC) equipped with a thermal conductivity conductor. The column was a 8 foot by 1/4 inch stainless steel (SS) 350A Molesieve 13X 80/100. The operational temperatures of the injection port, the oven, and the detector were 100, 50, and 100°C, respectively. Nitrogen was used as the carrier gas with a flowrate of 75 mL/min.

Nitrogen , carbon dioxide, and methane were detected by comparing the sample biogas with a standard of pure nitrogen, carbon dioxide, and methane using a GOW-MAC Series 350 GC equipped with a thermal conductivity conductor. The column was a 8 foot by 1/4 inch SS 350B Hayesep DB 80/100. The operational temperatures of the injection port, the oven and the detector were 150, 50, and 100°C, respectively. Helium was used as the carrier gas at a flowrate of 140 mL / min.

For each bottle, hydrogen production was calculated by multiplying the amount of biogas produced by the respective hydrogen gas concentration at that sampling time. These values were summed after each sampling time to determine cumulative hydrogen production. The last or highest value is designated the hydrogen production potential (mL) while the hydrogen production rate (mL / hr) was determined by dividing the hydrogen production potential by the duration (hr) of the experiment (see Appendix).

Data analysis

Once P and R were calculated for each serum bottle, the Linest function on Microsoft Excel was used to model a regression equation for both P and R as these values varied with the sucrose concentration (gCOD/L) and pH. This equation was then used to develop contour plots in the Igor™ software package. The visual evaluation of these plots gives insight to the main effects and the interactions of the variables used in the design.

Results

The Linest regression equations for the hydrogen production potential and the hydrogen production rate for the three experiments are presented in Table 4. The labels high, medium, and low correspond to high, medium, and low ranges of sucrose concentration. The coefficients across the top of the table correspond to the coefficients of the regression equation where X*Y corresponds to the interaction effect between pH and sucrose concentration on P and R. Y^2 and X^2 correspond to the effects of the quadratic terms of sucrose concentration and pH on P and R, respectively. Y and X are the coefficients for the effects of the linear components of sucrose concentration and pH on P and R, respectively. In the last column, the correlation coefficient is given to give an idea of how well the model fit the experimental data.

Subsequently, each of these regression equations was used in Igor™ to give a visual picture of the effects of pH and sucrose concentration on P and R. Figures 1 through 3 are the contour plots for the effects of the high, medium, and low ranges of sucrose concentrations on hydrogen production potential. Each treatment was conducted at a slightly different pH range to determine the optimum pH value. The sucrose concentration is expressed as g COD/L on the vertical axis. The circled data points represent actual experimental values at a unique pH and sucrose concentration while the contour lines represent the fitted lines generated by Igor™. Similarly, Figures 4 through 6 show the effects of the high, medium, and low ranges of sucrose concentration on the hydrogen production rate, R.

It is the belief of this author than an insignificant amount of methane was produced in the batch experiments. A methane detection curve was generated by injecting 0.04 to 0.5 mLs of a methane, nitrogen, and carbon dioxide standard corresponding to 2.8 to 70% methane. The methane concentrations in the sample bottles are determined to be less than 2.8% by comparing the sample methane curves to those of the standard (data not shown).

Table 4. Linest regression equations potential and rate.

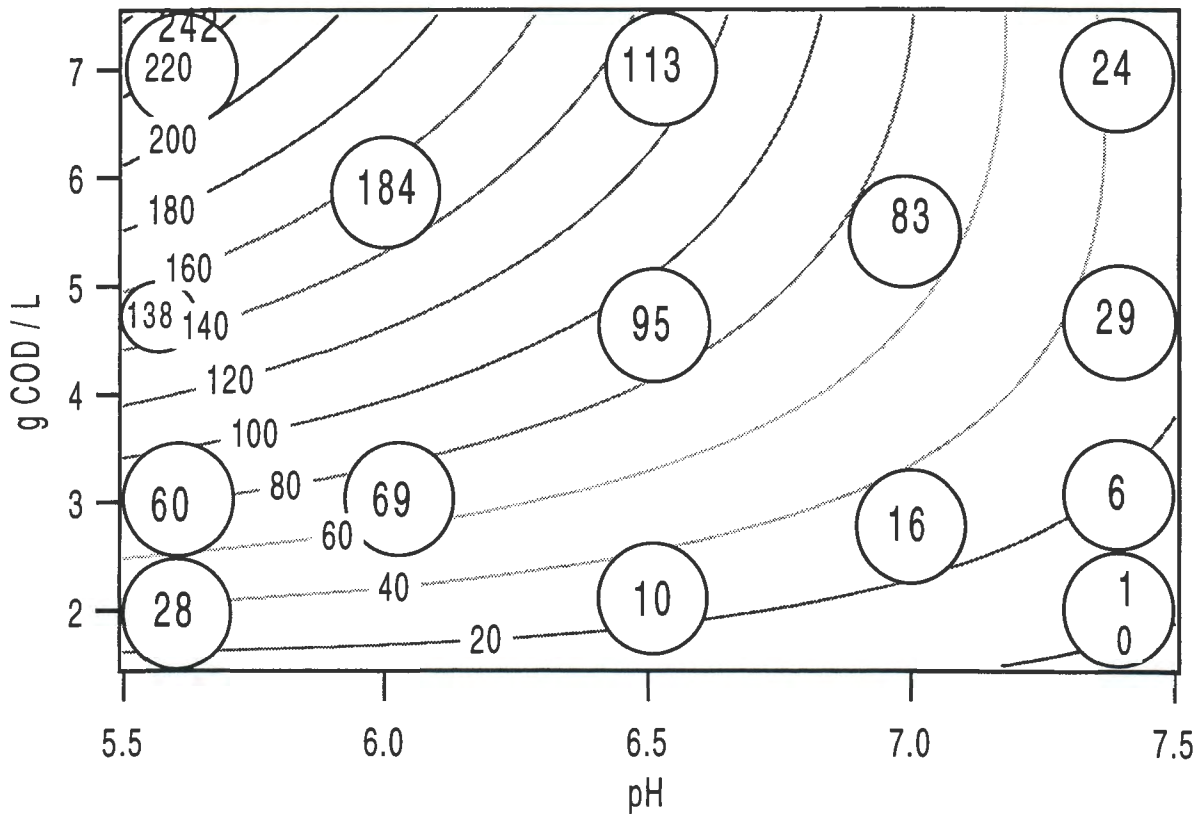|	X*Y	Y^2	X^2	Y	X	B	R^2
Potential							
High	-16.5	-1.7	-4.6	144.5	75.1	-335.2	0.96
Medium	-4.5	5.8	-11.8	14.0	148.1	-441.2	0.88
Low	2.7	2.5	-1.2	-13.5	10.7	-19.8	0.80
Rate							
High	0.52	0.17	1.71	-3.41	-24.91	88.47	0.81
Medium	-0.3	0.3	-0.8	1.3	10.0	-30.5	0.86
Low	0.18	-0.05	-0.03	-0.61	0.15	0.09	0.61

