Hydrogen production during fermentation of cellulose by the thermophilic bacterium *Clostridium thermocellum.*

by

Lauren Magnusson

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THE UNIVERSITY OF MANITOBA

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Lauren Magnusson

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirement of the degree

Of

MASTER OF SCIENCE

AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Biohydrogen production from cellulosic waste materials using dark fermentation is a promising technology for producing renewable energy. The purpose of this study was to evaluate residual cellulosic materials generated from local sources for their H_2 production potential and to characterize growth of *Clostridium thermocellum*, during fermentation of a cellulosic substrate under continuous culture conditions. *C. thermocellum* ATCC 27405, is a cellulolytic, thermophilic bacterium that has been shown to be capable of H_2 production on both cellobiose and cellulosic substrates. The complex cellulosic materials tested were dried distillers grain (DDGs), barley hulls (BH) and *Fusarium* head blight contaminated barley hulls (CBH). Several simultaneous batch fermentation experiments were conducted in order to quantify H_2 production, and to determine soluble end-product synthesis patterns. Overall, the dried distillers grain produced the highest concentration of hydrogen gas at 1.27 mmol H_2/glucose equivalent utilized. CBH and BH produced 1.18 and 1.24 mmol H_2/glucose equivalent utilized, respectively. Overall, this study indicates that hydrogen derived from a variety of cellulosic waste biomass sources is a possible candidate for the development of sustainable energy. Continuous hydrogen (H_2) production during fermentation of α-cellulose was established using a 5 L fermentor. Growth experiments were maintained for over 3000 hours. Substrate concentrations varied between 1 to 4 g L^{-1}. Continuous sparging with nitrogen gas was used to prevent clogging of the feed-line. The pH and temperature of the reactor were maintained throughout the experiment. At concentrations above 4 g L^{-1}, the delivery of α-cellulose was impaired due to feed-line clogging and it became difficult to maintain a homogenous suspension. The highest total gas (H_2 plus CO_2) production rate, 56.6 ml L^{-1} hr^{-1}, was observed at a dilution rate of 0.042 h^{-1} and substrate concentration of 4 g L^{-1}. Under these conditions, the H_2 production rate was 5.06 mmol hr^{-1}. Acetate and ethanol were the major soluble end-products, while lactate and formate were greatly reduced compared to production in batch cultures. Concentrations of all metabolites increased with increasing substrate concentration, with the exception of lactate. This data has increased knowledge of

H_2 gas production via direct fermentation of an insoluble cellulosic substrate under continuous culture conditions. These results show that H_2 production is proportional to substrate concentration, but product ratios remain constant within the loading rates tested.

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Chapter 1
Introduction

1.1 The need for alternative energy technologies

Since the advent of the internal combustion engine the economy has been inextricably linked with the production and consumption of oil. The abundance of cheap and readily available energy from oil for use in the transportation sector has affected not only how communities were designed but also how society developed. Innovations in automotive technologies and the ever-expanding fossil fuel infrastructure made it possible to live farther from work and other necessities than ever before, effectively creating commuter cultures and paving the way to the development of a suburban nation. The abundance of cheap oil also made possible trade with distant nations, further solidifying the link between the global economy and oil. Indeed the shape of society as we know it is largely to do with the booming oil revolution of the 20^{th} century.

The economic growth spurred by the development of the oil infrastructure seemed infinite until the oil crisis of the 1970s'. That market crash illustrated for the first time how volatile oil prices could be and how dangerous it was to be dependant on a fuel source when the production and distribution couldn't be controlled. This first crisis caused people to seriously think about the application and implementation of alternative energy sources. Since then many other issues surrounding the use of fossil fuels have come to light. Global climate change caused by anthropogenic carbon dioxide (CO_2) emissions and the inevitability of

reaching peak oil production have provided further reason to revisit the need for an alternative energy source (Turner 2004).

Currently world energy usage is increasing at a rate of 2% per year (Mason 2007). According to the Energy Information Administration the total world consumption of energy is projected to increase by 57% from 2004 to 2030, however nations within the Organization for Economic Cooperation and Development (OECD - Canada and the US included) are only forecasted to increase their energy usage by 24% whereas non-OECD countries are expected to increase their energy consumption by 95% in the same timeframe (EIA 2007). Therefore it will be developing nations that have the biggest impact on carbon dioxide emissions in the future. This projected increase in energy usage will have a negative impact on the price, availability and the pollution caused by combustion of transportation fuels. Analysis conducted by the EIA forecasts peak oil to occur between 2021-2067 depending on economic growth and advances in energy conservation, but many other studies have forecasted peak oil production to occur in the next several years (Simmons 2002). It is therefore necessary to advance research in the area of alternative fuel technology to a point where cleaner; more widely available and cheaper energy options become commercially feasible in the next 10 to 20 years.

1.2 Biofuels from starch based substrates

Biofuel production has garnered significant attention in recent times, attracting very polarized opinions from both the scientific community and the public. The Canadian and US governments both view the use of biofuels as a method of slowing greenhouse gas production

as well as a solution to help reduce dependence on foreign fuels while simultaneously supporting the local economy (Greene et al. 2004). New research in the area of food-grain starch based ethanol production has caused the validity of those assumptions to come into question. Currently industrial-scale biofuel production uses food grains such as wheat, corn and sugar cane to produce ethanol. There are dissenting opinions that argue against the environmental, economic and ethical benefits of growing food crops for use as fuels (Fargione 2008; Searchinger 2008).

The issues with cultivating plants for use as fuel are numerous and complex. Currently the most widely used and promoted biofuel in North America is ethanol derived from corn. Over the last decade, the cost of corn has been rising steadily (Streitfeld 2008). While there are many factors that contribute to this rise, many are placing sole blame on the increased demand for corn-based ethanol. The media has had a huge impact on how the public views the economy of biofuels. Many articles attempt to connect the increase in demand for ethanol with hunger around the world, claiming that ethanol directly results in food shortages for the poor while providing transportation and recreation for the rich (Qualman 2003). Indeed, diverting crops from food to fuels is one of the most compelling arguments against biofuels produced from wheat, corn or oilseeds. Because of the relationship between food and fuel, biofuels produced from corn and wheat have not succeeded in the goal of creating a secure fuel source, rather they have put more pressure on an already stressed and unstable resource.

The claim that starch based biofuels will aid in abating greenhouse gas emissions has also been debated. New studies have been conducted on the carbon balance of the corn to ethanol fuel production system that take into account land-use change. The studies have found that

the amount of carbon that cropland absorbs is substantially less than that of the land it replaces, regardless of the type of land originally present (Rosenthal 2008). Another study found that the clearance of grassland released 93 times the amount of greenhouse gas that would have been saved by the fuel made annually on that land (Fargione 2008).

Despite the controversy both the U.S. and Canada continue to create programs and legislation to support the use of biofuels (Soloman and Banerjee 2006). In the U.S., an energy bill passed on December 19, 2007 mandates that 36 billion gallons per year of ethanol is blended into the nations fuel supply by 2022. Part of that bill stipulates that at least 3% of that ethanol comes from cellulose-based sources by 2012 and increases to 44% by 2022. It is evident that corn starch based ethanol alone cannot meet the energy demands of the country. Even if 100% of the total corn production in the U.S. were devoted to ethanol production, they would only be able to replace 20% of their annual fuel consumption (Soloman et al. 2007).

Canada has similar legislation that calls for 5% renewable content in gasoline by 2010. Programs such as the 'Capital Formation Assistance Program for Renewable Fuels Production' ($200 million), the 'Biofuels Opportunities for Producers Initiative' ($10 million) and the 'Agricultural Biproducts Innovation Program' ($145 million) were put in place in order to achieve that mandate. More recently the ecoEnergy for biofuels program was allocated $1.5 billion over 9 years to support biofuels development and production in Canada. Despite the increasing amount of funding, the biofuel programs will not be successful if they continue to completely source the feedstock from agricultural crops such as corn, wheat and canola. The amount of feedstock required to support such a large

displacement is similar to the U.S. In order to meet the 5% goal, 48-52% of the total corn seeded area, 11-12% of the wheat-seeded area and 8% of the canola-seeded area would have to be devoted solely to biofuel production (AAFC, 2008).

1.3 Biofuels from cellulose based substrates

It is clear that in order to meet government-mandated requirements for biofuels, alternative methods of biofuel production will need to be employed. Most literature on this subject concurs that biofuel production from cellulosic sources, especially those derived as a by-product of an existing industry, have a net energy gain and do not cause any negative ancillary consequences (Lynd et al. 2006; Lynd et al. 2005; McLaughlin et al. 2002; Schmer et al. 2008; Sun and Cheng 2002). Although the technology used to produce fuel from raw lignocellulosic materials is still in its infancy, several pilot scale plants have been built that are able to use pretreated waste materials such as wood chips, wheat straw and corn stover to produce ethanol (Soloman et al. 2007). In the United States, the EPA has set an annual production goal of 3.8 hm^3 of cellulosic ethanol by 2015 (Service 2007).

1.4 Hydrogen as a fuel

Although ethanol has been the main focus of many government-funded programs and initiatives, in comparison hydrogen (H_2) for use as a transportation fuel has many environmental benefits. The use of hydrogen as a renewable fuel has been the focus of much discussion and research in part due to its high energy yield (2.75 times greater than hydrocarbon based fuels (Kapdan 2006)) and it's ability to deliver energy with no end-of-pipe emissions. One of the major setbacks to the commercialization of hydrogen for use as a

fuel is production. Unlike its fossil fuel counterpart, it isn't readily found in nature. This fact, however, hasn't been a deterrent to countries such as Iceland, who envision a completely hydrogen-based economy as soon as 2050. In fact, as of 2003 Iceland has completed the first goal of constructing a hydrogen fueling station and is currently operating several hydrogen-fueled busses. The second stage of the conversion began in 2007 and involves converting the massive fishing fleet, a substantial part of the local economy, to hydrogen fuel (Arnason and Sigfusson 2000; Ingason et al. 2008). Several other countries have begun to adopt hydrogen energy plans, perhaps most notably the state of California in the U.S.A. has initiated a hydrogen hiway networked dubbed "CaH2Net" (Ogden 1999).

There are several different methods of hydrogen production, but biohydrogen from dark fermentation is unique in that it is able to utilize waste to produce a fuel that has no detrimental emissions. Biohydrogen production by dark fermentation, rather than by bio-photolysis of water or photo fermentation, is ideal because of its ability to produce hydrogen efficiently. Other methods of hydrogen production such as electrolysis and steam reformation of methane require extensive amounts of energy and also are a source of polluting emissions, such as CO_2, CO, NO_x and SO_x (Turner 2004).

There are many obstacles to overcome before hydrogen can be introduced into society as a clean, efficient and most importantly, economical replacement to conventional fuels. Some of the road blocks to this goal include; H_2 collection, purification, distribution, storage and usage. The large-scale use of hydrogen as a fuel would require much research, thought and analysis of the practicality and economics.

Biological hydrogen production by dark fermentation must overcome several hurdles before industrial scale experiments are plausible. Continuous fermentation has been found to significantly increase hydrogen production, but barriers due to end-product inhibition, stability of the system and methanogenic bacteria contamination still pose problems. Many studies have been completed on bench-scale reactors to quantify and optimize these parameters, but no large-scale hydrogen production reactors have been built as of yet.

Hydrogen production by dark fermentation has been the focus of many recent studies (Nguyen et al. 2008; Oh et al. 2004; Surygala 2006; Zhang et al. 2006). The high yield of hydrogen per mole of glucose used is the reason for the enthusiasm surrounding fermentative hydrogen production. Theoretically one mole of glucose can produce 4 moles of hydrogen when acetate is the only product.

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 4H_2 + 2CO_2 \qquad \text{(Levin 2004)}$$

In practice, this high yield is never achieved as other end-products such and butyrate, formate, alcohols and lactic acid are produced. The key to achieving 4 mol hydrogen produced per mol of hexose degraded is to stop alcohols and reduced acids from forming while gearing the reaction towards the production of less reduced volatile fatty acids (VFA's) (Levin 2004).

The main goal of a large portion of the current research on biohydrogen production by fermentation is to increase H_2 production rate and yield enough that the process becomes economically feasible. Many studies have been conducted to evaluate the attributes of various substrates fermented by different species of bacteria (Chen et al. 2001; Fang 2002;

Minnan 2005). Variables such as temperature, pH, HRT and SRT have been tested to determine their effect on production rate. Physically, the process of fermentation and the bioreactor itself have been examined and altered by addition of fixed media to retain the bacteria and by the use of membrane biological reactors to both separate biogas from solution and as a means of retaining bacteria in solution (Liang 2002; Oh 2004). Most studies are currently being conducted using continuous culture because of the vast improvement in production rate over batch experiments.

Fermentative hydrogen production can be carried out using various sources of carbon. Studies have tested the hydrogen production using starch, cellobiose, lactose, glucose, sucrose, xylose and cellulose. The goal is to move away from lab grade substrates and begin fermenting complex lignocellulosic waste streams. Typically hydrolysis is necessary in order to ferment cellulose based waste materials. Bacteria find soluble substrates much easier to digest. As in conventional wastewater treatment processes, the degradation of insoluble constituents is the rate-limiting step.

1.5 Substrates for biofuels production from fermentation

1.5.1 Soluble substrates

Biofuel production by dark fermentation is not limited to cellulosic substrates. In fact, the majority of research conducted to date has been focused on fermentation of soluble substrates such as glucose, sucrose, lactose and xylose (Hernandez 1982; Lin and Chang 1999; Liu et al. 1988). These sugars are the hydrolysis products of cellulose and hemicellulose. Part of the reason for the abundance of studies on soluble substrates is that unlike cellulosic substrates,

hydrolysis is not required. This results in higher production rates and, in some cases, higher product yields. Additionally, working with insoluble substrates creates many operational issues in terms of product measurement and substrate delivery. Soluble substrates offer many process benefits compared to more complex and insoluble substrates:

1) Biomass concentration can be determined quickly by optical density (OD);

2) Substrate concentrations can be measured easily and accurately;

3) A low horsepower peristaltic pump can deliver the substrate consistently and reliably.

Despite the operational benefits of working with a soluble carbon source, the issues with using a substrate such as glucose as a model for biofuel production are numerous. The use of soluble substrate facilitates study on the kinetics and characterization of a bacterium, but an comercially feasible hydrogen producing process requires that the substrate, in most cases, be cellulosic and therefore insoluble.

Currently only a handful of studies exists that demonstrate continuous fermentation of an insoluble substrate (Desvaux et al. 2001a; Lynd et al. 1989; Pavlostathis et al. 1988). Cellulase production, cellulose hydrolysis and fermentation occur in a single process step. This represents a breakthrough that has the potential to significantly decrease the cost of biofuel production from cellulosic materials.

1.5.2 Lignocellulosic substrates

Cellulose is the most abundant organic compound on the earth (Lynd et al. 2006). It is found in plant cell walls, enveloped in a matrix along with hemicellulose and lignin. Typically 35

to 50% of native plant material is composed of cellulose (Lynd et al. 1991) 25-35% is hemicellulose (Ohmiya et al. 2005) and the remainder is composed of lignin. Hardwoods such as birch and willow are composed of approximately 40% cellulose, with the most abundant hemicellulose sugar being xylose (18-20%). Softwoods such as spruce and pine contain a slightly higher percentage of cellulose (approx. 45%) with mannose comprising the majority of hemicellulose sugar. Grasses like wheat straw, rice straw and corn stover have a similar composition to hardwoods (Wiselogel et al. 1996).

Cellulose is a polymeric compound built up of insoluble fibers of D-Glucose linked together by b-1,4-glucosidic bonds, with cellobiose as the smallest repetitive unit (Kumar and Wyman 2008). Native cellulose contains both loosely bound amorphous and highly organized crystalline regions. Intra and intermolecular hydrogen bonding is responsible for the formation of crystalline cellulose. The degree of polymerization (DP) of cellulose can be greater than 10000 (Jorgensen et al. 2007). Hemicellulose is a noncellulosic polysaccharide composed of pentoses (xylans, arabinans), hexoses (mannans, galactans and glucans) and sugar acids. Compared to cellulose, it is a shorter chain polymer with a higher degree of branching and a DP below 200. Lignin is a complex polyphenolic structure. Purified, it can be used for phenolic compounds extraction for pharmaceutical purposes (Kumar and Wyman 2008), or combusted and used as a source of heat or energy. Of the lignocellulosics, softwoods contain the highest amount of lignin (roughly 30%), hardwoods contain less (22-24%) while grasses contain the least (12-24%) (Hayn et al. 1993). The presence of hemicellulose and lignin in complex substrates makes them less available to enzymatic degradation, and very resilient against microbial fermentation.

1.5.2.1 Cellulosic Waste material

There is tremendous potential in Canada for biofuel production from a variety of biomass sources. It has been estimated that the energy contained in biomass across the country is approximately 2.58 PJ per year (Levin 2007). Taking into account the other fractions of waste, there is the potential that 456 PJ are available from biogas production. Comparing this to a total energy usage of 7,324.4 PJ per year used in all sectors, it can be seen that biogas production could meet 16% of Canada's energy needs. However, not all biomass will be technically suitable or economically feasible for biogas production. It is important to identify and evaluate locally generated and available sources of waste biomass for their energy production potential.

Complex cellulosic substrates can be grown as a crop or sourced as a waste by-product from industry. Cellulosic and lignocellulosic materials account for a large portion of the waste that ends up in landfills. Paper and paper sludge make up approximately 45% of landfills, around 3 million dry tons per year in the U.S. alone (Lark, 1997). Agricultural, industrial and forest residues all produce waste that is high in cellulose materials, about 180 million tons per year globally (Demain et al. 2005). Considering all sources of waste cellulosic and lignocellulosic materials, the estimated yearly biomass production is approximately 1.5 trillion tons (Kim and Yun 2006). The structure of lignocellulosic materials makes them resistant to the conventional methods of biofuel production by fermentation with *Saccharomyces cerevisiae*. Several stages of pre-treatment are required to expose the cellulose and enzymatically hydrolyze it to its sugar components. These additional steps make the process more expensive and less feasible on an industrial scale.

The number of continuous studies conducted using untreated cellulosic waste as substrate is few, with the majority focusing on ethanol production (Fan and Lynd 2007a; Fan and Lynd 2007b). Most of the studies conducted using high percent solids waste streams use intermittent feeding regimes to facilitate substrate delivery. For example, paper waste sludge has been converted to ethanol by simultaneous saccharification and fermentation using a semi-continuous system fed every 12 hours (Fan et al. 2003). Because a pure cellulosic substrate was used, that process did not require chemical or thermal delignification prior to fermentation. Several studies have utilized pre-treated cellulosic substrate (hydrolysate) for continuous hydrogen production, or waste sources that are not cellulosic. Although several batch studies have illustrated fermentation of a complex lignocellulosic material without pretreatment, continuous fermentation has not yet been demonstrated. A summary of current research in continuous and semi-continuous fermentation of waste substrates for hydrogen production is presented in Table 1.1.

