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The Performance Of Clostridium Phytofermentans For Biofuels Production From Lignocellulosic Biomass

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Abstract

THE PERFORMANCE OF *CLOSTRIDIUM PHYTOFERMENTANS* FOR BIOFUELS PRODUCTION FROM LIGNOCELLULOSIC BIOMASS

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Ethanol produced from lignocellulosic biomass is an alternative transportation fuel with the potential to lower greenhouse gas emissions and increase energy security. Source-separated organic waste (SSO) from the city of Toronto was investigated for feasibility as a lignocellulosic ethanol feedstock. *Clostridium phytofermentans* is a mesophilic, cellulolytic and ethanologenic species with potential application for ethanol production from lignocellulosic biomass. *C. phytofermentans* was considered for biofuels production through experiments on a variety of substrates including soluble sugars and pure celluloses. Results from this study found that *C. phytofermentans* produced 73% of the theoretical ethanol yield on cellobiose but grew poorly on glucose and xylose. In addition, *C. phytofermentans* grew marginally on microcrystalline and ball-milled cellulose, but with supplemental enzymes produced 55% of the theoretical ethanol yield.

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List of Acronyms

AFEX	ammonia fibre expansion
ARP	ammonia recycle percolation
ATP	adenosine tri-phosphate
CBP	consolidated bio-processing
CLSM	confocal laser scanning microscopy
DMC	direct microbial conversion
E10	blend of ethanol and gasoline with 10% ethanol
E85	blend of ethanol and gasoline with 85% ethanol
EPS	extra-cellular polymeric substance
NREL	national renewable energy laboratory
OFMSW	organic fraction of municipal solid waste
SHF	separate hydrolysis and fermentation
SSF	simultaneous saccharification and fermentation
SSO	source-separated organic waste

Chapter 1
Introduction

Climate change, energy scarcity and the desire for energy independence are increasing the pace and intensity of biofuels research and commercialization. Since the late 1990s global ethanol production has been steadily on the rise, with new and modified micro-organisms, pretreatments, process configurations and technologies improving conversion efficiencies and decreasing production costs. In first-generation biofuels, plant sugars and starches from food crops are fermented to ethanol by yeast. The advent of second-generation biofuels broadened the feedstock base to include non-food lignocellulosic biomass by incorporating chemical or enzymatic hydrolysis in various process configurations. Third-generation biofuels employ enzyme-producing ethanologenic micro-organisms to both hydrolyze plant polymers and ferment the resulting sugars. The Canadian government supports and encourages the development of biofuel production capacity and new technologies through a $2 billion commitment in the 2007 Federal Budget. The city of Toronto is actively expanding its collection rate of source-separated organic waste (SSO). SSO has potential application as a lignocellulosic feedstock for biofuels production and is particularly attractive because it has a negative cost – Toronto currently pays a $70 / ton tipping fee for processing. A project was proposed to investigate the potential of producing biofuels via direct microbial conversion of SSO using the bacterium *Clostridium phytofermentans*. This study contributes to the project by investigating the growth and behaviour of *C. phytofermentans* on a variety of substrates.

1.1 A History of Biofuels

1.1.1 First-Generation Biofuels

The boom in the North American biofuel industry during the late 1990s was fuelled by production of ethanol from corn. Meanwhile, Brazil provides for a substantial amount of its domestic fuel by

producing ethanol from sugar cane. Because both sugar cane and corn offer an easily accessible source of sugar for conversion to ethanol, these two ethanol production feedstocks dominate global ethanol production.

The most well-established and time-tested method of ethanol production is by yeast fermentation. Yeasts, such as *Saccharomyces cerevisiae*, can not only produce near-theoretical yields of ethanol from sugars, but also have a very high tolerance for ethanol in their environment (Chu and Lee 2007). Ethanol from sugar and starch crops is considered to be the first generation of biofuels, relying upon easily accessible sugars and yeasts capable of prolific ethanol production.