Figure 1. Hydrogen production potential (mL) at the high range of sucrose concentration (1.3 – 6.7 g / L).
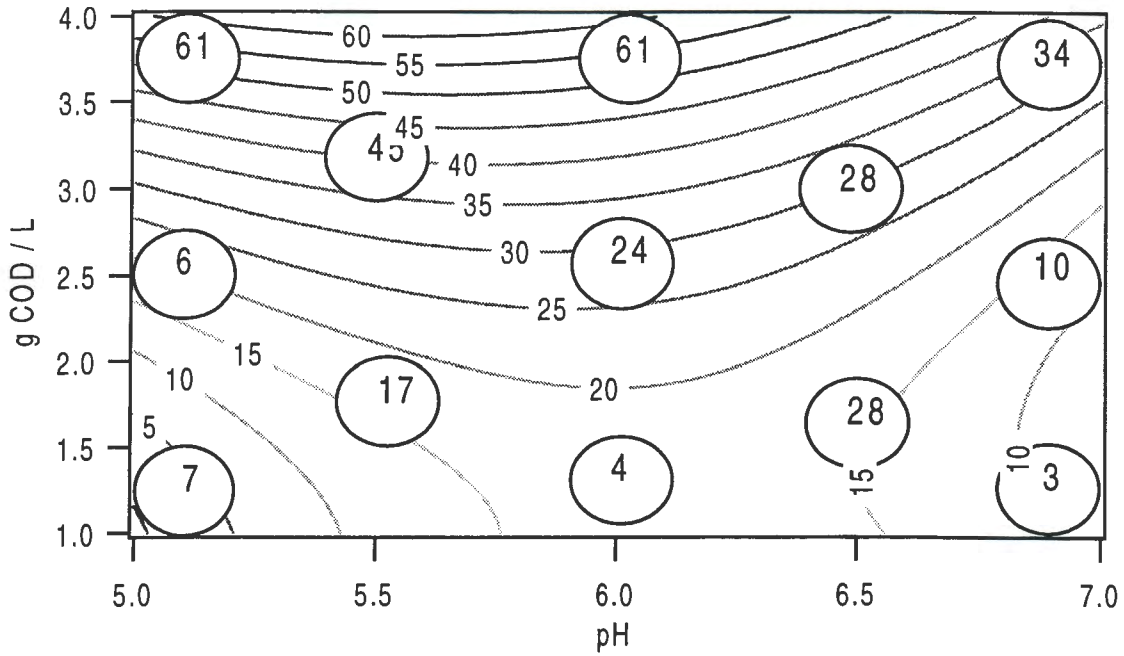
Figure 2. Hydrogen production potential (mL) at the medium range of sucrose concentration (0.9 – 3.5 g / L).
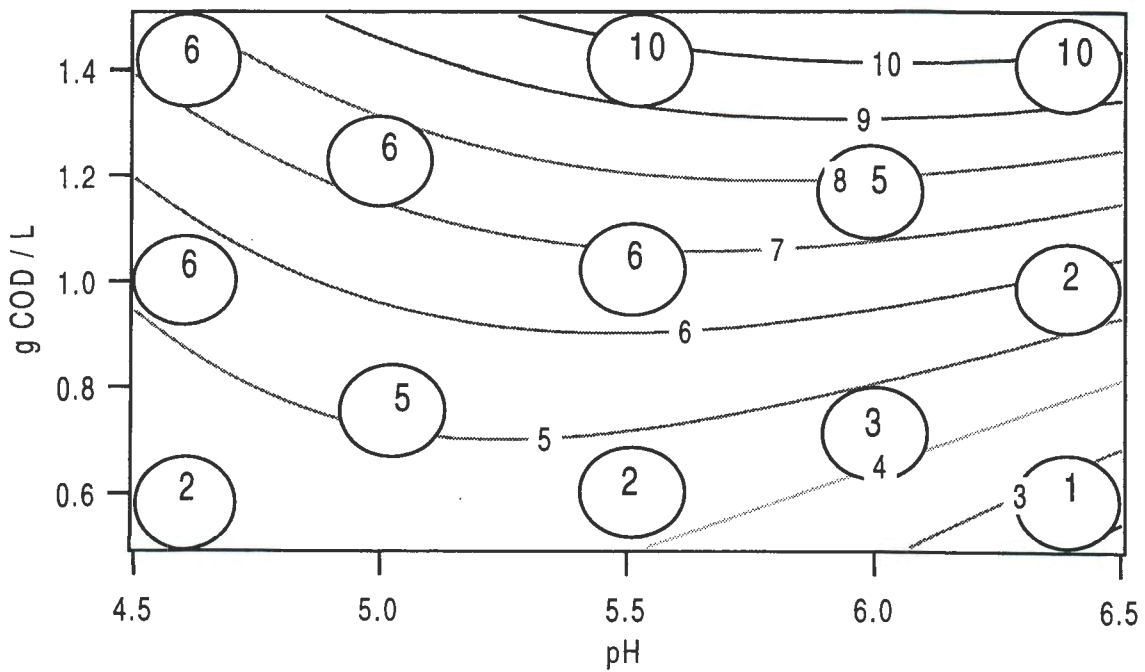

Figure 3. Hydrogen production potential (mL) at the low range of sucrose concentration (0.4 – 1.3 g / L).

Discussion

Sucrose was added to the cultures at different concentrations for a number of reasons. At low initial sucrose concentrations less volatile acids would be produced and the decrease in pH would be small. Enough buffering capacity was used to maintain the pH long enough to determine the initial rate of hydrogen production. It is assumed that during this time, the pH did not decrease more than the 0.5 pH increment used in the design. Second, it was the goal that all of the sucrose would be utilized giving hydrogen production potential a true meaning. In the first study using unbuffered cultures, the initial sucrose concentration was too high for the amount of buffering capacity available and undegraded sucrose remained. Third, since the amount of compost remained the same throughout the experiments, the F/M ratio could be optimized in subsequent research once the actual biomass concentration is known in the compost.

Figure 1 shows hydrogen production potential values at the high range of sucrose concentrations. Experimental values (circled values indicated in the figure) show that the highest potential occurred at a pH of 5.5 and at the highest sucrose concentration (7 g COD / L). The second highest potential occurred at a pH of 6.0 with a sucrose concentration of 6 g COD / L. The contour lines suggest that the potential increases as the pH decreases from 6.5 to 5.5. The values of potential at the pHs of 7.0 and 7.5 were much less than those at pH 5.5 showing that the optimal pH range is 5.5 to 6.5 with a peak at 5.5 (242 mL H_2).

Figure 2 shows hydrogen production potential values at the medium range of sucrose concentrations. Experimental values show that the highest potentials occurred at pH values of 5.0 and 6.0 at the highest sucrose concentration (4 g COD / L). The contour lines suggest an optimum pH range from 5.0 to 6.0.

Figure 3 shows hydrogen production potential values at the low range of sucrose concentrations. Experimental values show that the highest potentials occurred at pHs of 5.5 and 6.5 at the highest sucrose concentration (1.4 g COD / L). The contour lines suggest an optimum pH range from 5.5 to 6.5.

Even though the solution was buffered there were significant decreases in pH among the experiments. The buffering capacity was limited due to cation concentration (K^+). The concentration of potassium was 2,400 mg/L while the moderate inhibition threshold of potassium for typical anaerobic systems is 2,500 mg/L (McCarty, 1964). Those bottles with the highest sucrose concentration (as well as the highest hydrogen production) showed the greatest decline in pH as expected by the production of volatile acids and hydrogen from sucrose. The average decrease in pH among the high, medium, and low sucrose concentrations were 0.75, 0.54, and 0.4. Considering the high and medium ranges of sucrose concentration, this drop in pH is greater than the 0.5 pH step change used in the design. It is assumed that the drop in pH occurred when the cultures produced volatile acids. These acids are produced the same time hydrogen is produced. Consequently, the drop in pH coincided with hydrogen production. Hence, only a range of pH values can be considered optimum. The range in this study appeared to be from 5.0 to 6.0. However, upon completion of biogas production, the pH was not determined immediately. The bottles were put back in the incubator to allow for further hydrogen production. Hence, the bottles had as much as five to ten hours to sit and ferment before the final pH was checked. In hindsight, the bottles should have been checked for pH when the biogas production or the hydrogen gas concentration dropped to a certain level.

The highest hydrogen concentrations in the biogas for the high, medium, and low sucrose concentrations were 50%, 35%, and 24%, respectively. This is in agreement with earlier research in the previous chapter in that a higher sucrose concentration yielded higher concentrations of hydrogen in the biogas. However, since in the low sucrose concentrations the headspace was not completely replaced with biogas (the total biogas production was less than 100 mL), the percentage could be higher although this value was not calculated in this research.