Table 1.1 – Summary of semi-continuous hydrogen production studies fermenting waste products using a consortium of bacteria

Substrate	Temperature °C	Reactor configuration	pH	HRT hr	Rate L/Ld	Yeild mL/VS added	Reference
Food Waste	35	ASBR	5.3	24	2.71	61.7	Kim et al. 2008
Garbage with paper waste	60	CSTR	5.8-6	28.8	5.4	46.3	Ueno et al, 2007
Food waste	55	CSTR	5.5	120	1	125	Shin and Youn, 2005
Household solid waste	37	CSTR	4.8-5.2	48	1.61	43	Liu et al., 2006
Bean curd manufacturing waste	35	CSTR	5.5	6	1.2	24	Noike et al., 2005
Brewery waste	37	CSTR	5.5	18	2.68	37.9	Fan et al., 2006
Jackfruit peel waste	27	Contact filter	5.1	288	0.42	189.5	Vijayaraghaven et al., 2006

1.5.2.2 Cellulosic Crops for biofuels production

Switchgrass is a native, perennial prairie grass. Compared to other crops used for biofuel production, its yield is very high, it has lower nitrogen runoff (~40% less than corn and ~60% less than soybeans), very low erosion due to it's extensive root structure and increased soil carbon (Schmer et al. 2008). If harvested correctly, it can also provide wildlife habitat. It has been estimated that crops like switchgrass and hybrid poplar could generate 1.3 billion tons of biomass by the mid-twenty-first century.

Winter rye, oilseed rape and faba bean straw pretreated by wet oxidation have been studied for their ethanol and biogas yields (Petersson et al. 2007). All crops require high soil nitrogen availability, so planting in an area where manure is spread to fix N and prevent nitrogen runoff would be beneficial to the overall process. Additionally, synthetic nitrogen fertilizer production is very energy intensive. In fact, the application of fertilizer to a field accounts for a large portion of the total energy required to grow a crop. This can have a significant effect on the energy balance of producing biofuel from a dedicated crop (Wiselogel et al. 1996). 15 to 25% of complex substrates are made up of lignin. Far from being a wasteful by-product, lignin can be converted to fuel by thermochemical gasification and used to provide heat and energy to the fermentation process (Jorgensen et al. 2007).

1.6 Methods for biofuel production from lignocellulosic substrate

Biofuel production using lignocellulosic materials is conventionally seen as a process requiring several steps of pretreatment. The prevailing technology involves removal of

hemicellulose and lignin followed by enzymatic hydrolysis of cellulose and subsequent fermentation of the resulting cellodextrins. There are several processes used that can accomplish pretreatment of lignocellulosic products to produce soluble sugars, all of which increase the cost of biofuel production due to the requirement for additional materials, chemicals and energy. Figure 1.1 illustrates the differences between the various stages involved in separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF) and consolidated bioprocessing (SBP).

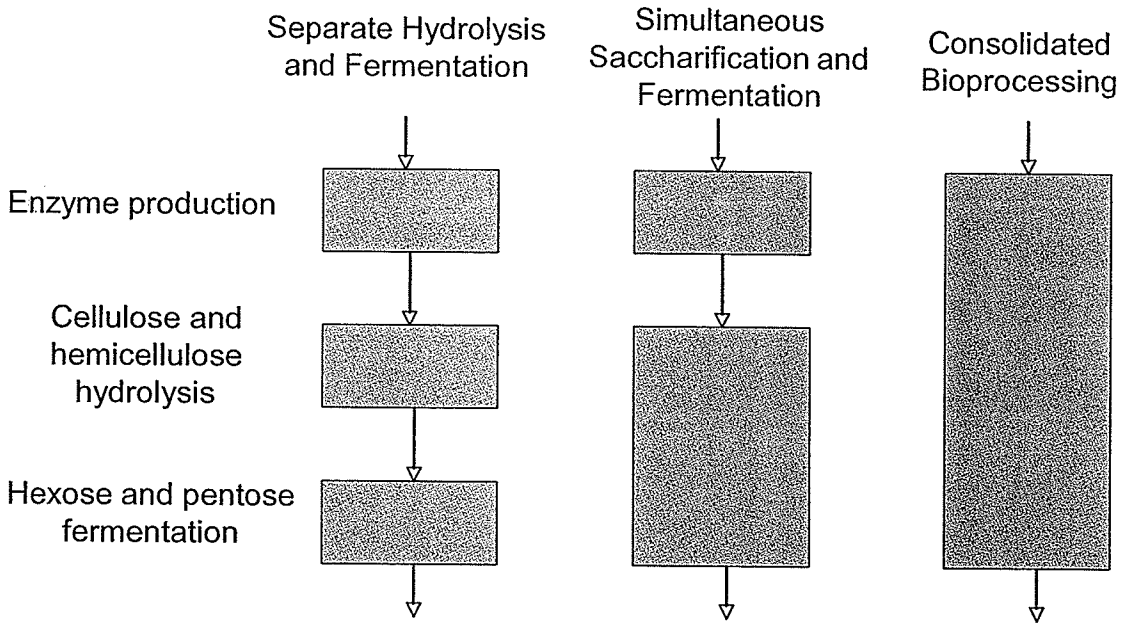


Figure 1.1 – Process flow diagram of the most common methods of biofuel production from lignocellulosic materials.

Cellulose can be hydrolyzed by chemical or physical reactions with varying concentrations of ammonia, acid or base and a combination of heat treatment and/or pressure. The aim of

pretreatment using dilute acid, steam explosion or acid-catalysed steam explosion is to hydrolyze and solubilize the hemicellulose fraction. The lignin is redistributed along the surface of the cellulose fibers. Alkali treatments function only to release the lignin, leaving the hemicellulose intact (Jorgensen et al. 2007). Enzymatic hydrolysis using cellulases and hemicellulases is required to further hydrolyze the substrate into cellobiose, glucose and other sugars (Hahn-Hagerdal et al. 2006). Removal of the lignin prior to treatment by enzymes eliminates the non-specific binding of the cellulase to the lignin. After complete hydrolysis of cellulose, up to 70% of the enzyme added has been found to be attached to lignin (Berlin et al. 2005). Proteins and surfactants are currently being developed that will help to reduce the unproductive binding of cellulases to lignin (Tu et al. 2007). Lignin also inhibits water adsorption and fiber swelling of the cellulose, which further limits the amount of sites enzymes have to attach to (Pu et al. 2008).

There are several types of bacteria that have been found to ferment cellulose using cellulase systems. Most research in the area of enzymatic cellulase hydrolysis systems involved the fungus *Trichoderma reesei* and the bacteria *Clostridium cellulolyticum* and *Clostridium thermocellum* (Bayer et al. 1998). *T. reesei* contains noncomplexed cellulases, whereas the cellulase systems of the mesophile *C. cellulolyticum* and the thermophile *C. thermocellum* are aggregated into a complex termed the cellulosome (Felix and Ljungdahl 1993).

The digestibility of a sample of complex substrate is influenced by several parameters. Cellulose and lignocellulose materials are highly resistant to both chemical and microbial degradation. The properties that contribute to its recalcitrance are its crystallinity, hydrogen bonding and hydrophobic outer layer. The aim of pretreatment is to increase the accessibility

of the substrate to the cellulases and to decrease the DP. Improvements in digestibility of a substrate after acid pretreatment can be explained in part by the removal of xylans, one of the main constituents of hemicellulose. Removal of non-fermentable pentose sugars helps to increase the availability of cellulose to attack by cellulases (Jeoh et al. 2007). Hydrolysis and subsequent dissolution of hemicellulose can be achieved by dilute acid pretreatment (Schell et al. 2003). Changes in porosity can also affect the fermentability of a substrate because their are more sites available for enzymatic attack (Esteghlalian et al. 2001) and the decreased DP (Knappert et al. 1980). Water content influences the swelling, crystallinity and digestibility (Grous et al. 1986). The opposite occurs when the substrate is dried. Internal surface area is decreased permanently.

Product inhibition caused by accumulated glucose and cellobiose reduces the effectiveness of enzymatic treatment. This problem has been addressed by developing a process termed simultaneous saccharification and fermentation (SSF). Enzymatic hydrolysis and fermentation occur in a single step, so glucose and cellobiose are metabolized as they are produced (Figure 1.1). However, the optimal conditions for fermentation and enzymatic hydrolysis do not typically coincide. Cellulases typically operate at temperatures around 50°C (Berger et al. 2007), therefore mesophilic fermentations are not favorable for enzymatic hydrolysis. Consolidated bioprocessing (CPB) using thermophilic organisms is able to achieve higher hydrolysis rates due to enzyme-microbe synergy (Lu et al. 2006), therefore a process using CPB to produce biofuels has the potential to be lower cost and higher efficiency than separate hydrolysis and fermentation (SHF). Pretreatment and cellulase production accounts for approximately 20% of the total capital investment cost, so removing

this step from the process would increase the feasibility of commercialization (Soloman et al. 2007).

1.7 Clostridium thermocellum

Clostridium thermocellum is a gram positive, acetogenic, thermophilic, strictly anaerobic and spore-forming bacterium that has the capability of breaking down cellulose in a single step fermentation process. Products of its fermentation include acetate, ethanol, carbon dioxide (CO_2), hydrogen (H_2), lactate and formate, see Figure 1.2 (Islam et al. 2006; Levin et al. 2006; Sparling et al. 2006). The cellulolytic and ethanogenic properties of the bacterium make it particularly suitable for converting waste lignocellulosic biomass into a useable fuel source. In addition to its ability to degrade lab-grade substrates such as cellobiose, crystalline and amorphous cellulose, it can also ferment lignocellulosic feedstocks and their hydrolysates. It is also capable of growth on glucose and other hexose sugars depending on the conditions in which it is grown and after an adaptation period of several days (Demain et al. 2005; Strobel 1995). *C. thermocellum* displays one of the highest rates of cellulose degradation owing in part to the presence of a extracellular cellulase system called a cellulosome that functions to adhere the bacterium directly to the cellulose (Demain et al. 2005; Lynd et al. 2002).

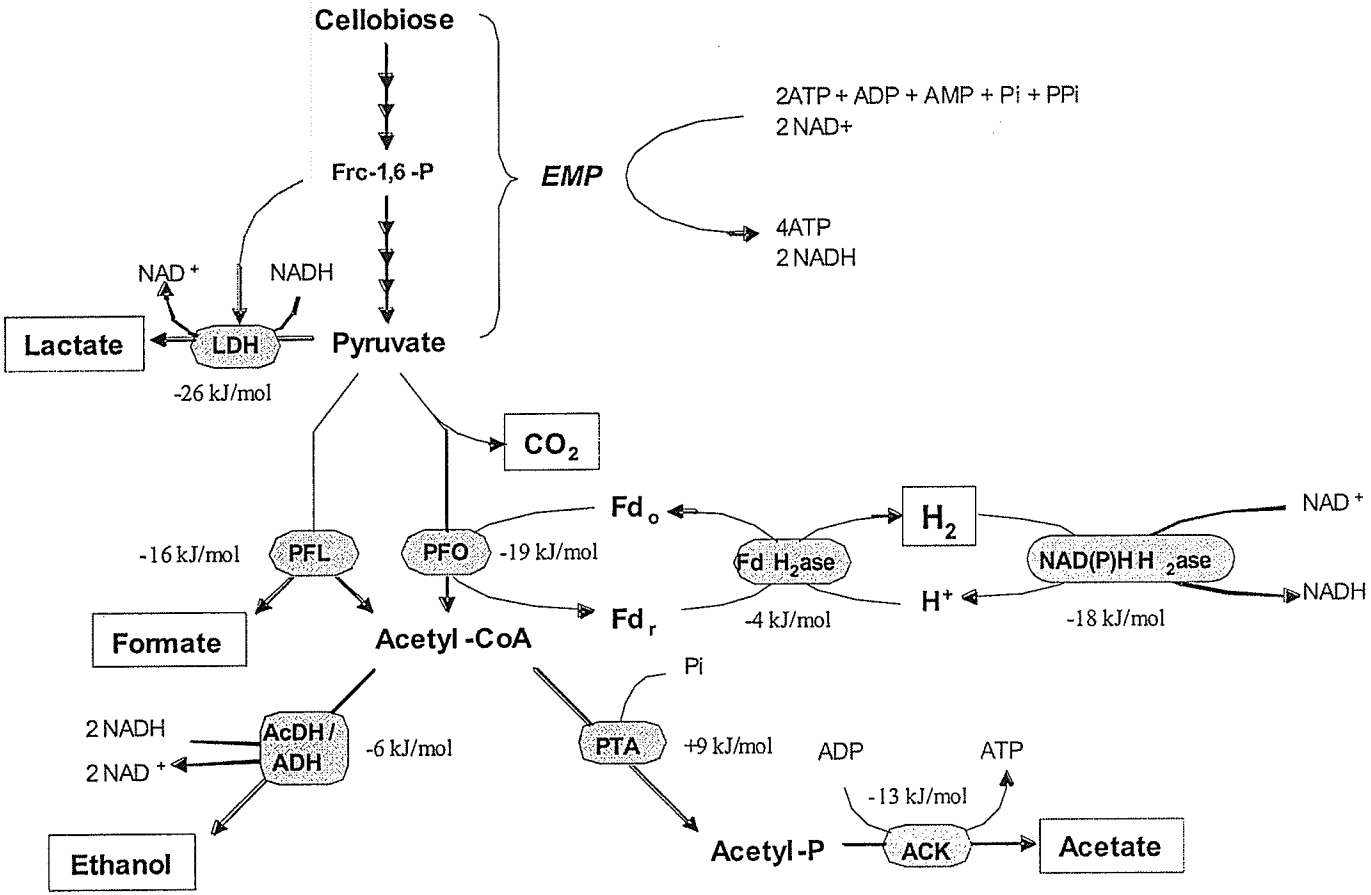


Figure 1.2 - Pathway-model of different metabolites based on literature review of cellulose fermentation by *Clostridium thermocellum* (Lynd et al. 1989; Sparling et al. 2006; Weimer and Zeikus 1977).

In addition to the high H_2 production rates associated with *C. thermocellum*, its growth conditions offer many other ancillary process benefits. The bacterium is most productive at 60 - 65°C (Krieg and Holt 1984), precluding growth of many contaminating organisms. This means that if used in an industrial application it may be possible to forgo the addition of expensive antibiotics and the energy intensive process of sterilization. The high growth temperature also facilitates ethanol removal by distillation. Keeping the concentration of ethanol in the reactor at a low concentration is desirable both from a thermodynamic perspective and because of the low ethanol tolerance of *C. thermocellum* (Herrero and Gomez 1980; Ng et al. 1981; Ng et al. 1977). The low cell growth yield of anaerobic bacteria results in higher specific production rates of metabolites (Ohmiya et al. 2005). The anaerobic nature of the bacterium also negates the need for costly aeration processes.

Several strains of *C.thermocellum* have been isolated and characterized from a variety of sources including soil, compost, manure, wastewater and geothermal springs (Bayer et al. 1983; Bayer and Lamed 1986; Stainthorpe and Williams 1988). *C. thermocellum* JW20 was isolated from de-seeded cotton bail which also contained *Klebsiella* and *C. thermosaccharolyticum* (Wiegel and Dykstra 1984). *C.thermocellum* growing *in situ* will typically be surrounded by host of other bacteria functioning in a symbiotic nature (Ng et al. 1977). Although *C. thermocellum* is not able to degrade hydrolytic products of hemicellulose, it contains many hemicellulose-degrading enzymes like xylanase, mannanase, lichenase and chitinase (Schwarz 2001). This could be due to a synergistic relationship with other bacteria that can use the products of hemicellulose hydrolysis in nature, or the function

may be to remove the hemicellulose fraction to expose the preferential cellulosic substrate (Bayer and Lamed 1986). Sporulation has been found to occur when the bacterium is grown on cellobiose and cellulose, but is not common when grown on xylose and glucose (Freier et al. 1988).

1.7.1 Hydrogen production

The ability of *C. thermocellum* to produce hydrogen from cellulose and its hydrolytic products has been studied in both batch and continuous culture (Bothun et al. 2004; Islam et al. 2006; Lamed et al. 1988; Levin et al. 2006; Sparling et al. 2006). The highest H_2 yield produced by *C. thermocellum* (2.32 mol H_2 per mol hexose degraded) was reported using delignified wood fiber as substrate at a concentration of 0.1 g L^{-1} which corresponded to carbon limited conditions (Levin et al. 2006). Table 1.2 summarizes the body of research that has been conducted on hydrogen production by *C. thermocellum*. In general, lower substrate concentrations seem to result in higher hydrogen production per mole of hexose degraded.

Table 1.2 - Summary of measured H_2 yields produced by several strains of *C. thermocellum* on various substrates in batch culture

Strain	Substrate	Variable	Substrate concentration (g/L)	Yield (mol H_2/mol hexose)	References
LQ8	cellulose		9.72	0.852	Wiemer and Zeikus, 1977
ATCC 27405	Cellobiose		4.5	0.98	Islam, 2005
JW20	Cellulose	No agitation	1	0.68	Freier, 1988
		100 RPM		1.23	
	Cellobiose	No agitation		0.61	
		25 RPM		0.73	
		75 RPM		0.94	
		200 RPM		1.02	
ATCC 27405	Cellobiose		0.1	1.63	Levin, 2006
			1.1	1.73	
			4.5	1.13	
	DLW[a]		0.1	2.32	
			1.1	1.47	
			4.5	0.99	
Wild type	Cellulose	pH 6.5	47	0.44	Tailliez, 1989
		pH 7.2		0.34	
LD1[b]		pH 6.5	47	0.23	
		pH 7.2		0.16	

[a] Delignified wood fiber

[b] Asporogenous and ethanol-tolerant mutant

1.7.2 Ethanol tolerence

The ethanol tolerance of *C. thermocellum* has been investigated extensively. *C. thermocellum* has a low alcohol tolerance for growth (<2% [vol/vol]) (Burdette et al. 2002). Ethanol exerted an inhibitory effect on fermentation of cellulose by *C. thermocellum* and caused a cessation of gas production (Weimer and Zeikus 1977). Inhibition of *C. thermocellum* by ethanol has been attributed to a blockage in glycolysis associated with ethanol-induced changes in the cell membrane (Herrero and Gomez 1980). These results indicate that removal of ethanol produced in the fermentation process could accelerate the growth rate and cellulose degradation. The maximum concentration *C. thermocellum* has been found to grow at is 60 g L^{-1} by an ethanol tolerant strain (Strobel and Lynn 2004). Another study has shown production up to 26 g L^{-1} (Lynd 1996).

1.7.3 Cellulose Degradation

C. thermocellum utilizes hexoses but not the pentose sugars generated from cellulose and hemicellulose (Demain 2005). Zhang and Lynd (2005) found that cellodextrins up to cellohexaose (G6) can be utilized by *C. thermocellum*. Using radiolabeled cellulose Zhang and Lynd also found that *C. thermocellum* preferentially assimilates cellodextrins with n~4 during growth on cellulose.