As the global demand for ethanol has increased with government policies, subsidies and the desire for energy independence, new sources of starches and sugars have been adopted for ethanol production: wheat, sugar beets, sweet potatoes and potatoes are among the crops currently in use as feedstock for industrial ethanol production. However, the use of food biomass for fuel production has the potential to affect food supplies, food prices and agricultural land use, and has been the focus of much controversy (Rutz and Janssen 2008). While the food versus fuel debate rages on and economists argue about whether or not corn ethanol is indeed responsible for the near doubling of corn prices from 2004 – 2007 (Olive 2008), it has generally been accepted that continued growth of the ethanol industry requires exploration of new materials and technologies.

It is apparent that first generation biofuels have some inherent limitations. In a 2007 Library of Parliament report, Frederic Forge (2007) estimates that "Canada would have to use 36% of its farmland to produce enough biofuels to replace 10% of the fuel currently used for transportation." There is an obvious limit to the amount of agricultural production that can be diverted to fuel production. It is simply not possible for first generation biofuels to displace significant fossil fuel consumption without seriously disrupting food networks.

1.1.2 Second-Generation Biofuels

As climate change issues came to the forefront in recent years, many biofuels proponents have peddled the idea that the production and consumption of any and all biofuels leads to reduced green house gas emissions. However, complete life cycle analyses of various forms of ethanol, from the agricultural production of the feedstock to the final use as fuel in an automobile, have shown that this broad assumption of the environmentally benign biofuel is simply not true (Forge 2007, Spatari *et al.* 2005). In response to these environmental and food equity concerns, researchers in ethanol production began to look elsewhere for suitable feedstocks: they found lignocellulosic biomass. Thus the second generation of biofuels was born.

Lignocellulosic biomass is the technical term for all of the woody and herbaceous plant material found all over the earth. It is abundantly available, often considered as a by-product or waste, and it doesn't need prime agricultural land to grow. The structural components of lignocellulosic biomass include significant amounts of fermentable sugars. However, unlike the first generation feedstocks, the monosaccharides embedded in lignocellulosic biomass are present in polymeric forms of cellulose and hemicellulose, and are extremely difficult to access. The second-generation biofuels approach to ethanol production is to ultimately break down lignocellulosic materials into their component sugars, and then ferment these sugars using established fermentation technology. The two challenges for second-generation biofuel are the necessity of pretreatments to alter the structure of lignocellulosic biomass and expose its constituent parts to enzymatic attack, and the efficient enzymatic hydrolysis of the cellulose and hemicellulose polymers.

Ottawa-based Iogen Corp. is perhaps the most celebrated example of second-generation biofuels. Their 2 million litre per year demonstration plant has been operating since 1997, and produces cellulosic ethanol from wheat, oat and barley straw (Iogen 2009). However, the reality of large-scale second-generation biofuels production has been slow to arrive. At present, there are very few

facilities running outside of the laboratory. Although the feedstock is abundant and inexpensive, the costs of chemical pretreatment and enzyme manufacture are limiting the competitiveness of second-generation lignocellulosic ethanol. These cost challenges have lead to innovations that extract more value from the biomass: pentose fermentation primarily from the xylose in hemicellulose by native pentose-fermenting or genetically modified micro-organisms (Chu and Lee 2007), simultaneous saccharification and fermentation by yeasts and enzymes (Kádár *et al.* 2004), and an increasing focus on co-products and fine chemical production (Taylor 2008).

1.1.3 Third-Generation Biofuels

Third-generation biofuels (Carere *et al.* 2008) employ a single organism to produce the enzymes required to hydrolyze cellulose and to simultaneously ferment the resulting sugars to ethanol. With this technology, the need to manufacture enzymes and hydrolyze the biomass with enzymes prior to fermentation is eliminated. Third-generation biofuel production is still in the initial research stages, with researchers such as Lee Lynd, Arnold Demain and Susan Leschine at the forefront of the research. Some researchers use the term consolidated bioprocessing (CBP) (Lynd *et al.* 2005, Lynd *et al.* 1996) while others refer to the process as direct microbial conversion (DMC) (Carere *et al.* 2008, Demain *et al.* 2005).