The amount of methane in the biogas was determined to be insignificant. In the experiment involving the medium range of sucrose concentration, no methane was detected in the biogas for fourteen of the fifteen bottles. The fifteenth peak (1mm compared to 8cm of the standard) was considered insignificant (<< 2.8% methane in the

biogas) although this *is* evidence that all the methanogens were not destroyed during the heat shock pretreatment.

The highest conversion efficiency based on H_2–COD to sucrose–COD was 15% at the high sucrose concentration range. This is 5% higher than the conversion efficiency determined in the previous chapter using compost as the seed source. It is the opinion of this author that less organic matter was used in this series of batch tests since the seed source was sieved leaving behind grass and leaves. The average conversion ratios for the high, medium, and low sucrose concentrations were 1.6, 0.9, and 0.5 moles of H_2 produced per mole sucrose, respectively.

Typical S – shaped or sigmoidal hydrogen production curves were not generated since the hydrogen was produced so quickly compared with the batch tests of the previous chapter. It seems that sieving the compost my have selected for a higher biomass concentration in the seed source. A lag phase was not detected in these batch tests either. Since, a sigmoidal curves were not generated, typical rate data found in scientific literature could not be determined in this study. Even so, a 'rate' of 7.3 mL / (hr * 150 mL bottle) was sustained for 26 hours and it appears to be linear over this duration. Even more so, this rate of '7.3' is rather conservative, since if a lag phase were detected, it would only tend to increase the steepness of the curve (See Appendix Figure A – 10).

Conclusion

In this study, the optimum pH range for hydrogen production potential and hydrogen production rates occurred from 5.0 to 6.0. The highest hydrogen production potential and rate values occurred at an initial pH of 5.5. The results show that the higher sucrose concentration range (1.5 to 7.5 g COD / L) resulted in the highest hydrogen production potential (242 mL H_2), the highest conversion efficiency (15%), and the highest conversion ratio (1.6 mole H_2/mole sucrose). In comparison with earlier work in the previous chapter, where the sucrose concentration varied from 15 to 45 g COD / L, this study shows a higher hydrogen production rate of 1168 mL / (L reactor volume *

day) compared to 768 mL/ (L reactor volume*day) using compost from the same facility. The only differences between the two studies were sucrose concentration, buffering, and compost sieving. It is the conclusion of this study that buffering the growth medium results in higher hydrogen production. It is difficult to maintain the pH in batch cultures since the bottles are not opened or removed from their incubating environment for extended periods. In subsequent experiments, confirmation of the effect of these experimental conditions should be done in a continuous reactor where the pH and the F/M ratio can be controlled over extended periods of time.

CHAPTER 5 CONCLUSIONS

In conclusion to this research, biohydrogen production can be achieved using naturally obtained bacterial seed sources. These sources contain mixed bacterial species and are easy to obtain even in large quantities. Heat shock pretreatment was used to reduce hydrogen-consuming bacteria in this research. Heat shock pretreatment served this purpose well in that little methane was produced in any culture throughout the study.

Although the actual bacteria responsible for the hydrogen production were not identified other than that they were likely sporeformers, there were differences in hydrogen production among the cultures inoculated with the various seed sources. Cultures with some seed sources produced more hydrogen than others, yet this difference may not be attributed to a particular group of bacteria owing to the variety of other factors that could have been responsible. These other factors may include organic content of the seed source, the buffering capacity of the seed source, or the initial biomass concentration in the seed source.

The use of a factorial experimental design and contour plots as a visual tool helped in noticing any main effects of pH and sucrose concentration or their interaction on hydrogen production. The effects of pH and sucrose concentration are easier to notice visually than by looking at raw data. Once the hydrogen production potential and rate values were known, it was easy developing regression equations by using Linest on MS Excel™ and using these equations in the Igor™ software package. The hydrogen conversion rate determined by the Gompertz equation can be correlated to a simple linear regression model. A simple linear regression model ($y = mx + b$) was also used to calculate the hydrogen production curves so the average hydrogen production curves could be compared to other research on the subject. Despite the differences in the rate prediction, the Gompertz equation was valuable in discovering differences among each seed source since it is believed that the Gompertz model predicted the rate of each curve to the same extent.

Some of the experiments were repeated several times while others were repeated only once. Those repeated showed high reproducibility and those that were not repeated showed higher hydrogen production rates and potentials than those that had replicates.

For example, those experiments utilizing potato soil as the seed source always seemed to have a hydrogen production potential around 100 mL and a rate around 2 mL / hr and they all seemed to last around one and a half weeks. Fortunately, the results happened in this way. In other words, there were not any wide swings in hydrogen production from an individual seed source and those seed sources that achieved the highest hydrogen production rate, did so with just one treatment. However, replication is always a good idea so those experiments with just one estimate should be viewed as just a single estimate. In addition, since only nine combinations were used out of the possible twenty-five in the design, any outliers should be carefully scrutinized because they would have a significant effect on the resulting plot.

pH became the critical factor in hydrogen production as demonstrated by this research. In the early stages of this research, the two factors under consideration were initial pH and initial sucrose concentration. Defining hydrogen production potential according to initial pH is acceptable with the understanding that the pH will change upon production of volatile acids in an unbuffered media. The hydrogen production potential should be defined according to pH and this pH should be maintained throughout the experiment. If the pH is left unchecked, an inhibitory condition may set in and the parameters that were originally intended to quantify may have errors in them. Subsequent analysis would have to be performed to find the extent of the inhibition. In the middle stages of this research, a series of experiments were conducted where the pH did become inhibitory. Some good results were obtained but the true potential and rate of hydrogen production was not determined. These studies did not show how much or how fast hydrogen could be produced since the bacteria stopped producing hydrogen before all of the substrate was depleted. In the last stages of this research, buffering the growth medium was used to maintain pH although it was not strong enough to maintain a constant pH for the duration of the experiment. Even so, results concluded a much higher hydrogen production rate (it increased from 672 to 1790 mL / (L reactor volume*day)). These two experiments differed in only three areas. The second experiment was buffered, was inoculated with 30 grams of sieved compost, and contained 1 gram of sucrose instead of 4 grams in the first experiments. Therefore, the increase in the

hydrogen production rate could be due to buffering or due to an increase in biomass concentration in the second experiment owing to the sieving process.

A pH of 5.5 and a substrate concentration of 7.5 g COD / L were shown to be the optimum pH and substrate concentration for hydrogen production in the medium used. Optimum values in the buffered experiments occurred in the 5.0 to 6.0 pH range more often than not and the optimum hydrogen production rate occurred at a sucrose concentration of 1 gram / 150 mL bottle (7.5 g COD / L). The sucrose concentration ranged from 0.066 grams per bottle to six grams per bottle. Hence, there could be a higher sucrose concentration that would give a higher hydrogen production rate if the pH would hold in the range of 5.0 to 6.0. In this study, the amount of seed source was always kept the same - 30 grams/bottle. Therefore, in future research, if the biomass concentration could be quantified, a F/M ratio could be used to start a continuous reactor partially based on these results.

Among the experiments using unbuffered cultures, the use of compost as the seed source produced the highest hydrogen production rate, yet the use of potato soil and the use of boiling as the heat shock treatment produced the highest hydrogen gas percentage in the biogas at 82%. Boiling may be a more intense or vigorous form of heat shock treatment selecting for highly efficient hydrogen producers. As mentioned earlier in this thesis, the higher hydrogen production potentials, rates, or biogas percentages could be due to the specific biomass in the seed source or due to the characteristics of the seed source. Characteristics of the seed source include the buffering capacity, biomass concentration, or the amount of organic matter in the seed source. Therefore, if each seed source consists of the best hydrogen producer available in nature, in theory, a reactor at a given set of operating conditions conducive for this best hydrogen producer could be inoculated with any seed source and still produce hydrogen at the optimum rate. This stated on the assumption that *Clostridium*, the suspected hydrogen producer in this thesis, is present in potato soil, soybean soil, and both kinds of compost

Specific hydrogen production potential (mL H_2 / g COD*L) and specific hydrogen production rate (mL H_2/ hr*ΔVSS) were the two main parameters to be studied in this research. They give more meaning to hydrogen production in the real world than just

potential and rate since these parameters relate hydrogen production according to the amount of the COD degraded and the change in biomass concentration, respectively. An operator of a hydrogen-producing reactor, given the amount of COD in the influent, would expect a certain volume of hydrogen from this COD. Also, given the biomass concentration, the operator could know how fast it would be produced. In turn, both parameters would enable the operator to operate the reactor at an optimum rate. However, due to the pH inhibition discussed earlier these two parameters have not yet been optimized.