1.7.4 Cellulosome

The cellulosome is a extracellular cellulase complex that consists of multiple components acting synergistically to link the bacterium to the cellulose and provide concentrated

enzymatic activity (Bayer et al. 1998; Kumar and Wyman 2008). The presence of a cellulosome is not unique to *C. thermocellum.* Several other species including *C. cellulolyticum* also contain complexed enzymatic units .

Production of the cellulosome is greatly dependant on the conditions in which *C. thermocellum* is being grown. Transcription of the most abundant exoglucanase CelS is controlled by the substrate as well as the growth rate under carbon limiting conditions (Dror 2003).

C. thermocellum has been reported to excrete a yellow affinity substance when grown on cellobiose and cellulose (Freier et al. 1988; Ljungdahl et al. 1983) Higher rates of cellulase activity were shown in cultures that were frequently transferred without letting the pH drop below 6.4 (Freier, 1988). Continuous culture conditions simulate frequently transferred conditions, which can explain why we see YAS in continuous culture, but not in batch.

The cellulosomal structure is affected by the stage of growth as well as the type of substrate it degrades (Mayer et al. 1987; Morag et al. 1992; Shoham et al. 1999). During the exponential phase of growth the majority of cells are attached to cellulose fibers, whereas during stationary phase they become detached (Wiegel and Dykstra 1984). Attachment is inhibited in the presence of glucose & cellobiose.

C. thermocellum for the biodegradation of crystalline cellulose. These authors showed that the activity of this cellulase system toward cotton was at least 50 times higher than that of the extracellular cellulase system from T. reesei (Lamed et al. 1988; Tomme et al. 1995).

One of the major organizational roles of the scaffolding protein of the cellulosome is to bring several catalytic subunits into close proximity (Lamed et al. 1983). The cellulosome of *C. thermocellum* contains multiple types of interacting scaffoldins, which increases the number of enzymes that can be present in the complex (Bayer et al. 1998).

1.7.5 Temperature

The thermophilic nature of *C. thermocellum* results in several operational and thermodynamic advantages over mesophilic processes (Ng et al. 1977). Maintaining culture purity of continuous cultures that are operated at lower temperatures can be difficult due in part to the abundance of mesophillic organisms. The continuous culture apparatus is inherently susceptible to contamination because of the many opportunities for entry during regular maintenance of media, pH control or effluent analysis. The larger scale the fermentation, the more difficult it is to maintain sterility. Therefore, executing a scale-up experiment of a pure mesophilic culture may be more problematic than it's thermophilic counterpart (Demain, 2005).

1.7.6 Agitation

In batch culture it has been shown that agitation decreases the ratio of ethanol to acetate and increases the cellulose degradation rate (Lamed et al. 1988). Stirring also increased the hydrogen production (Table 1.3). The shift in the ethanol to acetate ratio was greater in cellulose grown cultures than cellobiose. The stirring did not completely remove dissolved H_2 from the broth, but the effects of supersaturation were overcome at rates above 150 rpm.

However, during the initial growth period the culture must be left to settle or the cellulosome will not attach to the cellulose (Freier, 1988)

Table 1.3 - Effect of stirring on the production of H_2 by *C. thermocellum* grown on cellulose and cellobiose (Lamed, et al.,1988)

Substrate	Strain	Initial H_2 (µmol)	Stirred H_2 (µmol)	Improvement
Cellulose	YS	215	600	179%
	AS-39	420	770	83%
	LQRI	490	900	84%
Cellobiose	YS	340	530	56%
	AS-39	400	650	63%
	LQRI	400	900	125%

1.8 Continuous Fermentation

Continuous fermentation of organic substrates for hydrogen production has been successfully achieved with both mixed and pure cultures (Brosseau and Zajic 1982; Kim et al. 2005). In general, continuous fermentation produces significantly more hydrogen than batch processes because it is continually operating at the cells most productive stage and there is no down time required. The density of cells in batch culture is restricted by either the depletion of available substrate or by accumulation of inhibitory end-products.

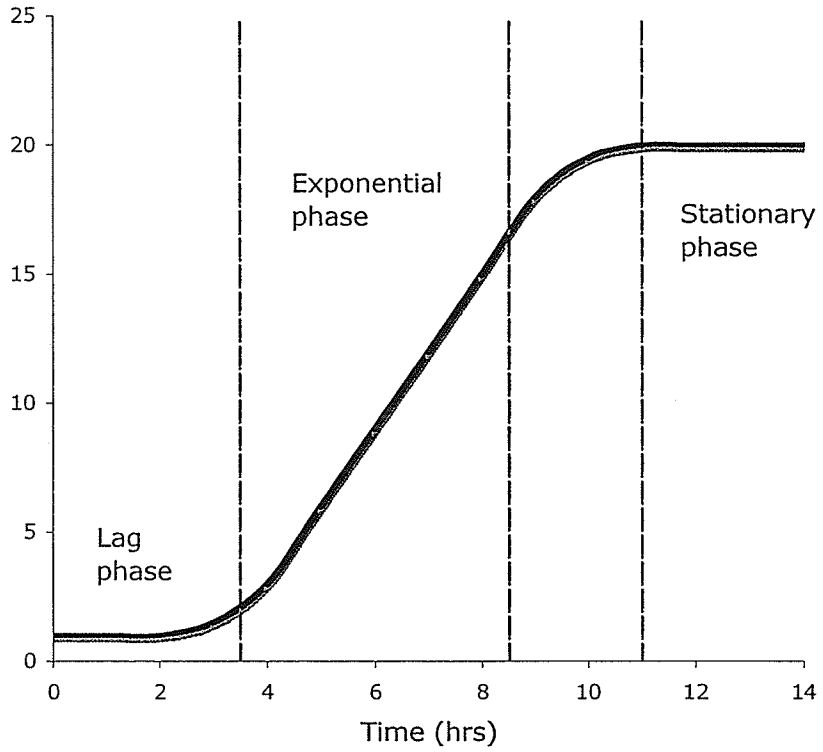


Figure 1.3 - Plot of the ln of biomass concentration vs. time during batch fermentation of a typical microbial culture

Figure 1.3 illustrates the typical batch fermentation plot of the ln of biomass concentration over time. The equation that describes biomass formation in typical batch culture fermentations is:

$$x_t = x_o e^{\mu t}$$

Where: x_t = the biomass concentration at time, t

x_o = the initial biomass concentration

μ = the specific growth rate

When operating in continuous mode, cells are constantly being exposed to fresh media and substrate, while waste products are removed. This allows the cells to grow for a theoretically infinite period of time in their most productive phase. The doubling time of the cells and hydraulic residence time of the chemostat reactor restricts cell densities. If a perfusion type or cell retention/recycle reactor is employed, much higher densities and substrate utilization rates are achievable. Some typical hydrogen growth rates from continuous fermentation studies are summarized in Table 1.4. Substrates used in previous studies have ranged from municipal waste to pure glucose or cellulose. In most cases there may be insufficient nutrients in the substrate for the bacteria to grow, so nutrient enrichment media is commonly added to the to the growth medium. Studies have also been conducted using many different types of bacteria. Mixed culture inoculant can be obtained from anaerobic sewage sludge digestors, or even compost heaps. Typically this material is heat treated at 100°C for at least 15 minutes to kill any non-spore forming bacteria. Spore formers such as *Clostridium* and *Bacillus* are typical hydrogen producers that are most often present in mixed cultures (Levin 2004). When mixed cultures are used, it is necessary to ensure that methane-producing bacteria do not have the opportunity to form within the system because these bacteria will consume H_2. Other studies have focused on using pure culture to determine the hydrogen production potential of a specific type of bacteria (Christophe Collet 2004; Collet et al. 2004; Yokoi 1997).

Table 1.4 – Summary of mesophilic continuous fermentation studies on soluble substrates using mixed microflora

Substrate	Concentration (g/L)	OLR[a] (g/L/day)	Reactor configuration	Temp °C	pH	HRT (hr)	H_2 Production ($l\ l^{-1}\ d^{-1}$)	Yeild mol H2 / mol	Reference
Sucrose	20 g COD l^{-1}		Immobilized	35	6	6.0	43.20	2.00	Wu and Chang, 2006
Sucrose		80	CSTR	35	5.2	12.0	0.50	1.15	Kyazze et al. 2006
Sucrose		82	Sequencing batch				0.42	0.94	Lin and Jo, 2003
Sucrose		854	Biofilm				7.40	1.59	Lee et al. 2003
Sucrose		1708	Stirred granular sludge				15.09	1.59	Wu et al. 2006
Sucrose	1.7		CSTR		6.9	3.0	26.90	1.17	Chen and Lin, 2003
Sucrose	12	48.6	UASB		5.5	4.1		2.10	Fang and Liu, 2001
Sucrose			CSTR[b]		4.4			0.99	Ren et al. 1995
Glucose	18.75	37.5			5.7	6.0	16.00	1.76	Lin and Chang, 1999
Glucose	8	16			5	2.0		1.13	Horiuchi et al. 2002
Glucose	10	27	N_2 Sparging	35	6	8.5	4.77	1.43	Mizuno et al. 2000
Glucose	9.37	68.1	Membrane Bioreactor		5.5	7.3		1.00	Oh et al. 2004
Glucose	15.95	42.5			5.5	2.7		1.12	Lee et al. 2004
Glucose	10		CSTR	37	5.5	10	6.11	1.95	Zhang et al. 2006
Glucose	10		CSTR	37	5.5	6	7.77	1.88	Zhang et al. 2006
Lactose								1.50	Collet et al. 2004
Molases							0.20		Ren, 2006

[a] Organic loading rate

[b] with solids retention

Most studies on continuous culture are focused on optimizing the system conditions in order to maximize hydrogen production and substrate utilization. It is widely accepted that reducing the concentration of dissolved H_2 and CO_2 in the broth results in increased H_2 yield and production, but the reason for the increase is still being debated (Kraemer and Bagley 2006). The hydraulic residence time (HRT) plays a key role in both H_2 production and substrate degradation. In many cases continuous experiments are performed on chemostat reactors (Lynd et al. 2002). This means the contents are assumed to be completely mixed, therefore the HRT is equal to the solids residence time (SRT). An important consideration for these types of systems is washout of bacteria. As the HRT is decreased, substrate is passed through the system at increasing rates. The bacteria are able to convert the substrate to product up to the point where they cannot reproduce fast enough, and are washed out of the system. It is therefore of interest to find a way to maintain bacteria in the system while keeping the HRT at a high rate. Some methods that have been suggested include fixed-bed, solid matrices, spin filters and various immobilization techniques. The following is a discussion of continuously operated bioreactors for the purpose of hydrogen production.

1.8.1 HRT

Depending on the substrate, the optimal HRT for hydrogen production can vary substantially. 8 hours on sucrose (Chen and Lin 2003), 12 hours for glucose (Lin and Chang 1999) and 18-24 hours for brewery wastewater (Yu et al. 2003). One study using *Clostridium thermolacticum* and lactose as substrate found that higher HRTs were best if complete degradation of lactose is desired, but the lowest volumetric production of hydrogen was also observed at these values. The maximum amount of hydrogen in the gas phase was 86%

(v/v), occurring at an HRT of 5.3 hours. When pH was increased, more hydrogen was observed in the gas phase. Decreasing the HRT had an effect on the amount of CO_2 diffused into the gas phase. The faster the medium was renewed in the bioreactor, the greater amount of bicarbonate ions were washed out, and lower amounts of CO_2 diffused to the gas phase (Collet et al. 2004). Another study using a hyperthermophilic bacterium tested HRTs between 2.5 and 1.1 hours. The highest production of hydrogen was observed at an HRT = 1.25 hours, cells were washed out at 1.1 hours (Kanai 2004). When cells are immobilized, the HRT can be decreased beyond what would usually cause washout. Hydrogen production using immobilized cells has been reported to increase from 8 to 30 times by increasing the dilution rate from 5.88 to 0.5 hours (see Table 1.4) (Wu 2005).

1.8.2 Immobilization of bacteria

Continuous fermentation can be run using many different configurations and variables. Some continuous systems use a technique to immobilize cells within the bioreactor, which allows for higher dilution rates and lower hydraulic retention times (HRTs) than suspended cell systems. This has the potential to produce greater volumes of hydrogen at increased substrate utilization rates, meaning the system is more efficient and therefore more economical.

1.8.2.1 Fixed Bed

A study completed by Chang (2002) used fixed bed bioreactors and porous media such as loofah sponge, expanded clay and activated carbon to evaluate the effectiveness of using a support matrix to retain the hydrogen-producing bacteria. This study used a mixed culture

bacteria sourced from acclimated sewage sludge with sucrose as substrate. The study found that substrate utilization was above 80% for all HRTs tested, and in many instances was found to be above 90%. The maximum hydrogen generation rate of 1.320 l/h/l occurred when using activated carbon as support, with an HRT of 1h and corresponding to a substrate utilization efficiency of 87.3%. Lowering the HRT to 0.5 hours resulted in cell washout (Chang 2002).

Another study completed by Kumar and Das (2001) looked at immobilizing a pure culture of *Enterobacter cloacae* on lignocellulosic materials. In this case rice straw, bagasse and coir were tested for their effectiveness as support matrices. The substrate used was glucose. They found the most productive support media to be coir, which when using a dilution rate of 0.9 h^{-1} resulted in a hydrogen generation rate of 62 mmol/L/h. When they changed the configuration of the bioreactor from tubular to rhomboid (to decrease gas hold up) and introduced cell recycle, they found that at a dilution rate of 0.93 h^{-1} the hydrogen generation rate increased to 75.6 mmol/L/h. In general, their conversion efficiencies were much lower than the previous study. The highest conversion efficiency recorded was 75% and dropped as low as 25% (Kumar 2001).

Other methods of immobilizing the bacteria include biofilm formation on poly vinyl alcohol (PVA), granulation by polymer addition (Kim 2005), and immobilization on silicone gel (Wu 2005). Immobilization on silicone gel was accomplished by mixing the acclimated sewage sludge with activated carbon. This mixture was combined with silicone gel and extruded to produce the immobilized sludge. The HRT was varied from 0.5 to 6 hours, and the substrate concentrations tested were 10, 20, 30 and 40 g COD L^{-1}. The highest specific hydrogen

production rate, 0.439 L h^{-1} g^{-1}-VSS occurred at a sucrose concentration of 30 g COD L^{-1} and 0.5 hour HRT. The highest hydrogen production rate, 15.09 L/h/L occurred at 40 g COD L^{-1} and 0.5 hours HRT.

1.8.2.2 Spin Filters

Spin filters are another method of retaining biomass in the bioreactor and increasing substrate utilization by preventing washout of bacteria at higher dilution rates. They operate based on physical sieving of particles. They help to reduce feedback inhibition of end products by removing them from the system as they are being produced, before they reach prohibitively high concentrations. The spinning action of the filter helps to keep biomass from accumulating on the surface and clogging the pores. For bacterial cell culture, speeds up to 1000 RPM are typical to help prevent biofilm formation on the surface of the filter (Mulchandani 1993). An important design consideration is the optimum rotational rate of the spin filter. Operating at a slow rate will increase the opportunity for bacterial contamination; however operating at too fast a rate will decrease the driving flux across the filter due to an increase in centrifugal force. Spin filter tests conducted on *E. coli* found that fouling that occurred below 500 RPM could be reversed by increasing the rotational speed, however fouling that occurred above this value was irreversible. With an enclosed pressurized spin filter backflushing with gas can be an effective method of removing biofilm deposits, but after long periods of operation EPS tends to foul the filter irreversibly (Naja 2006). The in-situ nature of spin filters creates limitations based on fouling. External cell recycle filters have increased flexibility in terms of cleaning and replacement during long-term continuous

operation. Typically spin filters are constructed from stainless steel. Polymeric membrane microfilters can also be wrapped around the stainless steel to improve biomass retention.

1.8.3 Membranes

Another important parameter that has an effect on hydrogen production is the partial pressure of hydrogen in the bioreactor. Many studies have shown that by continuously removing hydrogen, the reaction will be more productive. One study showed a continuous gas release yielded 43% more hydrogen gas than an intermittent gas release when using glucose as the substrate (Logan, 2002). The reduced hydrogen production with the intermittent release method is attributed to the high hydrogen partial pressure in the reactor. This results in dissolved hydrogen in the liquid, which is suspected to be consumed by methanogens, if they are present within the system.

Liang et al. (2002) completed a study on the use of silicone rubber membranes in removing the biogas from solution. This method of removing gas is effective because not only is the partial pressure of the gas in the headspace reduced, but the amount of hydrogen and CO_2 in solution is reduced as well. Removal of CO_2 from solution can be important because it is suspected that dissolved CO_2 can contribute to a reduction in pH, which inhibits end-products formation due to poor cell growth conditions. This study found that by adding the membrane to the system they were able to improve the hydrogen evolution rate by 10% and the hydrogen yield by 15%. They also found that when operating with excessive amounts of carbon and a low HRT the bacteria that were most prevalent under these conditions tended to

produce CO_2 over H_2. This indicates that when using a mixed culture it is important to optimize substrate dosing and HRT.

Membranes can also be used in a hydrogen production system to reduce methanogens by decreasing the hydraulic residence time (HRT). Methane-producing bacteria generally grow slower than hydrogen producing bacteria. By decreasing the HRT, the methanogenic bacteria have less time to grow. A study conducted by Oh, Iyer, Bruns and Logan (2004) found that when using an MBR with a mixed culture no methane was produced. They also found however that gas production was only increased in comparison with the system without the MBR when using a relatively short HRT (3.3hrs). Therefore, for a larger system with longer retention times it would be more efficient to run the reactor as a chemostat. In terms of removing methane producing bacteria, MBRs seem to be a possibility.

1.8.4 Methane inhibition

Production of methane can be a problem for continuous biohydrogen reactors when mixed cultures are used. One method of controlling hydrogen consuming methanogens is by addition of inhibitory substances. A study completed by Sparling et al. (1997) examines several different methods of inhibiting methanogenesis in small-scale batch reactors. The goal was to reduce the amount of methane produced and to encourage hydrogen production. Three methods of methane inhibition were compared: oxygen, 2-bromoethanesulfonate (BES) and acetylene. The substrate used was shredded Kleenex, and the bacteria tested was both *C. thermocellum* and an undefined consortium taken from anaerobic digesters. It was found that both BES and acetylene work well to inhibit methanogenisis, while supporting

hydrogen production using *C. thermocellum* and bacteria from the anaerobic digester (Sparling 1997). On an industrial scale, the addition of chemicals to control methanogenesis may prove to be a simpler and cheaper solution than sterilization or use of a membrane. This would be dependant on the cost of the chemicals. Another study found that acetylene was a better option than BES because the trials run using acetylene produces slightly greater amounts of hydrogen, and it is cheaper and easier to work with (Valdez-Vazquez 2005). This paper supports the need for research into continuous hydrogen production in that when hydrogen production reached a plateau, removing the gas from the reactor and continuing with the fermentation more hydrogen was generated. Three fermentation phases were used in this research, and they found that it was worth it to continue removing hydrogen and fermenting up to this point. After 3 phases, the hydrogen produced was almost double that produced in the first phase. This illustrates the inhibitory affect that hydrogen has on its own production. Continuous production and removal of hydrogen are the best conditions for maximum production. This study also pointed out the benefits of using a mixed culture over pure culture; less energy is needed because sterile conditions are not necessary, and since methane production can be inhibited with acetylene, it is not as large a problem. They also point out, however, that the hydrogen yield may be higher when using pure culture because the mixed culture could contain various types of bacteria that may consume a portion of the hydrogen generated.