Although information is scarce in the competitive environment of emerging energy biotechnology, companies have been formed to commercialize third-generation DMC processes. Qteros (formerly Sun Ethanol) was formed to exploit *Clostridium phytofermentans*, an anaerobe characterized by Thomas Warnick and Susan Leschine (2002). Warnick and Leschine (2007) have also filed a patent application for a CBP system using this species. Both the second and third generation have shortcomings in terms of cost effectiveness. However, both are able to overcome the two major problems with first generation biofuels: Biofuels from lignocellulosic materials do not necessarily

impinge on the production of food, and they present an opportunity to significantly reduce greenhouse gas emissions.

1.1.4 Biofuels in Canada

Canada is an automobile-dependent society with an estimated 19 million cars and light trucks on the road and 476 billion passenger-kilometres travelled per year (Statistics Canada 2008). According to one Statistics Canada report, the percentage of Canadians who "go everywhere in their cars" has been steadily on the rise for decades (Turcotte 2008). The Canadian transportation sector is responsible for 29% of Canada's total energy use (Cuddihy *et al.* 2005) and on-road fuel use accounts for 27% of Canadian total GHG emissions (Spatari *et al.* 2005).

The Canadian Federal Government has dedicated considerable resources to the development of biofuels through federal and provincial policy. A long-standing federal and provincial excise tax exemption on renewable fuels such as ethanol and bio-diesel has encouraged renewable fuels to be competitive with gasoline. This exemption was nullified as of April 1, 2008 in favour of programs that support biofuels development in the earlier capital-intensive stages (Federal Budget 2007). A proposed Renewable Fuel Standard, announced in December 2006, will require 5% of all on-road Canadian fuel to come from a renewable resource (Government of Canada 2006). While the renewable fuels that are in the mix that will go toward meeting these standards include biodiesel, waste vegetable oils, bio-butanol and hydrogen gas, the overwhelming majority of this renewable content will be from biologically derived ethanol.

By 2005, the federal government's Ethanol Expansion Program (EEP) had overseen $118 million distributed among 11 ethanol producers across Canada in two rounds of competition. According to the federal government's figures, the new ethanol facilities produced under the EEP should increase Canadian ethanol production capacity by a factor of seven from its 2002 levels, to a total of 1.4

billion litres of ethanol per year (Natural Resources Canada 2005). The Canadian government is taking the search for renewable fuels seriously and has made financial commitments to that end in the Canadian Federal Budget (2007), committing up to $1.5 billion for operating incentives and $500 million for next-generation renewable fuels.

In Canada, 99.9% of the 1.6 billion litres per year of existing and planned ethanol production capacity will come from corn and wheat ethanol (Canadian Renewable Fuels Association 2008). The remaining 0.1% is produced by Ottawa-based Iogen Corp, which operates the only existing cellulosic ethanol plant in Canada. Canadian ethanol production capacity will continue to expand to an anticipated 2.5 billion litres per year by 2020 (Klein 2005). The ethanol industry will need to expand its feedstock base to meet this production capacity without eating up a significant amount of Canada's agricultural land area and food production, and lignocellulosic conversion processes will play an important role in this continued expansion.

1.2 Source-Separated Organic Waste

Many cities across Canada have implemented organic waste collection programs, including Toronto, Edmonton, Hamilton, Halifax and Ottawa. In 2004, the total amount of organic waste being diverted from landfill in Canada was 1.7 million tons per year (Elliot 2008). The city of Toronto collects residential solid waste from approximately 510,000 single-family homes (Butts 2005). Based on a city of Toronto 2000 / 2001 waste audit (Wheatley 2000) those single-family homes generated an average of 299 kg / household / year of compostable materials and in 2002 Toronto implemented a separate collection stream for organic wastes. Of the estimated 150,000 tons of household organic wastes produced each year in Toronto by single-family homes, about 100,000 tons of source-separated organic waste (SSO) are collected through the Green Bin program (Butts 2005). In 2009

Toronto began to implement SSO collection to an additional 5,000 apartment buildings and condominiums (City of Toronto 2009) increasing the annual tonnage of SSO collected.