Theoretically, hydrogen production would not reduce the COD content of a wastewater below discharge levels. Therefore, an aerobic method or an anaerobic method with the production of methane would have to be in series with a hydrogen producing reactor. Methane production is well suited to follow hydrogen production in that low molecular weight volatile acids, precursors to methane, are produced in hydrogen production.

APPENDIX ONE
ADDITIONAL GRAPHS AND DATA

Figure A-1. Highest hydrogen production rate using potato soil as the seed source.
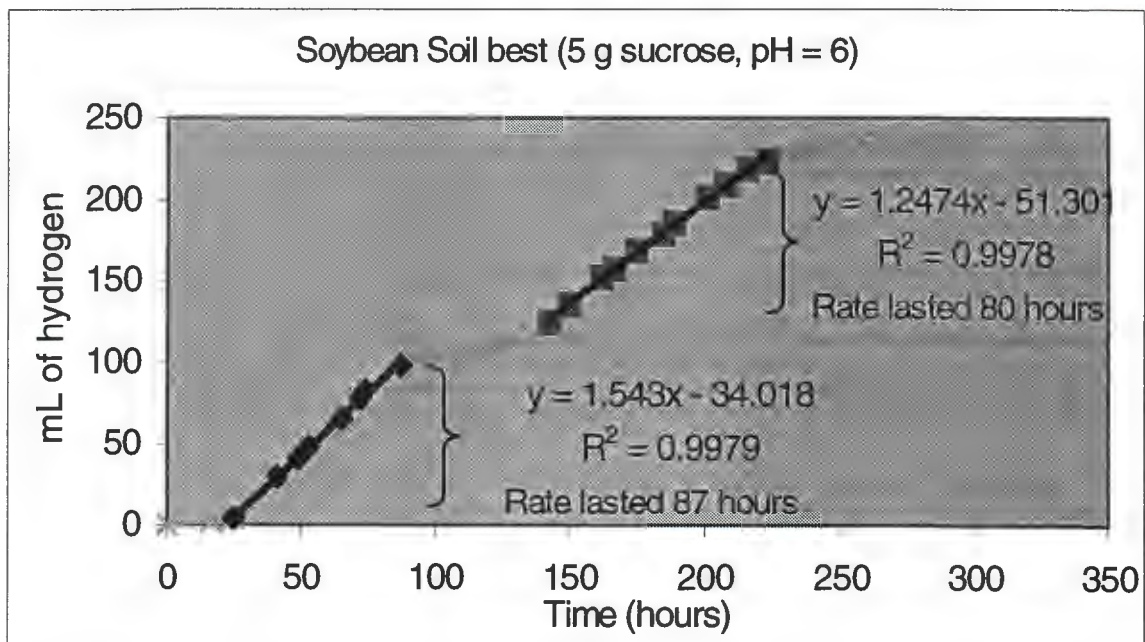
Figure A-2. Highest hydrogen production rate using soybean soil as the seed source.
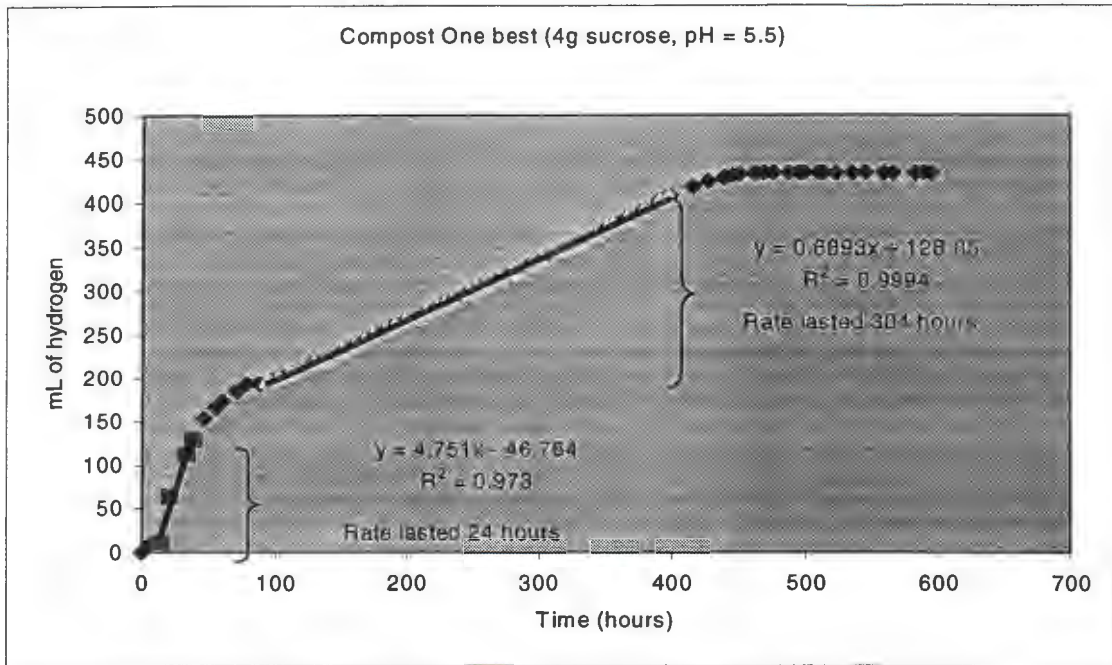
Figure A-3. Highest hydrogen production rate using compost one as the seed source.