Methanogenesis has also been repressed by operating the fermentation at low pH. A study using synthetic wastewater with sewage digester sludge managed the production of methane by controlling the pH at 5.0 (Kim 2005).

1.9 Methods of increasing the H_2 yield

The amount of H_2 produced per amount of hexose degraded is defined as the H_2 yield of the system. Ultimately for a single stage fermentation using H_2 producing bacteria, the maximum yield that can be achieved is 4 mol H_2 per mol hexose degraded if acetate and CO_2 are the only other products:

$$C_6H_{12}O_6 + 2H_2O \prod 2CH_3COOH + 2CO_2 + 4H_2$$

In practice, the yields are much lower than the theoretical maximum. Typically the reaction is a mixed acid fermentation producing other end-products such as butyrate, lactate and succinate, which reduces the theoretical maximum considerably (Kraemer and Bagley 2007). If butyrate is the main end-product produced, then the maximum possible H_2 yield drops to 2 mol H_2 per mol hexose consumed:

$$C_6H_{12}O_6 \prod CH_3\ CH_2\ CH_2COOH + 2CO_2 + 2H_2$$

Because other end-products are produced and super-saturation of gases impede the reaction from proceeding, methods to gear the reaction towards hydrogen production have been studied extensively.

Several studies have shown that maintaining a low concentration of dissolved H_2 in the fermentation broth increases hydrogen production. Sparging the liquid phase of the bioreactor with N_2 or CO_2 gas has been shown to increase the H_2 yield by between 20 and 120% (Hussy 2003; I. Hussy 2003; Kraemer 2006; Mandal et al. 2006; Mizuno 2000). Table 1.5 summarizes current studies on H_2 yield improvement by H_2 or CO_2 removal.

Table 1.5 – Comparison of H_2 yield improvements achieved through gas sparging, CO_2 scavenging and vacuum to reduce the H_2 partial pressure in continuous fermentation systems.

Bacteria	Substrate	Method	Sparge Rate (ml/min)	Initial Yield (mol H_2/mol hexose)	Improved Yield (mol H_2/mol hexose)	Improvement	Reference
Mixed culture	Glucose	N_2	160	1.3	1.8	38%	Kraemer, 2006
			58	1.26	1.87	48%	Hussy, 2003
			55	1	1.8	80%	Hussy, 2005
			110	0.85	1.43	68%	Mizuno, 2000
			630	1.23	1.65	34%	Kyazze, 2006
			300	0.77	0.95	23%	Kim, 2006
		CO_2	300	0.77	1.68	118%	Kim, 2006
		Biogas	100	0.77	0.86	12%	Kim, 2006
Heat treated sludge	Glucose	CO_2 Scavenging		1.4	2	43%	Park, 2005
Clostridium butyricum		N_2 sparging using a monolith		0.71	0.92	30%	Fritsch, 2008
Enterobacter cloacae DM11	Glucose	Vacuum		1.9	3.9	105%	Mandal, 2006

Various mechanisms have been credited for the increased yield. Based on Le Chatelier's Principle, and the effect of product concentration on the Gibbs free energy of reaction (Thauer et al. 1977), more hydrogen will be produced if H_2, CO_2 or acetate is removed from the system according to the equation;

$$K_c = \frac{[CH_3COOH]^2[CO_2]^2[H_2]^4}{[C_6H_{12}O_6][H_2O]^2}$$

A batch study using three strains of *C. thermocellum* and both cellulose and cellobiose as substrate found that when the culture grown on cellulose was stirred, the ethanol to acetate ratio decreased significantly and the hydrogen production increased between 80 and 180% (Lamed 1988). The effect of stirring was not as significant on the cultures growing on cellobiose. This suggests that because *C. thermocellum* attaches to cellulose particles, localized supersaturation of H_2 gas may be occurring when the cellulose is allowed to settle and remain stagnant. It is interesting to note that the strain that saw the highest improvement in yield when grown on cellulose (YS – Isolated from the sediment of a hot spring) saw the lowest improvement when grown on cellobiose. Conversely, the strain that achieved the best improvement when grown on cellobiose (LQRI) did not show the same increase in yield when grown on cellulose (See Table 1.3). The effects of stirring were negated when H_2 gas was introduced into the headspace at pressures above 1 atm. This is consistent with current knowledge regarding the partial pressures of H_2 required to impart a shift in the reduction potentials of NAD and ferrodoxin The maximum partial pressure of H_2 before the reaction becomes thermodynamically unfavorable can be calculated using the following equation:

$$P_{H_2 \max} \leq \exp\left[\frac{2F\left(E^o_{H_2} - E^o_x\right)}{RT}\right]$$

Where: E^o_x = redox potential of electron donor

$E^o_{H_2}$ = -414 mV, $E^o_{Fd} \approx$ -400 mV, E^o_{NADH} = -320 mV

F = Faraday's constant

R = Ideal gas constant

T = Absolute temperature

If ferredoxin is the electron donor, hydrogen production will occur if the H_2 partial pressure is less than 0.3 atm (3 x 10^{-4} Pa) according to the reaction;

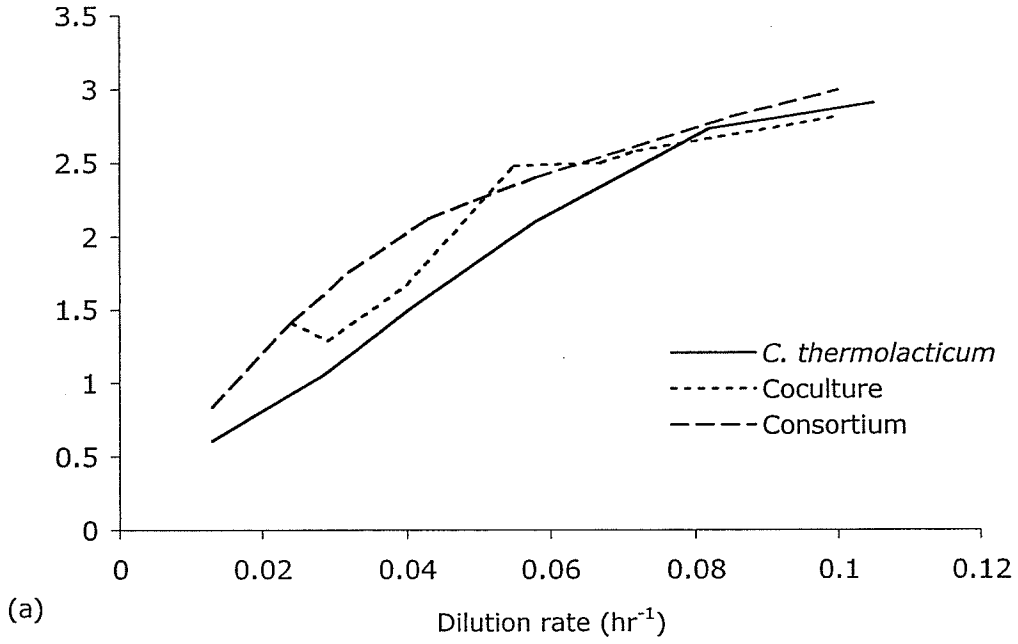
$2Fd(red) + 2H^+ \prod 2Fd(ox) + H_2$

If NADH is used as the electron donor, the H_2 partial pressure must be below 6 x 10^{-4} atm (60 Pa) for the reaction to be thermodynamically feasible (Angenent et al. 2004).

$NADH + H^+ \prod NAD^+ + H^2$

The amount of H_2 production in by *Clostridium thermolacticum* co-cultured with either *Methanothermobacter thermoautotrophicus* or a consortium containing *Moorella thermoautotrophica & M. thermoautotrophicus* (H_2 scavenging bacteria) was calculated to be twice as high as with *C. thermolacticum* alone (Collet et al. 2005). The specific productivity of acetate production (the amount of acetate produced per gram of biomass) did not differ greatly between the pure culture of *C. thermolacticum*, co-culture and consortium (See Figure 1.4a). The volumetric productivity of acetate (the amount of acetate produced per litre bioreator volume) however was much greater for the consortium, reaching a maximum

of 3.9 mmol L^{-1} hr^{-1} at a dilution rate equal to 0.058 hr^{-1} (HRT=17.2 hr) compared to 1.365 mmol L^{-1} hr^{-1} in the pure culture fermentation (see Figure 1.4b) (Collet et al. 2004). Higher acetate production in the presence of H_2 scavenging bacteria has also been reported for *C. thermocellum* cultured with *Methanobacterium thermoautotrophicum* (Weimer and Zeikus 1977) and *C. thermoautotrophicum* (Simankova and Nozhevnikova 1989). In both cases the calculated values of hydrogen produced in the co-culture were much higher than in the pure culture.



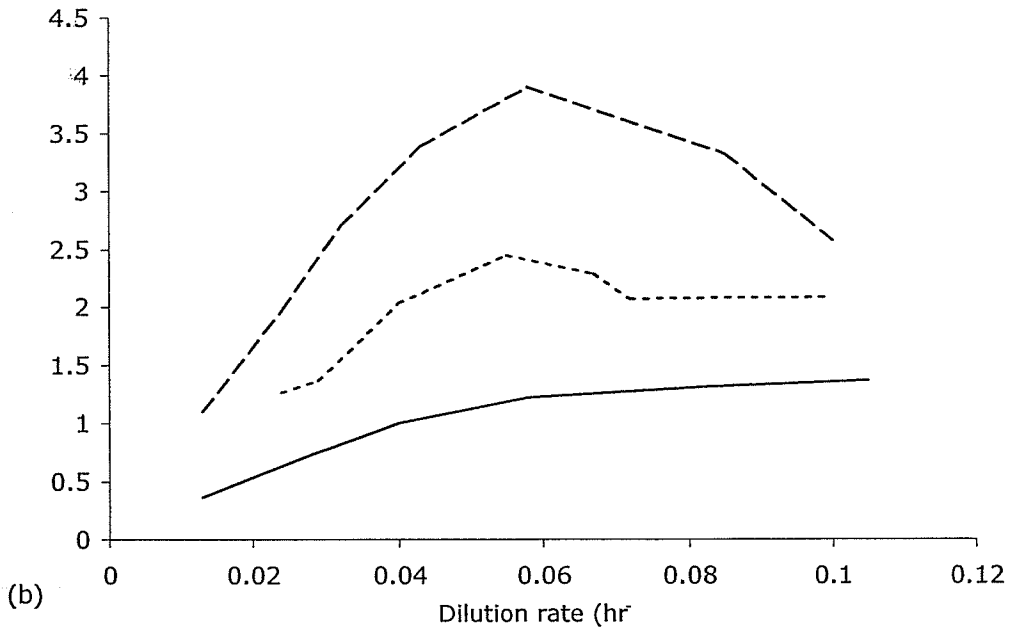


Figure 1.4 - Comparison of (a) acetate specific productivity and (b) acetate volumetric productivity in pure culture (*C. thermolacticum*), Co-culture (*C. thermolacticum* + *Methanothermobacter thermoautotrophicus*) or a consortium (*C. thermolacticum* + *Moorella thermoautotrophica* & *Meth. thermoautotrophicus*) adapted from (Collet et al. 2004; Collet et al. 2005)

The methods used to introduce the sparge gas into an anaerobic fermentation system have not yet been studied in detail. In order to improve the efficiency of the system, it would be of benefit to reduce the amount of gas introduced into the reactor by improving the method of dissolution. Sparge gas dilutes the product stream, necessitating costly processing downstream to separate H_2 from the other gases. No studies have currently been completed that evaluate various sparging techniques for removal of dissolved H_2 and CO_2. In order to evaluate the effectiveness of the design, the dissolved gas concentrations must be measured. Much research exists on processes such as aeration that are analogous to H_2 removal by gas sparging. Aeration, for example, is used in many industrial processes. In order for aeration to remain economically feasible, the process must be extremely efficient to reduce the cost of energy required facilitate the gas/liquid transfer. Gas stripping is used in industry to remove hazardous volatile compounds from liquid effluent. Both these processes require a degree of knowledge about gas to liquid transfer and the interactions between the gas and liquid interface.

1.10 Objectives of this research

Continuous fermentation of complex lignocellulosic substrates is a promising technology for biohydrogen production. The process has the potential to be both economically and industrially feasible due to the availability of the substrate coupled with the improvements in end-product production that is achieved with continuous fermentation. Various techniques for improving the efficiency of substrate degradation beyond that achieved by a continuously stirred tank reactor (CSTR) have been employed in lab scale bioreactors. Perfusion bioreactors, for example, use biomass retention to increase the solids retention time (SRT),

which increases the efficiency of the fermentation. Before this process becomes feasible on an industrial level, more research needs to be conducted on real-life substrates using larger scale equipment. The objectives of this research were to conduct batch tests with the goal of identifying possible alternative substrates for use in the continuous bioreactor and to evaluate the potential of *Clostridium thermocellum* to degrade cellulosic substrates in continuous culture.

Chapter 2
Materials and Methods

2.1 Microorganism, Preparation of Inoculum and Media

All chemicals and reagents for media and substrates were obtained from Sigma Chemical Co and Fisher Scientific. *C. thermocellum* 27405 was obtained from the American Type Culture Collection (ATCC). Inoculum was cultured in balch tubes (Bellco Glass Co.) with a working volume of 26 ml. Each tube was air sealed with butyl rubber stoppers and aluminum seals. They were reduced with 0.1 ml of NaH_2S solution after being gasses and degassed (1:4 min) four times with 100% nitrogen according to the protocol described in Daniels *et al.* (1986). 10% by volume of the actively growing culture was successively transferred into 50 ml then 500 ml containers containing 5 g L^{-1} filter sterilized cellobiose and 1191 media. This complex medium contained (per liter of distilled deionized water): KH_2PO_4, 1.5 g ; $Na_2HPO_4.12H_2O$, 4.2 g ; NH_4Cl, 0.5 g ; $MgCl_2 . 6H_2O$, 0.18 g ; Yeast Extract (BD 212750), 2.0 g ; Resazurin (0.1%), 1.0 ml; vitamin solution (10X), 0.50 ml; mineral solution (10X), 1.00 ml. Vitamin solution contained the following (per 1,000 ml): biotin, 20 mg; p-aminobenzoic acid, 50 mg; folic acid, 20 mg; nicotinic acid, 50 mg; thiamine, 50 mg; riboflavin, 50 mg; lipoic acid (thioctic acid), 50 mg; and cyanocobalamin, 10 mg. Mineral solution contained (grams per liter): trisodium nitrilotriacetate 20.2; $FeCl{\cdot}6H_2O$, 2.1; $CoCl_2{\cdot}6H_2O$, 2.0; $MnCl_2{\cdot}4H_2O$, 1.0; $ZnCl_2$, 1.0; $NiCl_2{\cdot}6H_2O$, 1.0; $CaCl_2{\cdot}2H_2O$, 0.5; $CuSO_4{\cdot}2H_2O$, 0.5; and $Na_2\ MoO_4{\cdot}2H_2O$, 0.5. Reducing solution was prepared under nitrogen

using sodium sulfide crystals in distilled water to a final concentration of 200 mM. Culture was grown at 60°C until it reached mid-late log phase.

2.2 Analytical Procedures

Product gas composition (H_2 and CO_2) was measured using gas chromatographs (Gow Mac model-580) with thermal conductivity detectors. The column used for H_2 quantification was a stainless steel (1/8 inch x 8 ft.) column packed with molecular sieve 5A (60/80 mesh) with nitrogen as carrier gas. All gas measurements were corrected by calculating their solubility in water and carryover amounts with inocula. Amount of CO_2 as bicarbonate equilibrium was taken into account for correction.

Acetate, lactate, and formate were measured using an IonPac AS11-HC anion-exchange column (Dionex Corporation, Sunnyvale, CA, USA). Ethanol concentrations were determined using the Enzymatic BioAnalysis UV-Test kit (R-Biopharm AG, Darmstadt, Germany) based on NAD^+ produced by alcohol dehydrogenase, which was measured spectrophotometrically at 340nm. Standards for acids and ethanol were prepared on 1191 medium to correct for the background.

Cellulose was measured gravimetrically by filtration through 0.2 μm glass fiber filter and dried overnight (Eaton 2005). Residual biomass was first washed off the cellulose pellet with NaOH following a modification of the Bradford method as reported previously (Sparling et al. 2006).

Chapter 3

Direct Hydrogen Production from Cellulosic Waste Materials with a Single Step Dark Fermentation Process

3.1 Introduction

Fermentative hydrogen production can be carried out using various sources of carbon. Studies have evaluated hydrogen production from starch, cellobiose, sucrose, xylose and cellulose. The goal is to move away from lab grade substrates and begin fermenting complex waste streams using specifically optimized and defined cultures to ferment raw naturally complex organic materials, especially insoluble substrates. Typically pre-hydrolysis is necessary in order to ferment cellulose based waste materials because most bacteria find soluble substrates much easier to digest (Datar et al. 2007; de Vrije et al. 2002).

The objective of this research was to evaluate local sources of waste biomass for their hydrogen production potential using *C. thermocellum*. Three potential candidates were identified. Dried distillers grain (DDG), which is a co-product of grain based ethanol fermentation and is produced locally from wheat. Barley hulls (BH) and fusarium head blight (a crop fungus) contaminated barley hulls (CBH) are currently unused waste products resulting from crop processing and are abundant locally. De-hulling of fusarium-contaminated barley is being considered for removing toxins generated by the fungus, yielding a usable barley stream (for animal feeding applications) and an un-usable hull stream (requiring disposal or post-processing). When using *C. thermocellum* for cellulose based fermentation, a number of competing organic end products may be produced

(including ethanol, formate and lactate; (Islam et al. 2006)). The purpose of this paper is to assess the degradability of the cellulosic wastes by *C. thermocellum* by determining the concentration of the various products generated from the fermentation under the conditions tested.

3.2 Materials and Methods

3.2.1 Media and Substrates

All chemicals and reagents for media and substrates were obtained from Sigma Chemical Co and Fisher Scientific. The fusarium head blight Contaminated Barley Hulls (CBH) and Barley Hulls (BH) came from Manitoba grown barley that was dehulled as part of a research project investigating the impact of de-hulling processes on fusarium related toxicity. The dried distillers grain (DDG) was wheat based, and also sourced locally. 5 g l^{-1} of each substrate was added to balch tubes containing 10 mL of 1191 medium made according to the procedures described in section 2.1.