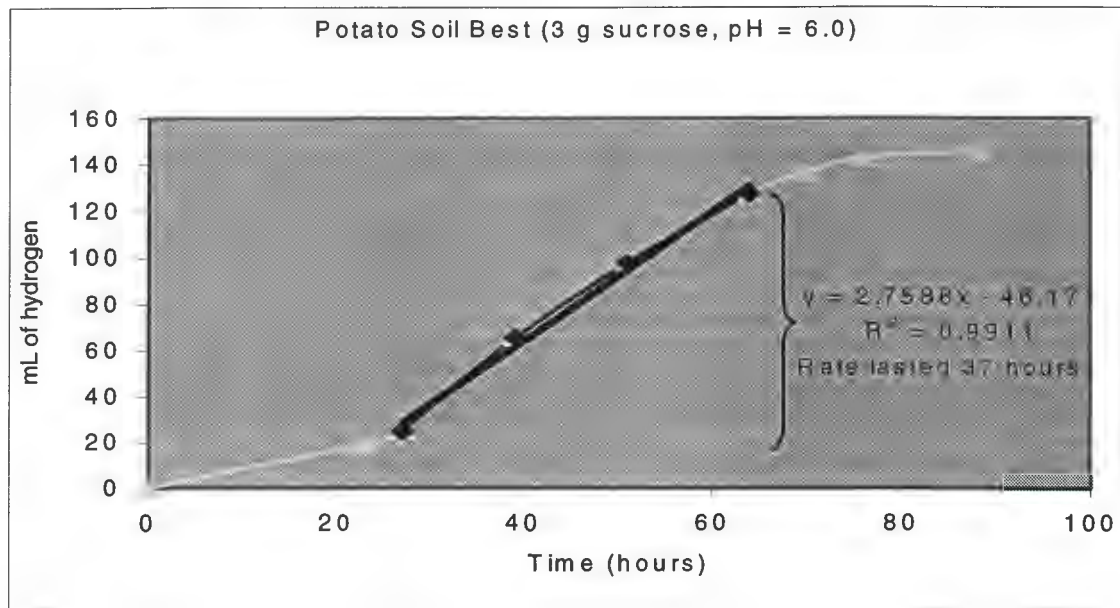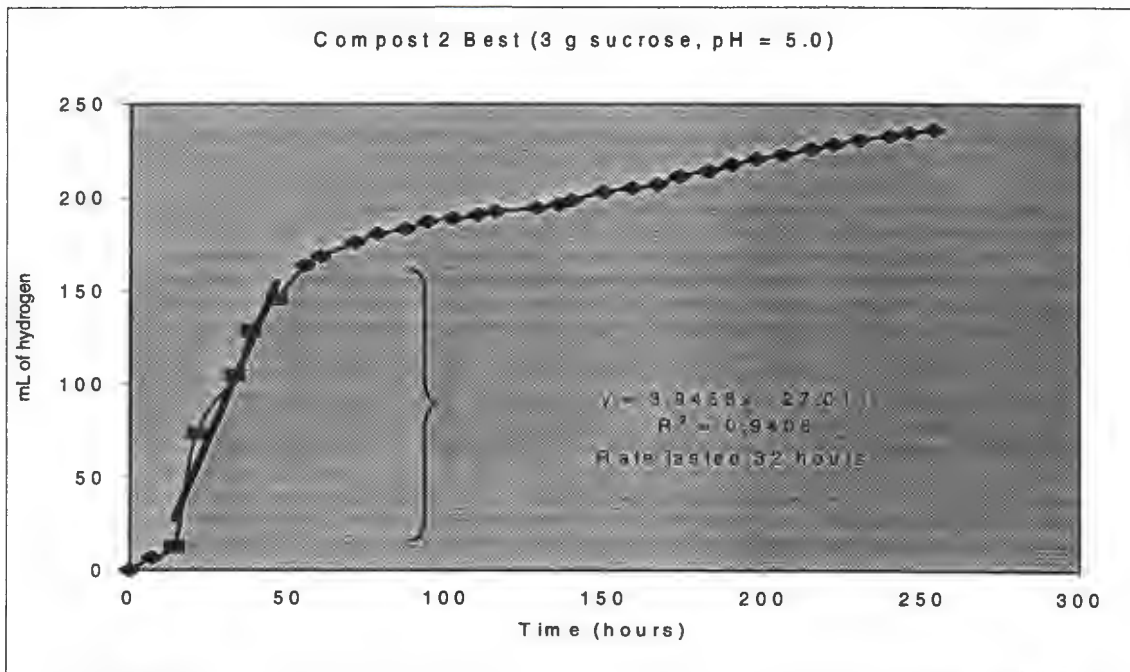**Figure A-4. Highest hydrogen production rate using compost two as the seed source.**
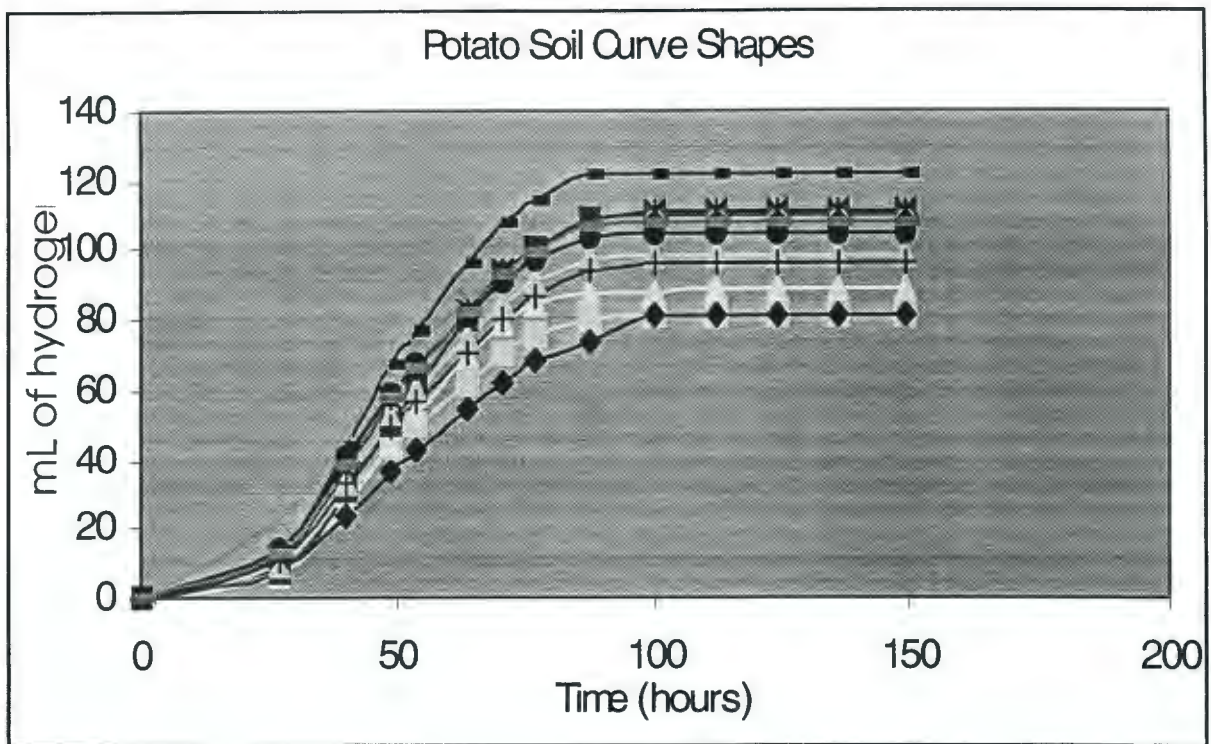
Figure A-5. Hydrogen production curves using potato soil as the seed source.
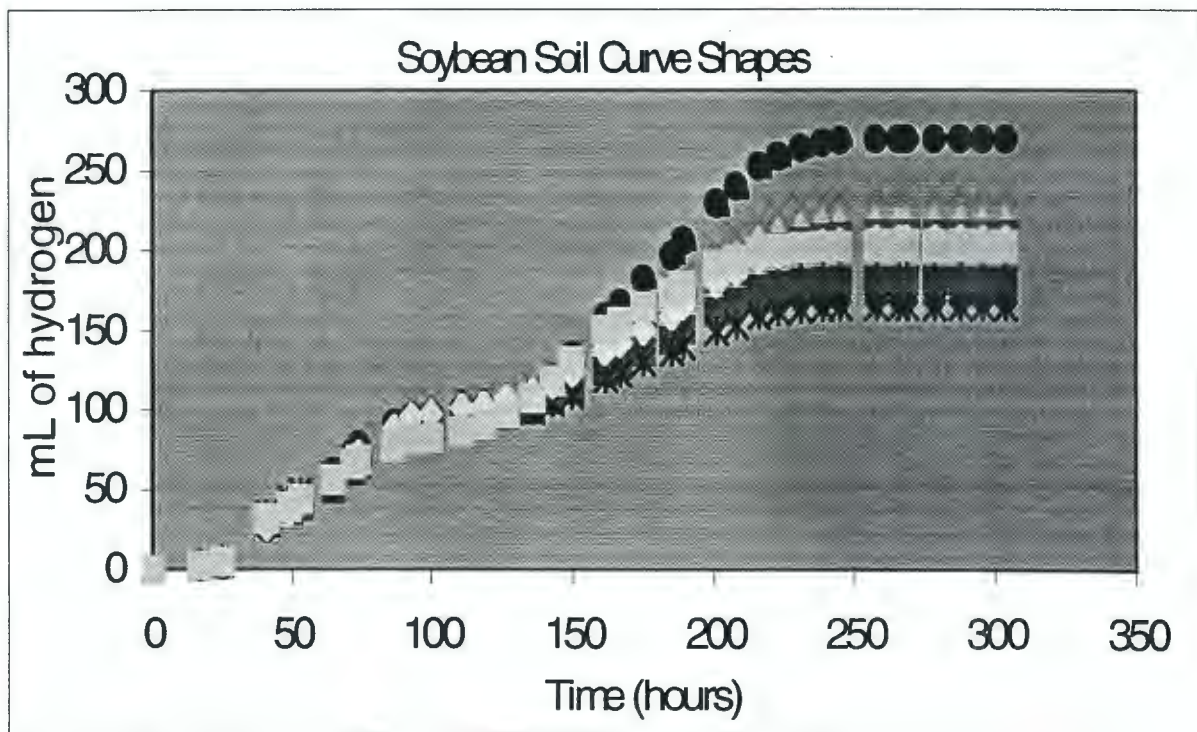
Figure A-6. Hydrogen production curves using soybean soil as the seed source.
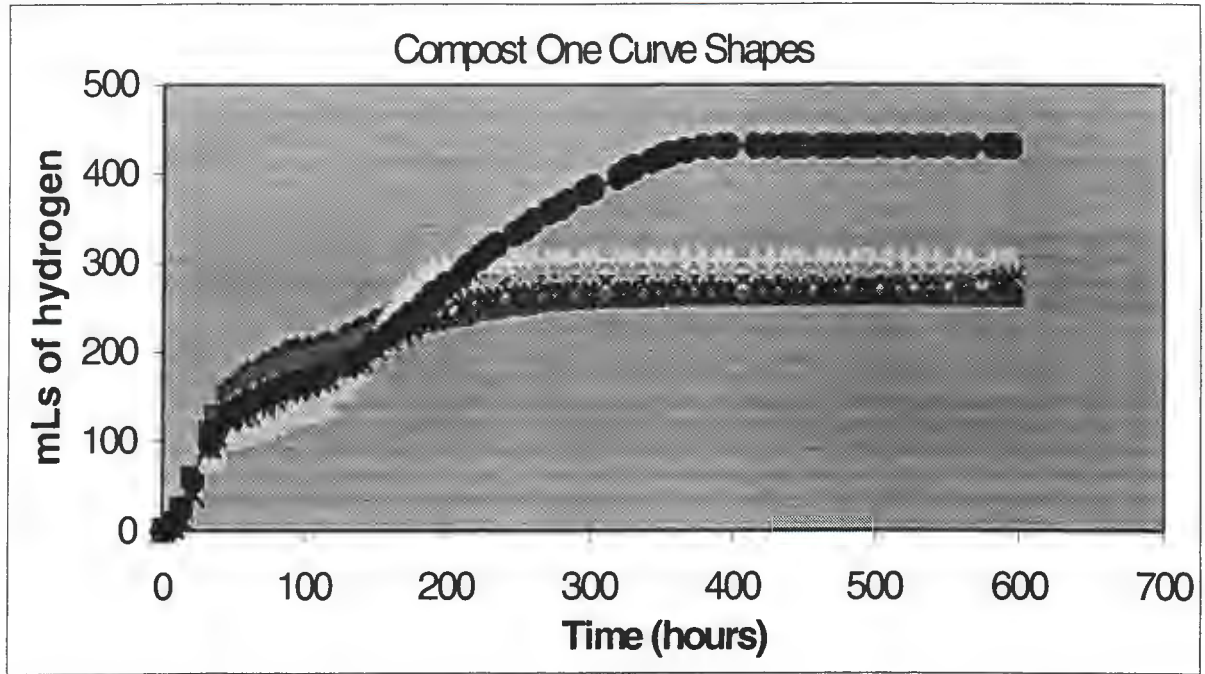
Figure A-7. Hydrogen production curves using compost one as the seed source.