3.2.2 Microorganism

The inoculum, C. *thermocellum* 27405 was continuously cultured on cellulose for 800 hours in a fermentation reactor system. At approximately 300 hours of operation a visual change in color was observed in the reactor. This could be attributed to an increase in the concentration of cellulosome. At this time, a sample was taken from the reactor and inoculated into serum bottles containing the alternative substrates.

3.2.3 Experimental Design

Batch experiments were designed with three different substrates, with initial dry weight concentrations of 5 g L^{-1}. Dried distillers grain (DDG), fusarium contaminated barley hulls (CBH) and barley hulls (BH) were fermented in balch tubes (Bellco Glass Co.) with a total volume of 26 ml. Each tube was air sealed with butyl rubber stoppers and aluminum seals. A control experiment was also carried out on 1.1 reagent-grade pure α-cellulose following the same procedure as described. They were reduced with 0.1 ml of NaS solution after being gassed and degassed (1:4 min) four times with 100% nitrogen according to the protocol described in Daniels *et al.* (1986). All batches were inoculated from freshly growing culture on 1191 medium in serum bottles (125 ml) at exponential phase.

3.2.4 Analytical Procedures

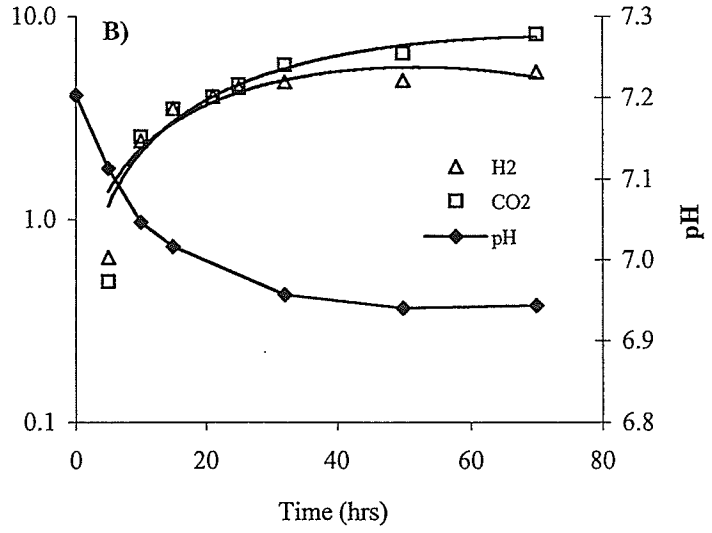
Product gas composition (H_2 and CO_2) was measured as reported in section 2.2. All gas measurements were corrected by calculating their solubility in water and carryover amounts with inocula. Amount of CO_2 as bicarbonate equilibrium was taken into account for correction. Acetate, lactate, and formate were measured as reported in section 2.2. Standards for acids and ethanol were prepared on 1191 medium to correct for the background. Yields of H_2 (Y_{H2}), CO_2, ethanol and organic acids were expressed as millimoles (mmoles) of H_2 produced per gram of substrate added in each tube.

3.3 Results

3.3.1 Gas Production

Figure 3.1 shows the gas production and pH drop of the cultures growing on DDG, CBH and BH. All three substrates produced similar amounts of gas, which was approximately 1/5 of the amount of hydrogen produced on the control of 1.1 g l^{-1} α-cellulose over the same period of time.

A drop in pH was observed within the exponential phase of the experiment (from 7.2 to 7.0). The drop in pH was much slower during stationary phase, reaching a final value of approximately 6.9 for all substrates (considering the standard deviation). The final pH for the α-cellulose experiment was somewhat lower than the alternative substrates at 6.7.


B)
10.0
1.0
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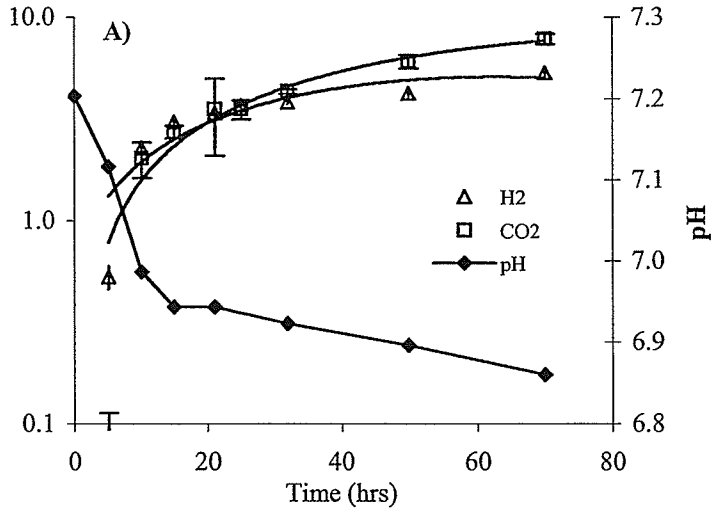
7.3
7.2
7.1
7.0
6.9
6.8
pH
H2
CO2
pH
0
20
40
60
80
Time (hrs)


A)
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60
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Time (hrs)

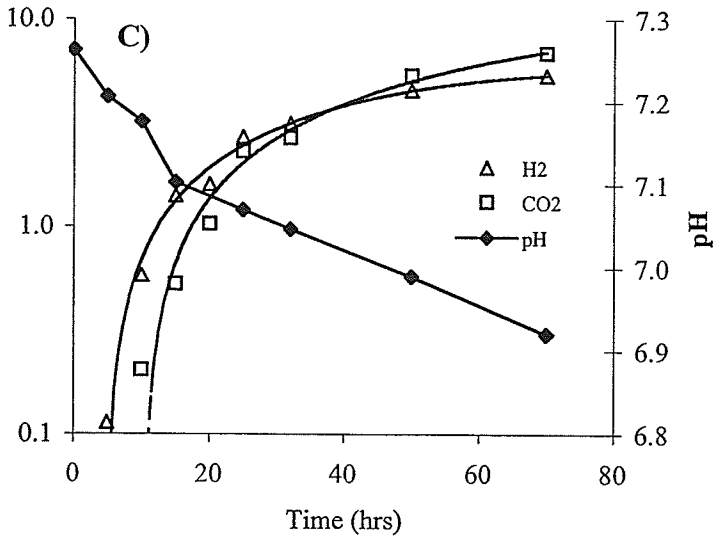
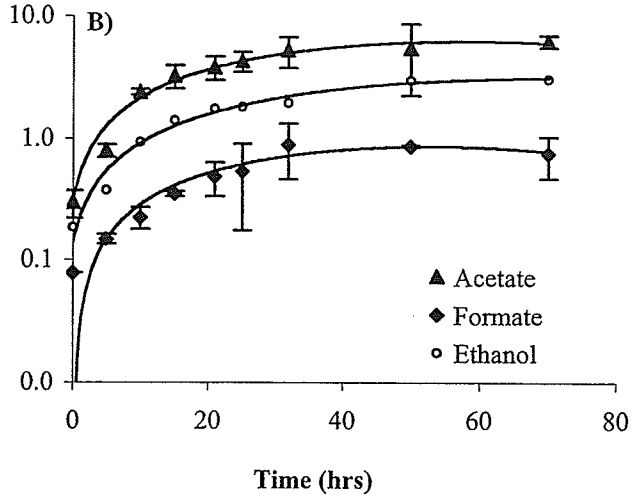


Figure 3.1 - Hydrogen and CO_2 production by Clostridium thermocellum ATCC 27405 cultured in 1191 media (10 ml) at 60°C on 5 g l^{-1} A) Dried distillers grain; B) Contaminated barley hulls; C) Barley Hulls

3.3.2 Acids and Alcohol Production

Acetate and ethanol were the major end products of fermentation by *C. thermocellum* for all 3 substrates (Figure 3.2). Formate was also produced in small amounts in each substrate. Lactate production was measured in BH and CBH, but was not produced in DDG. In the exponential phase, the rate of acetate production was 10 fold higher to that of formate in DDG. There was no significant production of formate in the exponential phase for CBH and BH. The rate of ethanol production was lower in all cases compared to acetate. The highest rate of ethanol production occurred in the CBH.


10.0
B)
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Acetate
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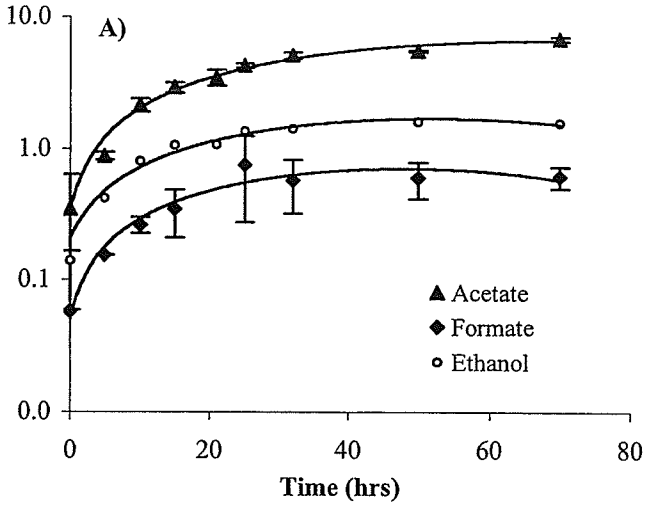
Time (hrs)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Acetate
Formate
Ethanol
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Time (hrs)

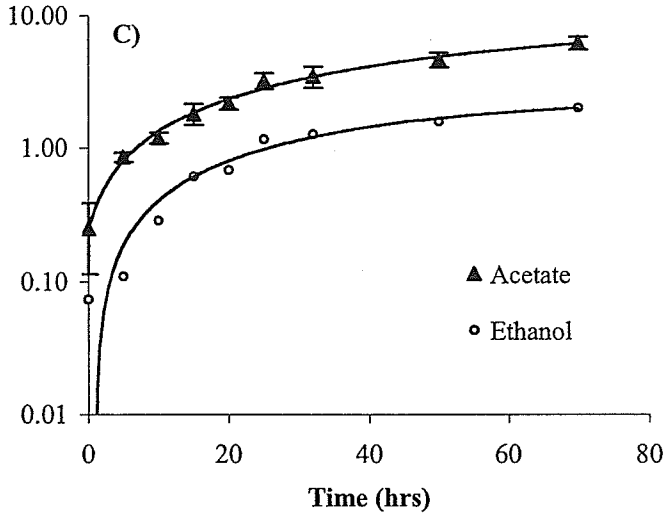

Figure 3.2 - Acetate, Formate and Ethanol Production by *Clostridium thermocellum* ATCC 27405 cultured in 1191 media (10 ml) at 60 C on 5 g l^{-1} A) Dried distillers grain; B) Contaminated barley hulls; C) Barley Hulls

3.3.3 Yields and Rates

Yields and ratios of H_2/CO_2 calculated based on the total measured gas produced for each substrate are presented in Table 3.1. The differences in H_2/CO_2 ratios were not substantial, varying from 0.9 – 1.15. The ratio for the control on 1.1 g l^{-1} was higher at 1.5 mole H_2 per mole CO_2 produced. The estimated ratios based on fermentation end-products were similar for all substrates.

Table 3.1 – Total Yields* of Lactate, Acetate, Formate, Ethanol, H2 and CO2 produced by Clostridium thermocellum ATCC cultured in 1191 media (10 ml) at 60 C on 5 g l^{-1} Dried distillers grain, Contaminated barley hulls and Barley Hulls.

Products	DDG 5.0 g/L	CBH 5.0 g/L	BH 5.0 g/L	α-cellulose 1.1 g/L
	millimoles of product per gram of substrate added			
Lactate	0.00	0.02	0.07	0.26
Acetate	1.36	1.25	1.25	3.61
Formate	0.12	0.08	0.03	1.11
Ethanol	0.32	0.53	0.41	2.33
A/E	2.89	2.22	2.99	2.53
A/F	6.93	8.12	-	3.34
Hydrogen	1.07	1.06	1.07	5.43
Carbon Dioxide	1.22	1.64	1.37	5.19
H_2/CO_2	0.93	0.90	1.15	1.50
H_2/GEP**	1.27	1.18	1.24	0.76
pH	6.86	6.94	6.92	6.70

*Calculations based of the final concentrations in the batch reactors in stationary phase. All values corrected for initial product present

**Glucose equivalent calculated based on end products produced

Yields were calculated based on the mass of substrates added to the culture. Among the alternative substrates the highest yields were found in DDG. Rates of production of hydrogen and carbon dioxide were similar between DDG and CBH, but much lower for BH (Table 3.2). All three substrates showed much lower rates of production of end products than the control on 1.1 g l^{-1}. The final amounts of hydrogen and carbon dioxide were very similar for all three substrates.

The ratio of H_2 to glucose equivalent utilized was higher in the alternative substrates than the α-cellulose controls. DDG showed the highest ratio of moles of hydrogen produced per glucose equivalent utilized at 1.27 followed by BH and CBH.

Table 3.2 - Rates of H_2 and CO_2 production in the exponential phase of the experiment produced by Clostridium thermocellum ATCC cultured in 1191 media (10 ml) at 60 C on 5 g l^{-1} Dried distillers grain, Contaminated barley hulls and Barley Hulls

	DDG 5.0 g l^{-1}		CBH		BH		α-cellulose 1.1 g l^{-1}	
	mmole of product produced per L of culture per hour							
Hydrogen	0.23	± 0.012	0.24	± 0.003	0.09	± 0.018	0.23	± 0.025
Carbon Dioxide	0.20	± 0.029	0.25	± 0.008	0.05	± 0.029	0.16	± 0.005

3.4 Discussion

Approximately 30% of DDG is composed of degradable fiber, which includes non-starch polysaccharides (cellulose, hemicellulose xylose, arabinose and pectin), lignin and glycoprotein (Slominski et al. 2007), indicating that out of the 5 g L^{-1} DDG added to the balch tubes, 1.5 g L^{-1} could be available for degradation by C. *thermocellum*. Therefore 1.1 g L^{-1} of α-cellulose (which had also been used in other published experiments in this group; Islam et al., 2006) was used as the control to compare the fermentation between a lab grade substrate and the alternative substrates under carbon limiting conditions. Experimental results showed that less of the alternative substrates (DDG, CBH, BH) were converted to end products than the 1.1 g L^{-1} α-cellulose (Table 3.1). It is possible that not all of the cellulose in the substrate is available to the bacteria, or simply that the alternative substrates do not contain the same amount of cellulose as the 1.1 g L^{-1} α-cellulose.

The degradable fractions of the BH and CBH were approximated using the analysis of neutral and acid detergent fiber completed at the University of Manitoba (Table 3.3). Acid detergent fiber (ADF) refers to the cell wall portions of the barley hulls that are made up of cellulose and lignin. The neutral detergent fiber (NDF) value is the total cell wall, which is

comprised of the ADF fraction plus hemicellulose. Therefore, the amount of hemicellulose in the alternative substrates is the difference between NDF and ADF. Both the contaminated and non-contaminated barley hulls contain approximately 25-30% hemicellulose. Considering that C. *thermocellum* cannot metabolize hemicellulose (Demain et al. 2005), the initial degradable amount of BH and CBH in the balch tubes are a maximum of 1.25g L^{-1} and 1.45g L^{-1}, respectively (depending on lignin content, which could not be determined).

Table 3.3 - Percentages of acid detergent fiber (ADF), neutral detergent fiber (NDF), protein and total fiber in contaminated barley hulls (CBH), barley hulls (BH) and dried distillers grain (DDG) (as determined by the Plant Science Department at the University of Manitoba).

	CBH	BH	DDG
NDF	56.98	53.49	26.6
ADF	29.1	25.47	-
Protein	-	-	41.3
Total Fiber	-	-	33.1

All of the alternative substrates produced similar final amounts of H_2, also comparable to the 1.1g L^{-1} α-cellulose control while the maximum H_2 production rates were significantly higher for DDG and CBH than BH. This suggests that during the exponential phase of growth substrate was available for fermentation, but the reaction ended due to lack of readily degradable substrate. So carbon limited conditions prevailed in the alternative substrates similar to the 1.1g L^{-1}α-cellulose control. However, per gram of fiber available for degradation, the complex substrates yield 22-34% less H_2 than the pure α-cellulose, indicating that omitting a pretreatment process will result in a reduction in overall efficiency. Rani et al. showed a 10% conversion efficiency of untreated paddy straw, sorghum stover

and deshelled corn cobs after 5 days incubation (Rani et al. 1998). Following delignification by alkali treatment, two strains of *C. thermocellum* tested (SS21 and SS22) were able to achieve above 50% degradation efficiency. In that case, the concentrations of cellulosic substrate tested were much higher (varied from 10 to 120 g L^{-1}), which will also affect conversion efficiencies.

After autoclaving, the DDG tubes contained high initial concentrations of lactate (close to 1 mM compared to insignificant levels in the control). This background concentration of lactate may inhibited lactate production in those tubes. Lactate production is higher when readily degradable substrate is abundant and the pH is low at the end of growth (Islam et al. 2006). These experiments show lower lactate production, indicating a lack of degradable substrate at the end of growth. When higher concentrations of α-cellulose were tested the lactate yield was higher, which supports this hypothesis. In addition, the pH drop in the alternative substrate experiments was lower than in the control (Table 3.1). This indicates that pH was not responsible for the shift from the exponential phase to the stationary phase, but is most likely caused by lack of readily available substrate.

In all substrates, acetate was the major end-product followed by ethanol, formate and lactate. In the alternative substrate experiments, the ratios of acetate to ethanol and acetate to formate were higher than the control (Table 3.1). High formate and ethanol yields for the α-cellulose controls showed that abundance of pure cellulosic substrate was shifting the metabolism away from acetate pathways. BH had the highest ratios of acetate to ethanol (A/E) and acetate to formate (A/F). Low formate production is indicative of low rates of degradation of readily degradable substrate (Islam et al. 2006). The rates of gas production were also lowest

in the exponential phase of BH fermentation relative to the 2 other complex substrates, yet the yield was similar to the DDG and CBH. Higher amounts of readily degradable substrate in the DDG and CBH would explain the higher gas production rates, as well as the lower ratios of A/E and A/F. Both the BH and the CBH produced a similar yield, implying that the same amount of material was degraded, but the initial degradation rate of the CBH was faster. This initial higher rate of production can be attributed to substrate being made available by the action of cellulosic enzymes; the fusarium contaminated CBH may have more available sites on which the bacteria can attach. Therefore it can be assumed that at the start of the experiment, tubes containing BH had less readily accessible substrate than those containing DDG and the CBH.