Figure A-8. Hydrogen production curves using compost two as the seed source.

Figure A-9. The figure depicts the relation between undegraded sucrose and the final volatile solids determination where a higher initial sucrose concentration resulted in higher final volatile solids.
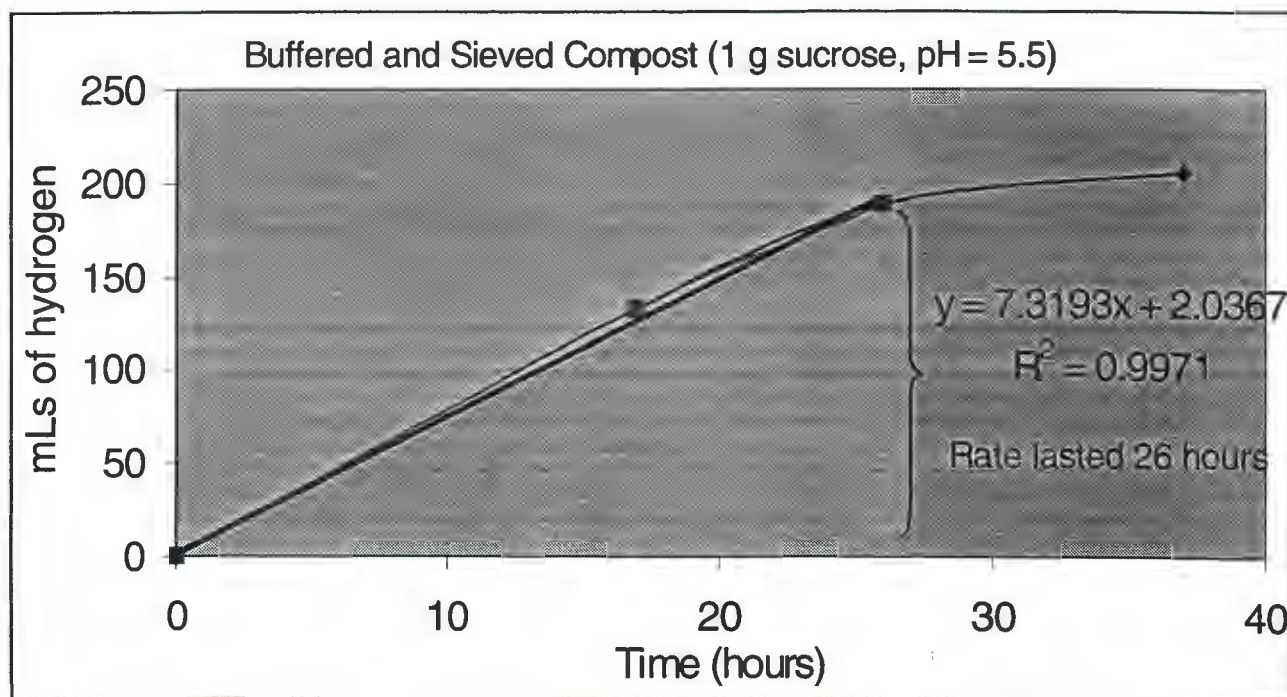
Figure A –10. Hydrogen production curve for the buffered sieved compost trial.

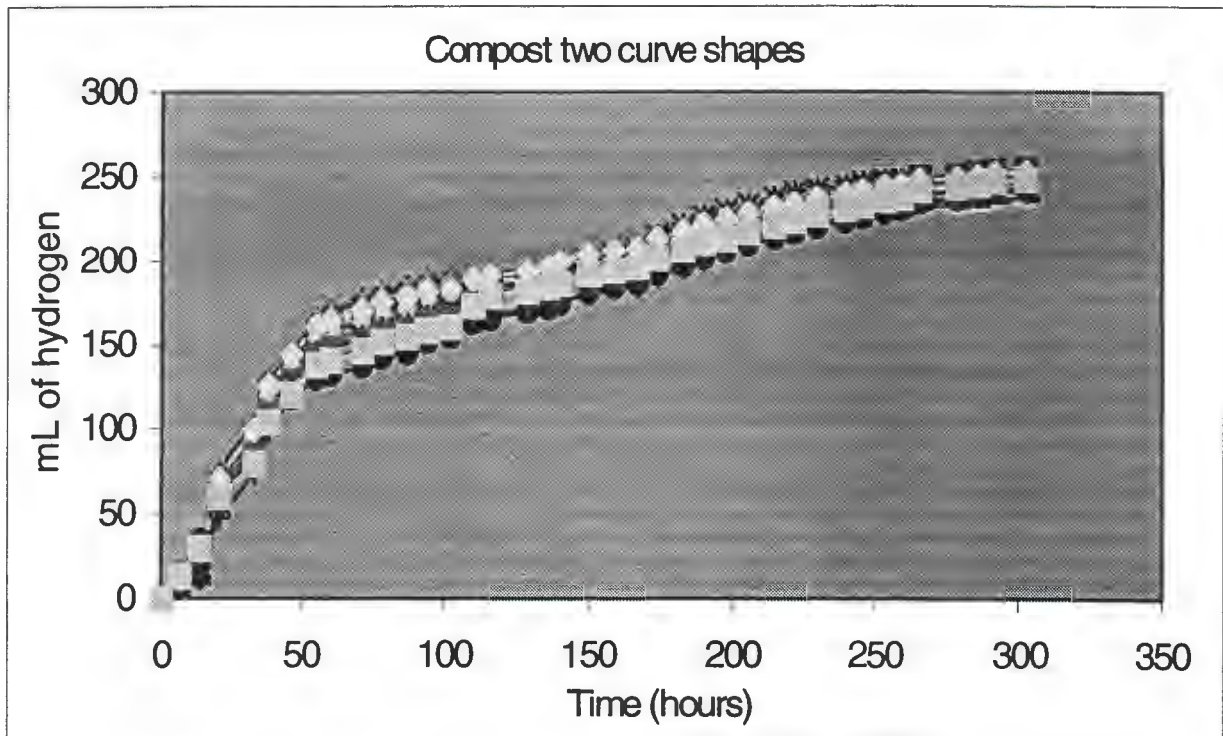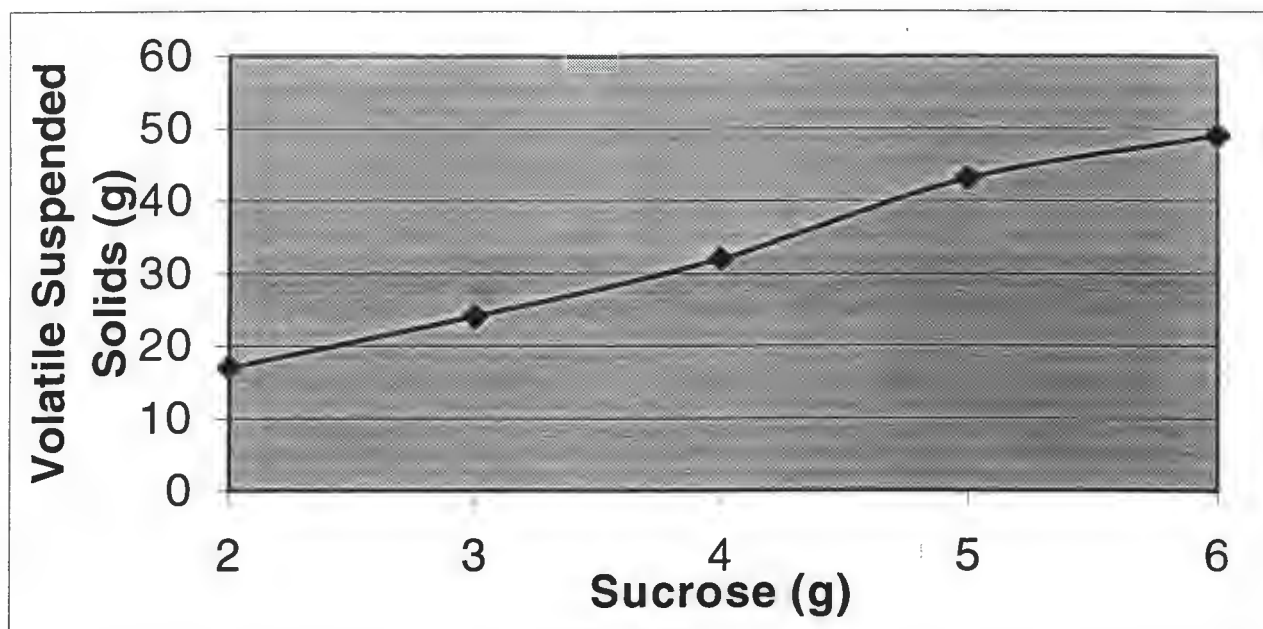The following is the raw data for the buffered high sucrose range experiment of Chapter 4. Included is the entire series of spreadsheets used to develop the regression equations that were used in the Igor™ plots. For most data sets, the line numbered 1 through 15 designates the bottle number. For most data sets, the left column is sampling time(0-37hrs). In instances where the data set would not fit on the sheet columns 12 through 15 were removed.

Design

Bottle	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Suc(g)	0.2	0.4	0.8	0.6	0.6	0.4	0.2	0.6	0.8	1	1	1	0.2	0.4	0.4
pH	7.5	6	6	7.5	6.5	7	6.5	5.5	7	7.5	6.5	5.5	5.5	5.5	7.5

Biogas

TIME	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
17	41	183	320	141	239	95	70	284	245	52	315	315	92	144	76
26	0	0	75	0	0	0	0	16	20	15	101	132	0	3.3	1
37	6	0	6.5	0	0	4	3.2	0	0	0	5.3	42	0	0	0

GC standard The top line represents 0.5 through 0.1 mL of pure hydrogen gas standard. 100 through 20 represents 100% through 20% hydrogen or the 'Y' used in the Linest regression equation of MS Excel™. The left column is sampling time $(0-37)$ while the array of numbers is the peak height (mm) of the standard or 'X' used in the Linest regression equation.

	0.5	0.4	0.3	0.2	0.1
	100	80	60	40	20
0	0	0	0	0	0
17	10.4	9.1	7.3	5.4	2.8
26	14.9	12.7	10.4	7.5	4.0
37	15.5	13.3	10.7	7.8	4.4

Regression of GC standard using Linest. After plugging in Y and X in Linest, the slope 8.81 was obtained for the standard curve. 0.96 is the R^2 value.

8.81	0.00
0.39	#N/A
0.96	6.56
89.03	4.00
3828.01	**171.99**

Sample GC. Below is the sample peak heights(mm) of each bottle at 17, 26, and 37 hours.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
17	0.23	4.25	5.25	2.35	4.50	1.85	1.57	5.25	3.60	4.40	3.04	5.60	3.50	4.60
26	0.00	0.00	7.25	0.00	0.00	0.00	0.00	6.40	4.43	4.80	4.45	8.00	0.00	5.70
37	0.50	0.00	7.00	0.00	0.00	0.50	0.30	0.00	0.00	0.00	3.80	8.10	0.00	0.00

Hydrogen Percentage. This is calculated by multiply the sample peak heights by the slope 8.81.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
17	2	37	46	21	40	16	14	46	32	39	27	49	31	41	8
26	0	0	45	0	0	0	0	40	28	30	28	50	0	36	5
37	3	0	42	0	0	3	2	0	0	0	23	48	0	0	0

Hydrogen Production(mL). This is calculated by multiply biogas (mL) by the hydrogen percentage.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
17	1	69	148	29	95	15	10	131	78	20	84	155	28	58	6
26	0	0	34	0	0	0	0	6	5	5	28	66	0	1	0
37	0	0	3	0	0	0	0	0	0	0	1	21	0	0	0

Cumulative Hydrogen Production(mL) or CH2 in the following simulation spreadsheet. This is calculated by consecutively summing up hydrogen production at each sampling time.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
17	1	69	148	29	95	15	10	131	78	20	84	155	28	58	6
26	1	69	182	29	95	15	10	138	83	24	112	221	28	60	6
37	1	69	185	29	95	16	10	138	83	24	113	242	28	60	6

Typical simulation spreadsheet modeling the experimental values with the Gompertz equation. The (0 to 40) in the Time column represents time in the Gompertz equation. The data in the Reg column represents fitted P, R, and L values in the VALUE column subjected to the MIN and MAX restraints using Solver. The REG-1 column represents P, R, L values fitted using the time in the TIME column. SE is the difference between the fitted and actual P, R, and L values. SSE is sum of square error. R^2 is the correlation coefficient and D is SSE/R^2.