The type of cellulose found in nature is not purely crystalline. Studies have shown that the degree of crystallinity of the cellulose is an important characteristic in determining the effect of enzymatic hydrolysis (Koullas et al. 1992; Koullas et al. 1990). Another consideration when accounting for the lower degradability of the alternative substrates in comparison to the α-cellulose is the content and organization of lignin within the sample. Beyond the fact that lignin has not been reported to be degraded by C. *thermocellum,* the characteristics and the structure of the lignin and how it is arranged within the cellulose and hemicellulose fractions will influence the overall degradability of the substance (Tong et al. 1990). For example, if the cellulose is contained within a lignin shell, then the bacteria will first have to degrade the lignin to access the cellulose. This is why the relationship between lignin content and biodegradability is not always linear.

3.5 Conclusions

The untreated natural substrates tested in this experiment showed good potential for hydrogen production and other valuable end products when compared with lab grade α-cellulose. All substrates produced similar amounts of hydrogen, which were comparable to those produced with the α-cellulose control. However, the overall amount of hydrogen produced per glucose equivalent utilized was higher for the alternative substrates. The fusarium fungus may have made the cellulose in the barley hulls more available to degradation, which could aid in increasing fermentation efficiency to *C. thermocellum*. This synergistic effort between the enzymes of both species could account for the high hydrogen production rates from CBH. On the other hand, the microbial activity that exists during DDG production may release some soluble sugars (from starch degradation) and could provide an explanation for the high hydrogen production rates with C. *thermocellum.* Overall, this study indicates that the source of biomass substrate will have a substantial impact on further processing with cellulose degrading organisms such as *C. thermocellum*. Hydrogen produced through C. *thermocellum* fermentation can be an attractive candidate for the development of sustainable energy from a variety of waste biomass streams.

Chapter 4

Continuous hydrogen production during fermentation of α-cellulose

4.1 Introduction

Biological H_2 production by dark fermentation must overcome several hurdles before industrial scale applications are plausible. Continuous fermentation has been found to significantly increase H_2 production (Brosseau and Zajic 1982), but barriers due to end-product inhibition, stability of the system and methanogenic bacteria contamination still pose problems (Valdez-Vazquez et al. 2005). Indeed, the main goal of the current research on biohydrogen production by fermentation is to increase H_2 yield. Many studies have been conducted to evaluate the attributes of various substrates fermented by different species of bacteria (Chen et al. 2001; Fang 2002; Minnan 2005). Variables such as temperature, pH, HRT and SRT have been studied to determine their effect on production rate (Collet et al. 2004; Lynd et al. 1989; Pavlostathis et al. 1988). Physically, the process of fermentation and the bioreactor itself have been examined and altered by addition of fixed media to retain the bacteria and by the use of membrane biological reactors to both separate biogas from solution and as a means of biomass retention (Liang 2002; Oh 2004). Most studies aimed at increasing the yield of H_2 are conducted using continuous culture because of the vast improvement in production rates over batch or semi-batch experiments (van Groenestijn et al. 2002).

The overall objectives of the research described in this chapter were to establish a continuous H_2 production system using α-cellulose as the carbon source and *Clostridium thermocellum*

ATCC 27405 as the fermentative organism. Specific goals included the characterization of *C. thermocellum* growth, H_2 production yields and soluble end-product synthesis patterns under continuous culture conditions at four different carbon-loading levels. Additionally the study aimed to evaluate long-term operational stability and overall robustness of the process.

4.2 Materials and Methods

4.2.1 Experimental Setup

Continuous culture fermentation was carried out using a 14 L New Brunswick Scientific Bioflow 110 fermentor. The apparatus was modified several times prior to achieving the final design because of insoluble cellulose clogging the feed line and media vessel contamination. The principal components of the final system were comprised of an α-cellulose feed tank, a media feed tank, a designated peristaltic pump for feeding the cellulose slurry, the Bioflo 110 bioreactor system (including 4 peristaltic pumps, pH, temperature and level controller, agitation device), a KOH reservoir, an N_2 delivery system, and a condensate trap and effluent gas line designed to capture volatile end products. A schematic of the final design of the system is shown in Figure 4.1

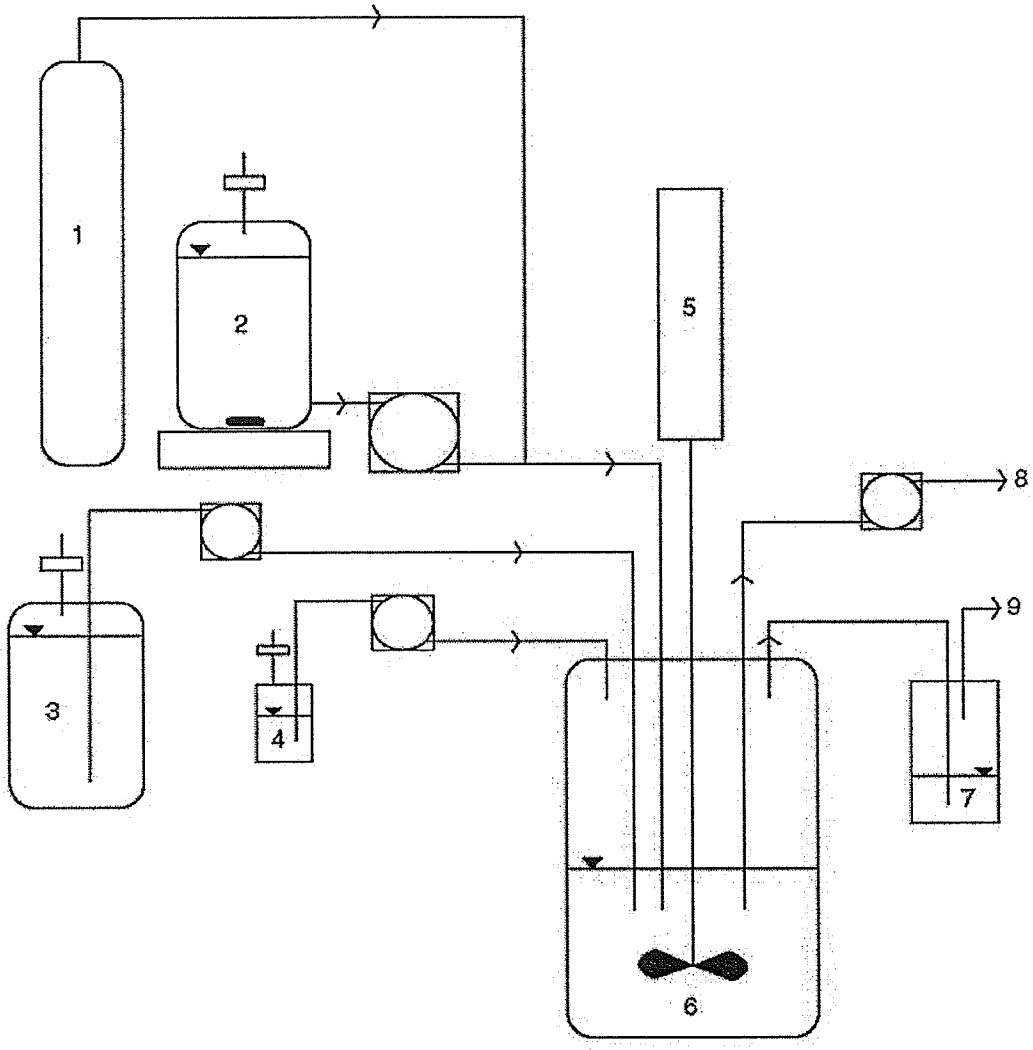

Figure 4.1 - Process flow diagram of continuous culture apparatus. 1) Nitrogen tank; 2) Cellulose feed vessel; 3) Media feed vessel; 4) KOH reservoir; 5) Rotor; 6) Bioreactor; 7) Condensate trap; 8) Liquid effluent line; 9) Gas effluent line.

To simplify the system, the cellulose and media feed tank were not kept under N_2 or CO_2 headspace, rather air was allowed to enter the tanks through a sterile 0.2 μm PTFE membrane filter. Despite the influent feed tanks being aerobic, the bioreactor maintained anaerobic conditions throughout the fermentation. Nitrogen (N_2) gas was constantly bubbled through the reactor at a rate of 0.53 (± 0.01) mL s^{-1}. The N_2 gas was introduced into the cellulose feed line just prior to the peristaltic pump, which, in addition to maintaining reduced conditions also helped to prevent clogging as the cellulose entered the reactor. A Masterflex L/S (1.6 to 100 RPM, 26 kg cm^{-1} horsepower) peristaltic pump with the easy-load II pump head supplied by Cole Parmer was used to move the cellulose slurry into the fermentor. The medium, KOH and effluent lines were fed to the fermentor through the peristaltic pumps that were supplied with the fermentor control module. The pH was kept at 7.0 by 2M KOH and a PID controlled pH probe. A level sensor set to maintain the fermentation broth volume at 5 L controlled the effluent pump. The fermentation broth was mixed at 75 RPM by an axial flow impellor and baffles helped sustain a homogeneous suspension of the soluble fractions of the fermentation broth. The bioreactor vessel as well as the α-cellulose and media feed tanks were autoclaved prior to startup of the experiment.

4.2.2 Experimental Procedure

The working volume for all continuous experiments was 5 L. To start the continuous experiment, 5 L of 1191 media and 5 g L^{-1} α-cellulose were added to the reactor and autoclaved for 20 minutes. While cooling to 60°C, the nitrogen flow and temperature control were hooked up to the reactor. The air in the headspace gas was replaced with nitrogen by

approximately 10 minutes of sparging. Sodium sulfide crystals (5 g) were added to remove any dissolved oxygen present in the broth. When the temperature reached 60 °C and the pH was adjusted to 7.2 with KOH, the reactor was inoculated with 500 ml of actively growing *C. thermocellum* culture. The 5 L reactor culture was grown in batch mode on 5 g L^{-1} cellulose until the pH reached approximately 6.9, indicating significant growth had taken place and the continuous culture could be started without washout occurring. *C. thermocellum* does not initiate growth if it is agitated during lag phase (Freier et al. 1988), therefore the agitation was not started until the continuous mode of operation commenced. The duration of the fermentation experiment was 115 days. Four different carbon-loading conditions (1.5, 2, 3 and 4 g L^{-1} of α-cellulose) were tested, while maintaining a constant dilution rate of 0.04 hr^{-1} (HRT = 24 hrs). Throughout testing, the pH and temperature were kept at 7.0 and 60 °C respectively, with the exception of the system upsets that were caused by power failure or line clogging.

4.2.3 Analytical Procedures

Gas samples were removed from the headspace of the bioreactor several times per day through the septum port using a stoppered syringe rated for gas. Product gas composition (H_2 and CO_2) was measured as outlined in section 2.2. All gas measurements were corrected by calculating their solubility in water and carryover amounts with inocula. Amount of CO_2 as bicarbonate equilibrium was taken into account for correction. The percentage of N_2, CO_2 and H_2 present in the headspace was deduced by comparison with a standard curve, and corrected for the daily temperature and pressure. No reliable method was found to physically measure the flow rates of gas production within the bioreactor since the rates of H_2 and CO_2

production were small in comparison to the rate of influent N_2. Therefore effluent gas flow rates were calculated using the flow rate of N_2 gas entering the reactor and the percentages of gases as determined by GC analysis. Liquid samples (1.5 mL) were removed from the completely mixed bioreactor twice daily. Ethanol, acetate, lactate, formate, cellulose and protein were measured as described in section 2.2.

4.3 Results and Discussion

4.3.1 End-product synthesis

During growth of *C. thermocellum* in continuous culture on α-cellulose, acetate, ethanol, lactate and formate were the main products in the liquid phase as observed previously in batch cultures (Islam et al. 2006). In the gas phase, only H_2 and CO_2 were detected. End-product results from the continuous fermentation of α-cellulose under the different loading conditions are shown in Table 4.1. All measurements reported were taken during steady-state operation, meaning conditions fluctuated less than 10%, there were no consistent increasing or decreasing trends over time and at least 2 bioreactor volumes had passed through the system since the last system upset (Zhang and Lynd 2005a).

Table 4.1 - End-product rates of production with respect to carbon loading conditions (± Standard deviation).

Parameters	α-Cellulose Concentration (g L^{-1})			
	1.5	2	3	4
Molar Rates [mmol $(hr)^{-1}$]				
H_2	1.47 ± 0.35	2.92 ± 0.59	3.76 ± 0.80	5.06 ± 0.93
CO_2	2.33 ± 0.59	3.78 ± 0.57	5.06 ± 0.96	6.51 ± 1.11
CO_2 (aq) + HCO_3^-	0.38	0.61	0.85	1.07
Acetate	1.62 ± 0.54	2.29 ± 0.51	2.85 ± 0.73	NM[a]
Lactate	0.12 ± 0.06	0.10 ± 0.08	0.06 ± 0.07	NM
Formate	0.24 ± 0.14	0.26 ± 0.15	0.30 ± 0.13	NM
Ethanol	0.34	0.58	0.79	1.11
Yield [mol H_2 (mol hexose)$^{-1}$]	0.88	1.48	1.38	1.16
O/R	2.28	1.91	1.95	1.83[b]
A:E	4.78	3.92	3.60	-

[a] not measured due to instrumentation problems
[b] calculated based on formate concentration at 3 g L^{-1}

The rates of formation of end-products were determined and compared with varying α-cellulose feed concentrations. H_2, CO_2, acetate and ethanol increased linearly with increasing α-cellulose concentration, while formate and lactate were washed out of the system at the given dilution rate (Figure 4.2). At 8 g L^{-1} α-cellulose concentration in the feed reservoir (4 g L^{-1} in the bioreactor), the magnetic stirring began to fail and the feed lines became clogged on a regular basis. Although a continued improvement of H_2, production yields could be expected at even higher carbon loading conditions, the system set-up did not allow for these tests because of concerns related to long-term operational reliability (primarily related to feed delivery and mixing).

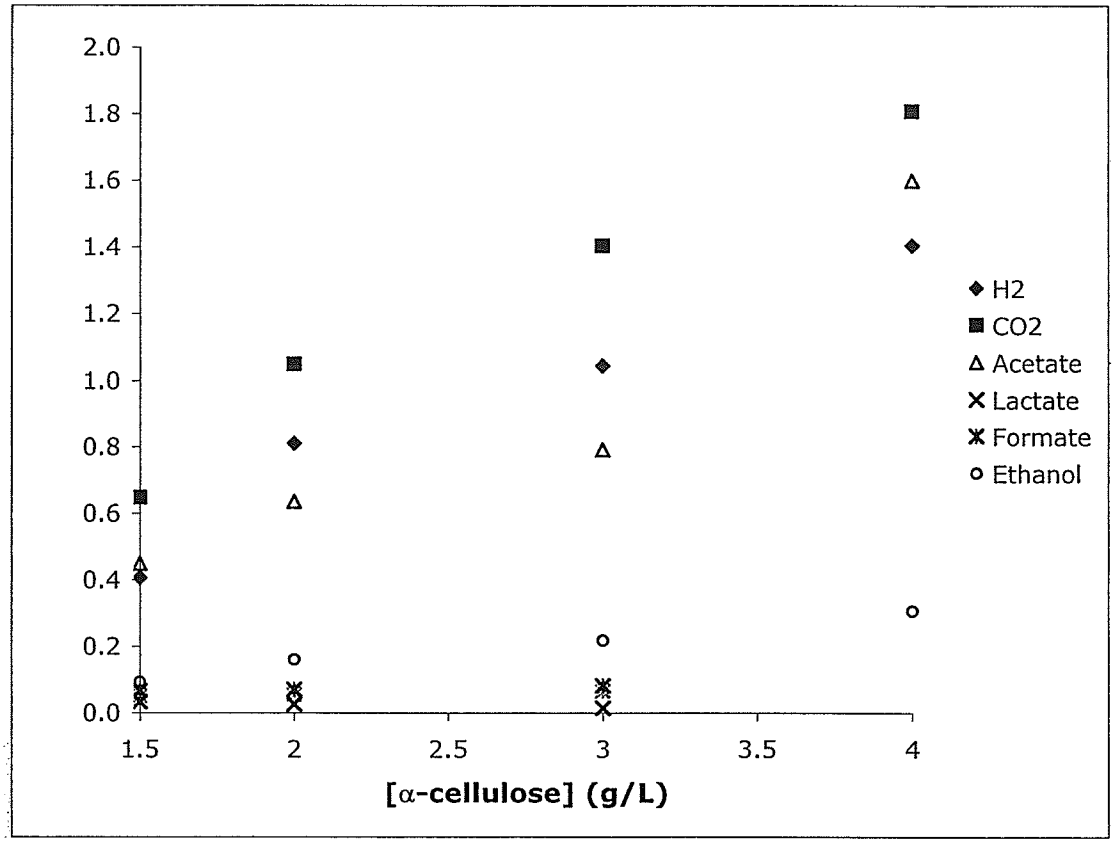

Figure 4.2 – Rates of production of H_2, CO_2, acetate, lactate, formate and ethanol by *C. thermocellum* continuously cultured on 1.5, 2, 3 and 4 g L^{-1} α-cellulose. Acetate concentration at 4 g L^{-1} was calculated based on other end-products produced. All values reported represent steady state conditions (standard deviations < %10).

CO_2 concentrations exceeded H_2 concentrations in the gas phase. Typically, similar amounts of H_2 and CO_2 have been reported as the main gaseous products of cellulose fermentation by *C. thermocellum* in batch cultures (Islam et al. 2006; Levin et al. 2006; Sparling et al. 2006). Since CO_2 excreted by the cells dissolved state, the bicarbonate concentration in the fermentation broth is a function not only of pH, but also the dilution rate. It has been reported that when the dilution rate is increased from 20 to 5 hrs, more bicarbonate is washed out of the system, resulting in less CO_2 in the gas phase (Collet et al. 2004). It is possible that

due to the low dilution rate used in this experiment, more CO_2 was allowed to accumulate resulting in a higher than anticipated CO_2 gas measurement.

When the concentration of cellulose is growth limiting, the production of the end-products are growth linked, as they are catabolic by-products of the fermentation (Pavlostathis et al. 1988). Since product ratios remained similar and synthesis of all by-products was linear with respect to substrate concentration (Figure 4.2), it can be assumed that cellulose was growth limiting during the course of fermentation. Exogenous glucose levels in the culture broth were below the detection limit of 2 mM.

Another organism that posses a cellulosome and has been studied extensively for it's ability to degrade cellulose for biofuel production is the mesophilic, anaerobic bacterium C. *cellulolyticum* (Desvaux 2005; Desvaux et al. 2001a; Desvaux and Petitdemange 2001). These studies showed that at concentrations above 7.6 g L^{-1} cellulose, the fermentation was not limited by the carbon concentration and the majority of cells were attached to the surface of the substrate. However when cellulose is limiting, a portion of the cells will be suspended in the broth (Desvaux et al. 2001b). In a growth-limited reactor with free cells suspended in the broth, any glucose or soluble sugars produced by the bacteria would be readily consumed. The highest H_2 production (5.06 mmol hr^{-1}; 24.8 ml L^{-1} hr^{-1}) occurred at the highest carbon-loading rate (8 g C per day) as shown in Table 4.1. Comparison of end-product production rates with other studies can be problematic because most continuous fermentation studies have used soluble substrates as a carbon source. Shorter chain sugars like glucose, cellobiose and lactose are more commonly used as substrates because they are easier to work with and more readily utilized by bacteria (Lynd et al. 2002). H_2 production rates using soluble

substrate fermentation are generally higher, since carbon utilization is improved due to the omission of a rate-limiting substrate hydrolysis step prior to cell metabolism. Among the studies that use cellulose or another insoluble product as substrate, the units used to report H_2 production vary widely, with some studies reporting only substrate degradation and omitting gas production.

A study conducted by Collet et. al. (2004) using *C. thermolacticum* and soluble lactose as the carbon source found a H_2 production rate of 73 ml L^{-1} hr^{-1} at optimized conditions. Although higher, this is comparable and in the same range as the highest H_2 production rate found in this study of 24.8 ml L^{-1} hr^{-1}. Considering that a complex substrate was used in this study and that the system was not yet optimized, the finding that H_2 production reported here is in the same range as that during the fermentation of a soluble substrate is promising. For comparison, a study conducted by Pavlostathis et al. (1988), using continuous fermentation of cellulose with the mesophilic bacteria *Runinococcus albus,* reported a maximum total gas flow rate of 4.25 ml L^{-1} day^{-1}. This is several orders of magnitude lower than the amount of gas produced in this study by *C. thermocellum* (1360 ml L^{-1} day^{-1}).

Desvaux et al (2001) operated a continuous fermentation experiment using *C. cellulolyticum* at concentrations ranging from 1.9 to 27.0 g of cellulose per liter and at a dilution rate of 0.048 hr^{-1} (HRT = 20.8 hr). Although gas production rates were not reported, it is possible to compare liquid end-product concentrations at similar carbon loading conditions. At an influent cellulose concentration of 3.8 g L^{-1}, acetate, ethanol and lactate concentrations were 7.59, 3.34 and 0.13 mM respectively. In the current study, at similar operating conditions of

3 g L^{-1} loading at the slightly longer HRT of 24 hrs, acetate, ethanol and lactate concentrations of 13.66, 3.8 and 0.27 mM, respectively, were obtained.

The high temperature of thermophilic fermentation offers ancillary benefits in terms of evaporation of the more volatile end-products. The effluent gas line passed through a condensate trap before gases were vented to atmosphere. Analysis completed on the contents of the condensate show that it is primarily composed of ethanol and water, reducing ethanol levels in the fermentation broth. Although *C. thermocellum* has been reported to be tolerant to ethanol of up to 1% (v/v) (Herrero and Gomez 1980; Tailliez et al. 1989), lower ethanol content in the fermentor could boost end-product formation.

4.3.1.1 H_2 production per hexose

The H_2 yield per mole hexose consumed was found to be 0.88, 1.48, 1.38, 1.16 for 1.5, 2, 3 and 4 g L^{-1} α-cellulose, respectively (Table 4.1). These numbers are typical for continuous hydrogen fermentation systems (Kraemer and Bagley 2007; Ueno et al. 2001) and very similar to those achieved in batch studies in this lab (Levin et al. 2006). In batch cultures of *C. thermocellum* using cellulose as substrate, it has been shown that stirring increases the ratio of acetate to ethanol and that H_2 increases in proportion to acetate (Lamed et al. 1988). Compared with previous unstirred batch studies completed in this lab, the acetate:ethanol (A:E) ratios found in the continuous system are higher, yet the H_2 yield per mole hexose degraded remained the same (Levin et al. 2006). N_2 and CO_2 sparging have also been shown to increase the yield of hydrogen produced in both continuous and batch cultures by between 18 to 188% (Hussy et al. 2003; Kraemer and Bagley 2006), which could partly account for

the higher A:E ratios. The O/R balance for each condition tested is above 1, indicating that not all electrons have been accounted for. Considering that the carbon balance is close to 100%, it is unlikely that the missing electrons can be accounted for by a deficiency in the CO_2, ethanol or formate measurements. Since H_2 is the smallest molecule and the lightest gas in the bioreactor, it is possible that some gas may be escaping. This would result in a lower H_2 measurement and cause the O/R ratio to be higher than normal. Using O/R = 1, which is the O/R ratio of glucose, the expected amount of hydrogen produced can be calculated based on the concentrations of other end-products.

Table 4.2 – Theoretical molar rate and yield of H_2 calculated based on an O/R ratio equal to 1

Parameters	α-Cellulose Concentration (g L^{-1})			
	1.5	2	3	4
Molar Rate [mmol $(hr)^{-1}$]				
H_2	4.22	6.64	8.82	11.09
Hexose consumed	1.67	1.97	2.73	4.35
Yield [mol H_2 (mol hexose)$^{-1}$]	2.53	3.37	3.23	2.55

Using the amount of H_2 calculated based on the O/R, the yield per mole hexose degraded increases substantially. If these H_2 values are correct, then the H_2 yield using a continuous culture system is approaching the theoretical value.

4.3.2 Carbon Balance and Utilization

Table 4.3 presents analyses of solids entering and exiting the reactor for all 4 carbon-loading conditions. The standard deviations of the effluent solids are high for typical steady state conditions, ranging from 16% to 42%. Due to the insoluble nature of the cellulose, some settling occurred in the bioreactor as well as in the effluent line. This resulted in a somewhat intermittent release of cellulose, skewing the effluent solids readings and subsequent standard

deviations. The average measurement, however, is representative of the cellulose exiting the bioreactor over several days.

Table 4.3 - Cellulose Utilization and Carbon Recovery for each carbon loading condition.

Influent Carbon (g/d)	Effluent Carbon (g/d) ± SD[c]	n[a]	Carbon Utilization	Carbon Recovery
3	0.67 ± 0.05	4	78%	92%
4	1.24 ± 0.20	6	69%	108%
6	2.18 ± 0.46	6	64%	102%
8[b]	1.91 ± 0.20	10	76%	98%

[a] n is the number of steady-state data points
[b] Carbon Recovery based on end-products calculated
[c] SD represents the standard deviation of the samples

A carbon balance was completed for all conditions to ensure that all end-products were accounted for (see Table 4.3). Sources of effluent carbon considered were; CO_2 (liquid and gas), ethanol (effluent line and condensate), lactate, acetate, formate and cellulose. Typical carbon balances for continuous fermentation of an insoluble substrate range from 68% (Lynd et al. 1989) to 97.4% (Desvaux et al. 2001a; Desvaux and Petitdemange 2001). Considering the large standard deviations, this study falls within range of reported values for carbon recovery. The calculated carbon recoveries of 92 to 108% indicate that the majority of end-products were accounted for. The carbon utilization varied from 64-78% (as shown in Table 4.3) exhibiting no increasing or decreasing trends over the four loading concentration tested. This suggests that the system was always operating under carbon-limited conditions.

Desvaux et al (2001) conducted continuous experiments of cellulose fermentation by *C. cellulolyticum* found carbon conversion efficiencies to end-products ranging from 67 to 81.5% depending on the HRT tested (Desvaux et al. 2001a). Lynd et al (1989) studied the fermentation of cellulosic substrates Avicel and pretreated hardwood by *C. thermocellum* in

continuous culture. At a dilution rate of 0.05 h^{-1} (20 hr HRT) they found a conversion efficiency of 77% for Avicel and 41% for the pretreated hardwood. At a dilution rate of 0.133 hr^{-1} these values dropped to 47 and 17% respectively. The carbon utilization rates reported here are consistent with reported values in other cellulosic fermentation studies.

4.3.3 Operational Stability and System Robustness

Continuous-feed fermentation encompasses a large number of process components that are susceptible to mechanical failure. During the course of the fermentation described here, several system failures occurred that changed the conditions in the reactor. Figure 4.3 demonstrates the performance of the fermentor at varying carbon-loading conditions in terms of gas production. In general, *C. thermocellum* has been shown to be resilient to changes in temperature and medium composition (Zhang and Lynd 2005b). The adaptability of *C. thermocellum* was shown to be exceptional under stressful conditions including interruptions in pH control, temperature maintenance, carbon and media feed.

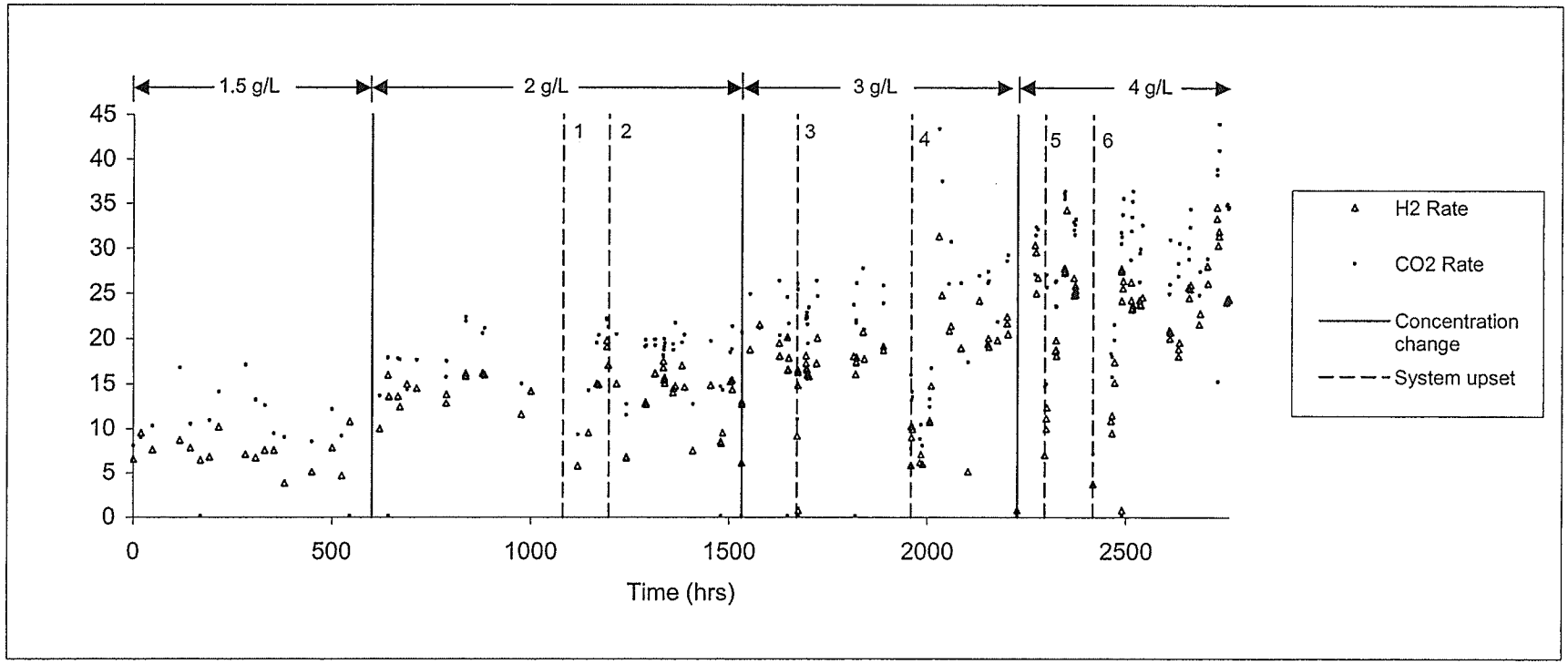

Figure 4.3 - Volumetric gas production rates at 1.5, 2, 3 and 4 g L^{-1} α-cellulose loading

Table 4.4 - System failures and robustness

#	Type of Failure	Duration (hrs)	Time to Recovery (hrs)
1	Cellulose - stir bar stopped	8	90
2	Cellulose - feed line clog	8	46
3	Media - pump failure	6	47
4	pH - no buffer added, pH to 5.21	48	170
5	Cellulose - stir bar stopped	48	22
6	Cellulose Media, pH, Temp	5	70
7	Cellulose - stir bar stopped	8	48
8	Cellulose - stir bar stopped	8	48
9	Media - pump failure	6	24

4.3.3.1 pH

The pH control of the system was interrupted at 1890 hours into the fermentation experiment for approximately 48 hours during the 3 g L^{-1} carbon loading conditions (Figure 4.3). During this time, no KOH was added to the reactor and the pH slowly dropped due to the production of volatile fatty acids, reaching pH 5.21 before the buffering was resumed. At this pH, there was a notable accumulation of cellulose in the reactor. Before the pH interruption, the H_2 production during the 3 g L^{-1} carbon loading condition fluctuated around an average of 3.8 mmol hr^{-1}. As a result of the pH drop H_2 production reached minimum levels of 1.2 mmol

hr^{-1}. However, less than 170 hours after restoring pH control, H_2 production rates more than doubled from the 3 g L^{-1} steady-state conditions to 6.4 mmol hr^{-1} (Table 4.4). These data are consistent with findings on sporulation and cellulase activity in *C. thermocellum*. Spore formation has been found to occur when the pH drops below 6.4, or the temperature drops to below 45°C (Freier et al. 1988; Wiegel and Dykstra 1984). Because of the presence of extra-cellular cellulases, cellulose hydrolysis to cellobiose continues, despite unfavorable conditions for bacterial growth (Demain et al. 2005). In fact the most abundant catalytic subunit of *C. thermocellum*, the endoglucanase CelS, displays optimal activity between a pH of 5 to 6 (Kruus et al. 1995). In addition to the effect on enzyme activity and bacterial growth, pH has also been shown to affect the H_2 partial pressure in the gas phase, volumetric productivity, H_2(gas)/CO_2(gas) ratio, and dissolved bicarbonate (Collet et al. 2004).

4.3.3.2 Power Outage

Temperature failures occurred twice during the course of the fermentation study. Each occurrence was caused by a total system power outage and therefore accompanied by cessation in carbon and media delivery, pH control and effluent removal. Essentially the fermentor operated in batch mode for the duration of the power failure, with the exception of the drop in temperature. The power was recovered after 5 hours, but carbon feeding was not re-initiated for several more hours. Sporulation of *C. thermocellum* has been reported to occur at temperatures at or below 45 °C (Freier et al. 1988). Since the power outage commenced and resolved overnight, however, the minimum temperature and pH reached

during the outage was not recorded. The system returned to steady-state production 70 hours after conditions were returned to normal (Table 4.4).

4.3.3.3 Carbon Feeding

Carbon delivery failures were the most frequent malfunction during the course of the fermentation tests. In almost all instances they resulted from complications due to working with an insoluble substrate. When the concentration of cellulose in the feed vessel reached 8 g L^{-1} solids, the magnetic stir plate was at times unable to maintain a homogenous suspension. In other instances, the delivery line from the feed reservoir to the bioreactor clogged resulting in the cessation of carbon delivery. The recovery time from the carbon limitation failures were the lowest among all the failures, varying between 22 to 90 hours (Table 4.4). This can be attributed to the properties of carbon utilization of *C. thermocellum*. Since carbon utilization during steady-state operation was between 64-78%, there was always some excess cellulose in the reactor for the bacteria to metabolize. The length of time that the stir bar was not functional or the feed line was clogged was generally restricted to a few hours, so in many instances the bacterial metabolism continued using unconsumed cellulose present in the reactor. During cellulose delivery failures all other bioreactor conditions remained the same, thus the time to full system recovery was based on how quickly cellulose was reintroduced into the system. The frequency of cellulose delivery failures is indicative of the need to develop a reliable insoluble substrate delivery system at higher substrate concentrations. Many industrial-scale systems use auger, solenoid or diaphragm pumps to deliver insoluble substrates at extremely high concentrations, but such systems are not

readily available for low-flow laboratory-scale applications. In fact, bench-scale fermentation research on insoluble substrates is often restricted to batch, fed-batch, or continuous delivery at low substrate concentrations (Lynd et al. 2002).

4.4 Conclusions

This study demonstrates that long-term continuous H_2 production using an insoluble substrate such as α-cellulose is feasible. The fermentation system was operated for over 3000 hours with no signs of contamination, as indicated by the lack of butyrate or methane production. The system was robust, displaying quick recovery from a variety of operational disruptions including failure to maintain pH and temperature as well as media and carbon delivery. During the pH failure the bacteria may have sporulated, which resulted in a longer recovery times but may have inadvertently helped to maintain a pure culture. Cellulose utilization varied between 64 and 78% and was not related to the carbon loading of the system. Carbon balances completed on each condition were close to 100% indicating all carbon-containing end-products were accounted for. At concentrations above 4 g L^{-1}, the delivery of α-cellulose was impaired due to feed-line clogging and it became difficult to maintain a homogenous suspension. The highest total gas (H_2 plus CO_2) production rate of 56.6 ml L^{-1} hr^{-1} occurred under these conditions.

Chapter 5
Conclusions

5.1 Continuous fermentation of complex substrates

H_2 production has been demonstrated in batch on complex lignocellulosic substrates and by continuous fermentation of lab grade α-cellulose. The H_2 yield using lignocellulosic substrates was found to be similar to the fermentation of pure α-cellulose, but because 40-70% is composed of hemicellulose and lignin it is not usable by *C. thermocellum*. To produce the same volume of H_2, more dry weight of substrate is required. This could pose problems for lab-scale continuous fermentation studies using complex substrates because of the operational issues involving stirring and pumping a slurry that contains high concentrations of insoluble materials. Using the continuous set-up as described in chapter 4, the mixing and delivery of the α-cellulose in the study was impeded when concentrations reached 8 g L^{-1} in the feed reservoir. To achieve the same concentrations of cellulose (and therefore H_2 production) using CBH as a carbon source almost 30 g L^{-1} of substrate would be required. In addition to the issues involved in delivering a high concentration of substrate to the reactor, it is possible there will also be operational issues involving the removal of the unused portion of substrate from the reactor. Considering a 5 L bioreactor operating at an HRT of 24 hrs, un-fermentable substrate would be accumulated at a rate of 110 g day^{-1}.

To achieve the highest H_2 yield it would be beneficial to use all of the available substrate, including the fraction of hemicellulose. The sugars produced by the hydrolysis of hemicellulose are not easily metabolized by *C. thermocellum*, if at all, but there have been

several other thermophilic anaerobic bacteria isolated that are able to utilize high concentrations of xylose to produce ethanol (Sommer et al. 2004). *T. ethanolicus* was able to produce 24 mM ethanol when grown on steam exploded birch wood hemicellulose (Wiegel et al. 1983), and *C. thermosaccharolyticum* produced 26 mM grown on D-xylose and pretreated oak sawdust (Liu et al. 1988). A co-culture using *C. thermocellum* and a hemicellulose-degrading organism may increase the efficiency of the continuous fermentation when using lignocellulosic materials as substrate and aid in reducing the amount of solids exiting the reactor.