TIME (HRS)	(ML)	REG	REG-1	SE			VALUE	MIN	MAX
0	0		15.903	2.5E+02	POTENTIAL	(ML)	241	0	250
17	155.4		173.292	3.2E+02	RATE	(ML/HR)	11	0	15
26	221.3		216.333	2.5E+01	LAG	(HR)	0	0	20
37	241.88		234.444	5.5E+01	SSE	6.5E+02			
					R^2	0.992839			
0	15.9				E	2.718282			
10	109.8				D	657.7241			
20	192.0								
30	225.7								
40	236.5								

Simulation curve fitting the Gompertz model to actual data

Kinetics Spreadsheet for all bottles.

	1	2	3	4	5	6	7	8	9	10	11
P	1	69	185	29	95	16	10	138	83	24	113
R	0	2	5	1	3	0	0	4	2	1	3
L	0	0	0	0	0	0	0	0	0	0	0
SSE	71.1	61.1	86.3	88.4	109.4	96.0	90.0	69.9	97.7	77.1	69.8
R²	0.997	0.998	0.997	0.997	0.997	0.997	0.997	0.998	0.997	0.997	0.998

Linest polynomial regression equation spreadsheet. The Z column represents all potential values for the batch at the particular pH(column X) and sucrose concentration (g COD/L)(column Y). The 'Y' values in Linest is column Z and the X values in Linest are columns X through XY. The first row in the bottom array is the regression equation with subsequent rows of associated statistics.

	S	Y COD	X PH	Z P	X	Y	X²	Y²	XY
1	0	0.22	7.5	0.03	7.5	1.49	56.25	2.23	11.20
2	0	0.45	6.0	1.85	6.0	2.99	36.00	8.92	17.92
3	1	0.90	6.0	4.99	6.0	5.97	36.00	35.68	35.84
4	1	0.67	7.5	0.79	7.5	4.48	56.25	20.07	33.60
5	1	0.67	6.5	2.56	6.5	4.48	42.25	20.07	29.12
6	0	0.45	7.0	0.42	7.0	2.99	49.00	8.92	20.91
7	0	0.22	6.5	0.26	6.5	1.49	42.25	2.23	9.71
8	1	0.67	5.5	3.72	5.5	4.48	30.25	20.07	24.64
9	1	0.90	7.0	**2.25**	7.0	5.97	49.00	35.68	41.81
10	1	1.12	7.5	0.66	7.5	7.47	56.25	55.75	56.00
11	1	1.12	6.5	3.07	6.5	7.47	42.25	55.75	48.53
12	1	1.12	5.5	6.54	5.5	7.47	30.25	55.75	41.07
13	0	0.22	5.5	0.76	5.5	1.49	30.25	2.23	8.21
14	0	0.45	5.5	1.61	5.5	2.99	30.25	8.92	16.43
15	0	0.45	7.5	0.16	7.5	2.99	56.25	8.92	22.40

XY	Y²	X²	Y	X	b	
0.65	0.20	1.89	-4.82	-27.15	96.39	#N/A
0.82	0.39	3.37	6.29	43.79	140.72	#N/A
0.37	5.74	#N/A	#N/A	#N/A	#N/A	#N/A
1.20	10.00	#N/A	#N/A	#N/A	#N/A	#N/A

APPENDIX TWO
FUTURE RESEARCH

In subsequent research, an attempt was made to discover hydrogen production rate at lesser initial VSS concentrations using domestic activated sewage sludge as a seed source and using 'enriched' hydrogen producing cultures. The enriched hydrogen producing cultures used compost and potato soil as the seed sources. In the first set of batch experiments of this thesis, undegraded sucrose was detected in the determination of the VSS concentrations giving an error in the biomass concentration. In the second set of batch experiments to estimate the increase in biomass concentration, the background VSS was still always too high to detect any significant increases in biomass concentration resulting from the degradation of sucrose. As a potential remedy to this situation, new batch serum bottle reactors were inoculated with an initial VSS concentration of 76 to 2000 mg / L.

In the enrichment procedure, a 1 L 'mother' reactor was used with the same F/M ratio stated earlier in this article. Here, F/M is defined as 4.7 grams of sucrose added to 140 grams of baked seed source. The reactor fermented for roughly thirty hours and the supernatant was poured off leaving a high solids portion behind. This supernatant was then boiled for fifteen minutes to destroy hydrogen consuming bacteria. Subsequently, the supernatant was centrifuged at 2,000 rpm for a half hour. The resulting supernatant (now fairly clear) was poured off leaving a solid portion behind. This solid (slurry) portion was washed (diluted with nanopure water / shaken / centrifuged / clear supernatant poured off) an additional two more times to wash away any VFA's that may have built up during the fermentation. This slurry was then used to inoculate a batch culture. This new F/M is defined as 0.5 grams of sucrose added to a 150 mL batch bottle consisting of a 2,000 mg VSS / L concentrated solution of enriched seed source. The table on the following page gives the highest rate results and the corresponding pH of several batch experiments that were conducted after the first portion of this thesis was written.

Seed Source	Initial VSS (mg/L)	pH	Sucrose (g)	Lag Phase (hr)	Rate (mL/hr)
Activated Sludge	963	6	0.5	37	1.7
Compost	76	5.5	0.5	72	3.3
Compost	439	6	0.5	34	3.9
Potato Soil	1367	6	0.5	81	3.5
Potato Soil	2000	5	0.5	64	3.8
Potato Soil	2000	6.5	1	27	3.0
Potato Soil	2000	6.5	0.5	24	3.0
Potato Soil	2000	5.5	1	27	3.0
Potato Soil	2000	5.5	0.5	42	3.4

In future research, the biomass concentration will be estimated using a fluorescence technique where a dye is added to a 1 mL portion of the batch bottle biomass slurry. In this technique, live bacteria are stained green while dead bacteria and plant material are stained red. After dyeing, the solution is 'fluoresced' and the resulting green and red colors are detected and 'enumerated'. This technique gives the number as well as the size of the bacteria in solution.

A semicontinuous reactor will be operated and the pH will be maintained in future research. This reactor will include a gas sampling port, a pH probe, continuous mixing, and an injection port to continuously add a buffer solution. It is the goal of this part of the research to maintain the pH at certain levels and then to compare the hydrogen production rates at these levels. It is another goal of this part of the research that any background VSS resulting from plant material will be passed through or eliminated from the system leaving just biomass. In this way, the biomass concentration could be calculated without any interference from background VSS.

Volatile fatty acids will be modeled in future research. The research group acquired a GC-FID to measure VFA's and alcohols. As noted in this thesis, some species of Clostridium are known to produce VFA's and hydrogen concurrently and some species of Clostridium switch to alcohol production once the pH decreases to a certain amount. It is important to model VFA's and hydrogen to understand the metabolic characteristics of the dominant biomass. It is also important to maintain a hydrogen producing culture. In earlier research by Dr. Lay using a hydrogen producing reactor fermenting starch, the culture unexpectantly 'shut' down and reverted back to their spore

form. The future goal would inevitably be to find out why this happened if it should ever happen again. Another goal is to compare the metabolism of hydrogen producers to typical acetogens in traditional methane production processes.

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