5.2 Bioreactor design

It has been shown that stirring increases the H_2 production due to a shift in thermodynamic conditions, yet growth of *C. thermocellum* can be inhibited by continuous stirring if the cellulosome does not have an opportunity to attach to the cellulose (Freier et al. 1988). A fermentation system that has the ability to intermittently stir and sparge followed by a settling and drawing period might provide optimal conditions for both hydrogen production and biomass retention. The fact that *C. thermocellum* adheres to the cellulose fibers can also help keep the culture pure. By increasing the HRT during periods of settling, any bacteria suspended in the broth may be washed out of the system.

Modified microbial fuel cells (MFCs) are being studied for their role in the complete oxidation of organic matter (Ditzig et al. 2007; Logan 2004a; Logan 2004b; Logan et al. 2005; Oh and Logan 2005). Combining fermentation and MFC could result in an H_2 yield of 8-9 mol H_2/mol hexose degraded (Liu et al. 2005).

5.3 Analytical methods

Restrictions imposed by constraints of the analytical methods and equipment used in these studies limited the amount of information gained. For example, we can tell by the accuracy of the carbon balance and the overestimation of the O/R ratio calculated in the continuous fermentation system that the amount of hydrogen produced was not accurately represented. It is likely that the amount of hydrogen produced was greater than that measured. In some studies, the biogas is trapped in a solution of H_2SO_4 and the volume is recorded along with the composition as determined by GC. It is not feasible to trap the entire volume of gas exiting the reactor because the rate of N_2 sparging used in these experiments was so much higher than the rate of production of H_2. If high sparging rates are employed, the error in the calculation of biogas produced should be kept to a minimum. This can be achieved by closer monitoring of the parameters that are used to calculate H_2 production. Headspace temperature, pressure and gas composition as well as dissolved gases and flow rate of N_2 are required to calculate H_2 production. If feasible, online measurements and data-logging of those parameters would aid in increasing the accuracy of the calculation.

The nature of a continuous system means that there is not always an operator on hand when something goes wrong. To better understand the effects of changes in the system and to explain the reaction of the bacteria to the disturbance, it is necessary to know information that is not currently being measured on a continual basis. The temperature in the broth and the headspace effects the calculation of the gas concentrations in both the headspace and the dissolved broth. Kraemer and Bagley (2004) found that for continuous systems, the calculation of dissolved gases is consistently over-estimated. Better methods of measuring

dissolved gases need to be developed. If the temperature fluctuates, gas concentrations are effected. The calculation of the molar rate of H_2 production is also based on the flow rate of N_2 entering the system, the pressure recorded in the headspace (typically at atmospheric) and the biogas composition. Although many of these parameters do not seem to vary significantly over the course of the experiment, small deviations in each measurement can lead to a much larger discrepancy in the amount of hydrogen calculated. Other studies have successfully measured ORP, pH, temperature, $\%CO_2$, $\%H_2$ and effluent gas flow rate (Kyazze et al. 2006).

The amount of biomass produced in both the batch study on alternative substrates and the continuous study on lab-grade α-cellulose could not be determined by the modified Bradford method. The method measures total protein using a colorimetric assay. In the case of the alternative substrates, the substrates themselves imparted a color on the supernatant, which rendered the method inaccurate. The reason behind the inaccuracies during continuous fermentation of the α-cellulose was less clear. It has been previously reported that biomass measurements in a continuous cellulose fermentation are problematic, but the reasons behind the difficulties were not discussed (Lynd et al. 2002). The protein concentrations in this study seemed to vary widely, and were unrelated to any concentration change or other system disturbance. It is likely that because the Bradford method does not distinguish between live and lysed cells, the concentration of the total protein in the system is entirely dependant on the HRT. In order to fully characterize *C. thermocellum* and its growth kinetics in a continuous culture system, a method of determining the amount of live cells is required.

For future continuous studies on complex substrates, better methods of characterization of the complex substrate both in the influent and effluent would help to understand what portion of the substrate is degraded and what sugars are produced by it's hydrolysis. Evidence suggests that *C. thermocellum* may mutate over time and be able to metabolize hydrolytic products of hemicellulose as the continuous fermentation progresses. It would be of use to know what enzymes it produces in response to being continuously cultured in an environment with hemicellulose and lignin.

There are many sources of cellulosic waste to utilize, all with varying amounts of cellulose, hemicellulose and lignin. To complicate matters further, the organization and the crystallinity of the substrate plays a major role in its availability to enzymatic and bacterial degradation. There needs to be a common methodology to determine the suitability of a substrate for bioenergy production. Currently it's hard to compare the H_2 production potential of various substrates in a meaningful way. It has been reported that differences in the hydrolysis rate of cellulose can result from the use of different cellulosic substrates with different crystallinity, allomorphism, porosity, capillarity and gross surface area (Desvaux 2005). To aid in comparison studies should report at minimum:

1) Initial and final concentrations of cellulose, hemicellulose and lignin
2) Crystallinity index
3) SEM to determine structural organization of the substrate

Additionally, in order to make the process commercially viable, the cost of fermentation should be minimized by:

1) Minimize the sparging to keep the biogas as pure and undiluted as possible

2) Minimize the stirring and heating to keep power costs down

3) Reduce pretreatment costs including enzymes and sterilization

4) Make use of all available substrate, including hemicellulose and VFA's

5.4 Engineering Significance

These studies have increased knowledge of both batch fermentation of various complex substrates and continuous fermentation of an insoluble substrate using the bacterium *C. thermocellum*. The goal of this project was to characterize the fermentation by determining the metabolic end-products of fermentation at various concentrations of α-cellulose from which further studies could be based upon. The highest production of H_2 found in this study was 24.8 mL H_2 per L bioreactor per hour. How does this translate in terms of a functioning hydrogen economy? An average hydrogen fuel cell car would use 108 scf of H_2 per day (Ogden et al. 1998). The volume of reactor required to power one car for one day would have to be 5 m^3. Bioreactors for ethanol production have already been built with a capacity of several thousand cubic meters. Twenty fermentors, each with a working volume of 3 million Liters (3000 m^3) were constructed for a bioethanol production facility in Jining, China (Bai et al. 2008). Considering the measured H_2 production in this study, a bioreactor this size could fuel 600 cars a day. If we use the calculated amount of H_2 produced based on an O/R = 1, the same bioreactor could fuel almost 1200 cars. In Manitoba there are 643,580 registered vehicles on the road (Canada 2008). Using the higher H_2 production estimate, it

would take 536 reactors to fuel all the cars in the province. Advances in increasing H_2 production on cellulosic substrates will lead to better and cheaper technology. To date, no large-scale hydrogen fermentation studies on insoluble substrate have taken place.

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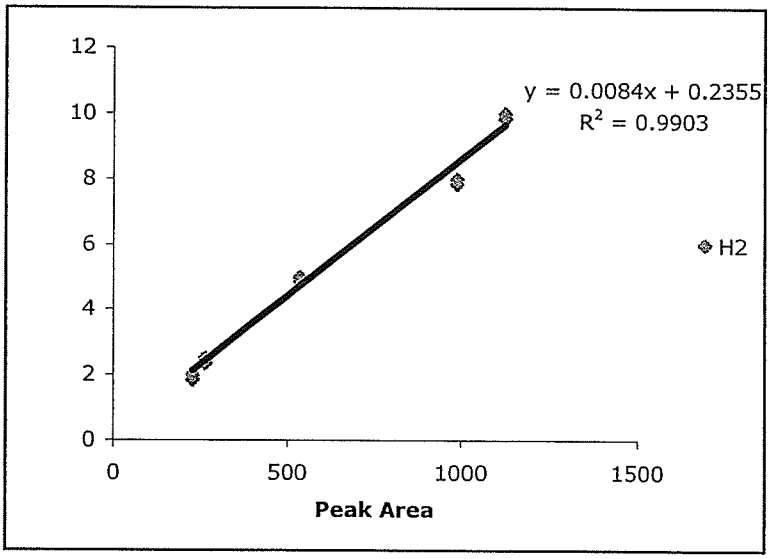
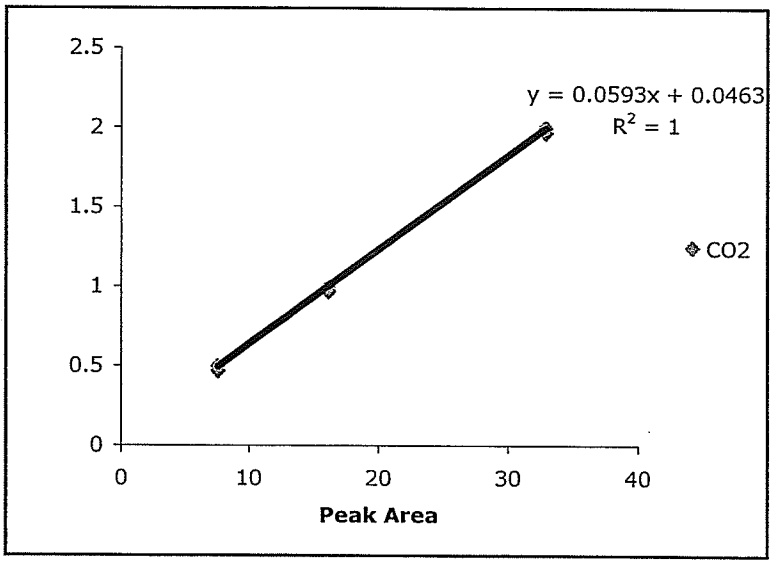
Appendix A

Sample Calculations

5.5 Gas Calculations

The percentages of CO_2 and H_2 in the headspace can be deduced by the peak areas found from the GC and the standard curves





5.5.1 Gas Flow Rate

$$Q_{H_2} = \%H_2 \bullet [(1-\%N_2) \bullet Q_{N_2} + Q_{N_2}]$$

$$Q_{CO_2} = \%CO_2 \bullet [(1-\%N_2) \bullet Q_{N_2} + Q_{N_2}]$$

Where: Q_x = Flow rate of gas (mL/s)

%X = Percentage of gas component in headspace, determined by GC

5.5.2 Volumetric Production Rate

$$VPR_x = \frac{Q_x}{V}$$

Where: VPR_x = The volumetric production rate of CO_2 and H_2 (mmol/hr)

V = Bioreactor working **liquid** volume (L)

5.5.3 Molar production rate

$$MPR_x = \frac{Q_x \bullet P}{R \bullet T}$$

Where: P = Pressure (Pa)

R = Gas constant, 8314 (mL Pa/K mmol)

T = Temperature (°K)

5.5.4 Concentration

$$[X] = \frac{VPR_x \bullet Q_x}{V_{headspace}}$$

Where: V = volume of bioreactor headspace

5.6 Liquid calculations

5.6.1 Dissolved CO_2 and bicarbonate

$$[CO_2]_L = P_{pCO_2} \bullet K_h$$

Where: P_{pco2} = Partial pressure of carbon dioxide in gas phase at 333°K

$= \%CO_2 \bullet P_T$

K_h = Henry's constant at 333°K

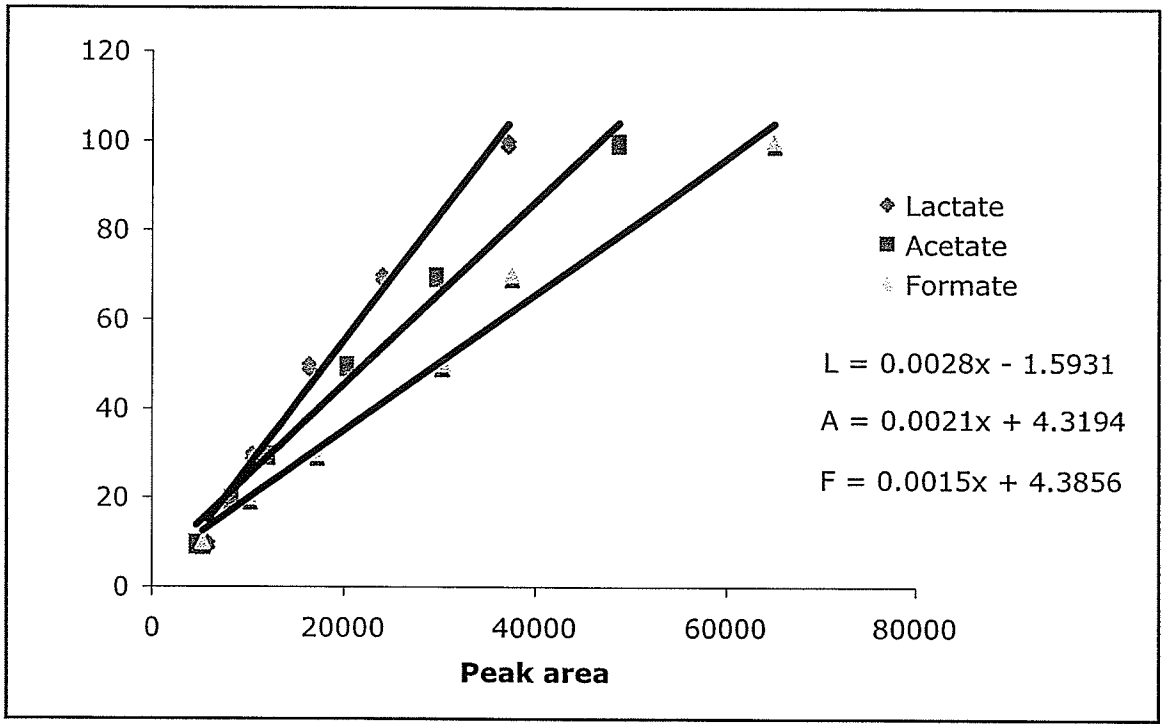
$$[HCO_3^-] = \frac{[CO_2]_L \bullet K_1}{10^{-pH}}$$

Where: $[HCO_3^-]$ = Concentration of bicarbonate

K_1= 1st something constant

5.6.2 Volatile fatty acids

The concentration of all volatile fatty acids including lactate, acetate and formate were determined by HPLC. Concentrations were calculated by comparison of peak areas with a standard curve.



Ethanol

$$[EtOH] = \frac{V \bullet \Delta A}{\varepsilon \bullet d \bullet v \bullet 2}$$

Where: [EtOH] = Concentration of ethanol

V= Final volume, 0.198 mL

v = Sample volume, 0.002 mL

d = Light path length, 0.8 cm

ε = Extinction coefficient of NADH at 340 nm, 6.3 L/mmol•cm

Cellulose

5.7 Hydrogen per hexose utilized

eg. 3 g/L α-cellulose feed

$\alpha\text{-cellulose}_{in} = 3$ g/L

$\alpha\text{-cellulose}_{out} = 1.09$ g/L

$\alpha\text{-cellulose}_{utilized} = 1.91$ g/L

Converting to hexose utilized,

$MW_{hexose} = 180$ g/mol

$MW_{cellulose} = 162$ g/mol

$$hexose_{utilized} = (\alpha - cellulose_{utilized})\frac{180}{162}$$

$\text{hexose}_{utilized} = 1.91 \text{ g/L} \bullet 1.11$

$= 2.12$ g/L

Converting to g/hr,

HRT = 24 hrs

$Q_L = 5\text{L}/24$ hrs

$Q_L = 0.208$ L/hr

$\text{hexose}_{utilized} = 2.12 \text{ g/L} \bullet 0.208 \text{ L/hr}$

$= 0.442$ g/hr

Converting to mol/hr

$$hexose_{utilized} = \frac{0.442 g/hr}{180 g/mol}$$

$hexose_{utilized} = 2.46$ mmol/hr

We have hydrogen production,

$H_2 = 3.76$ mmol/hr

therefore H_2 production per hexose utilized,

$$H_2 per\ hexose = \frac{3.76}{2.46}$$
$$H_2 per\ hexose = 1.53$$

Appendix B
Protocols

B.1 Microplate assay for ethanol

Boehringer Mannheim / R-Biopharm

Reaction Mixture 2* Preparation

- 3 ml of bottle-1 (rxn mixture 1)
- 1 tablet from bottle-2
- Mix

Assay Procedure

Wavelength: 340 nm

Light path length: 0.8 cm

Temperature: 20 °C

Total volume: 203 µl

Pipette into cuvettes	Blank	Sample
Reaction mixture 2*	180 µl	180 µl
Redist. Water or 1191 (blank)	20 µl	-
Sample solution	-	20 µl

Mix thoroughly. After 3 min read absorption of the solutions (A1)		
Start reaction by addition of suspension 3	3 µl	3 µl
Mix. After approx. 5-10 min read A2		

* With repeater pipette 175 µl is dispensed

Determine absorbance difference (A2-A1) for samples and blank

(A2-A1) should be at least 0.100

Calculation

$$[EtOH] = \frac{V \bullet \Delta A}{\varepsilon \bullet d \bullet v \bullet 2}$$

V = Final volume [µl]

v = sample volume [µl]

MW = molecular weight of the substance to be assayed [g/mol]

d = light path [cm]

ε = extinction coefficient of NADH at

340 nm = 6.3 [l x $mmol^{-1}$ x cm^{-1}]

Hg 365 nm = 3.4 [l x $mmol^{-1}$ x cm^{-1}]

Hg 335 nm = 6.18 [l x $mmol^{-1}$ x cm^{-1}]

For ethanol this relationship can be simplified to

C = (0.284 x 46.07 x ΔA) / (ε x d x 0.04 x 2 x 1000)

= 0.33 (ΔA / ε) g ethanol / l sample solution

B.2 Protein

Modified Bradford method)

- Centrifuge 1 ml culture at 14000 rpm for 10 min
- Separate supernatant from the pellet
- Re-suspend the pellet in 0.9% NaCl solution to wash it
- Discard the supernatant after centrifuge
- Re-suspend the pellet in 1 ml (same amount as culture taken) of 0.2N NaOH solution
- Boil in water bath for 10 min
- Centrifuge for 5 min
- Use the supernatant for protein assay

- Prepare BSA protein standards from (10 ug/ml to 100 ug/ml) on 0.2N NaOH

Bradford solution preparation:

In a 1000 ml glass container take 300 ml of milliQ water and dissolve the followings,

- Coomassie brilliant blue: 25 mg
- 85% sulfuric acid: 50 lm
- 95% ethanol: 25 ml
- Add water to bring the final volume upto 500 ml.

Keep the prepared **stock solution** in 4C and in a brown bottle.

Table: Compositions and concentrations of standards. Total volume of each standard solution will be 1000 micro liter.

No.	Stock + 0.2N NaOH composition	Vol (microliter)	BSA (mg/ml)	BSA (ug/ml)
1	20% + 80%	200 +800	0.2	200
2	10% + 90%	100 + 900	0.1	100
3	05% + 95%	50 + 950	0.05	50
4	03% + 97%	30 + 970	0.03	30
5	02% + 98%	20+ 980	0.02	20
6	01% + 99%	10 + 990	0.01	10

- Filter the Bradford reagent (stored at 4°c)
- Add 200 microliter of Bradford reagent to each well

- Add 20 microliter of sample (supernatant) or standards in each well
- Add 20 microliter of NaOH to the blank wells
- Wait 5 min for color development
- Measure the absorbance at 595 nm. The signal should be stable for 60 min.