Toronto's decision to collect organic waste was largely motivated by the closure of the Keele Valley Landfill, Toronto's major landfill site from 1983 to 2002. In 1998, in anticipation of the closure of the Keele Valley site, Toronto began to ship its landfill waste to Michigan at an average cost of $63 per ton (Estey 2006). During eight years of exporting its waste, Toronto has spent upwards of $300 million on hauling trash to Michigan. As the city of Toronto moves towards its goal of 70% waste diversion from landfill by 2010 (City of Toronto 2007), the need to find economically sound ways to process SSO is imminent. A portion of Toronto's SSO is currently treated by anaerobic digestion at the Dufferin Transfer Station, and the remainder is composted aerobically at various locations in Ontario and Quebec. At present, Toronto pays a tipping fee of $70 / ton SSO to have it processed, making SSO a potential negative-cost feedstock for biofuels production.

1.3 Project Description

Based on the potential of biofuels to address several current societal needs – renewable, domestic energy with reduced environmental impacts – and the identification of SSO as a potential low-cost or negative-cost lignocellulosic feedstock, a project was proposed to investigate the feasibility of producing biofuels from SSO. Ryerson University, represented by Dr. Grace Luk in the department of Civil Engineering, and Clean 16 Environmental Technologies Corp., represented by Jamie Bakos, partnered with the Ontario Centres for Excellence to carry out the investigation.

Clean 16 identified a thermal extrusion technology as a potential pretreatment for SSO and other lignocellulosic biomass to improve enzymatic digestibility. The thermal screw press pretreatment provides a high-pressure, high-temperature, shearing process that is known to dramatically increase

the compostability of biomass, and was therefore selected as a pretreatment. Clean 16 provided SSO from the city of Toronto pretreated with the thermal screw press for the project.

The bacterium *Clostridium phytofermentans*, characterized by Warnick *et al.* (2002), is a known cellulolytic and ethanol-producing species. Researchers (Leschine and Warnick 2007, Siezen and Wilson 2008) have identified this species as a potential micro-organism for direct microbial conversion of lignocellulosic biomass to ethanol. While DMC or CBP processes are still in development, this process configuration shows promise for simple and economical biofuels production from lignocellulosic feedstock (Lynd *et al.* 2005, Carere *et al.* 2008). Therefore *C. phytofermentans* was chosen as the cellulolytic and fermentative organism to be used in this project.

Preliminary studies performed as part of an undergraduate civil engineering thesis at Ryerson University (Faye and Percy 2007) indicated that there was potential to produce ethanol and hydrogen via direct microbial conversion of SSO by *C. phytofermentans*, and the decision was made to pursue further research on the topic.

There are three fundamental questions that form the foundation of the project. First: Is SSO an appropriate and feasible lignocellulosic feedstock for biofuels production? Second: Is *Clostridium phytofermentans* an appropriate micro-organism to accomplish the direct conversion of SSO into biofuels? Third: What is a feasible process configuration for the bioconversion of SSO to ethanol? To address the first question, the project will seek to provide a full characterization of the SSO. This will include: the structural composition of the biomass in terms of cellulose, hemicellulose, lignin, extractives and ash; the concentration of heavy metals and other contaminants; the extent of enzymatic digestibility after pretreatment with the thermal screw press; and the seasonal and geographic variability of all biomass characteristics. An investigation into the growth, behaviour and

metabolism of *C. phytofermentans* will seek to answer the second question, and provide insight into the third.

1.4 Study Objectives and Methodology

The contribution of this study to the project is to investigate the suitability of *C. phytofermentans* as a micro-organism for biofuels production by direct microbial conversion of lignocellulosic materials. *C. phytofermentans* is a known cellulolytic, ethanol and hydrogen-producing bacteria isolated from soil near the Quabbin Reservoir in Massachusetts by Thomas Warnick (2002). Literature regarding *C. phytofermentans* is very limited, and only two peer-reviewed publications (Warnick *et al.* 2002, Ren *et al.* 2007) provide any quantitative data regarding the growth and metabolic by-products of the bacterium. The scarcity of data on *C. phytofermentans* is not because it has been overlooked or dismissed as a potential micro-organism for biofuel production, but rather because it has only been recently isolated and is under active investigation for commercialization by the biofuels company Qteros. The objective of this study is to produce quantitative data describing the growth and metabolism of *C. phytofermentans* on a variety of substrates, and to analyze the findings for behaviours and trends relevant to biofuel production. Results from this study will improve the understanding of *C. phytofermentans* and provide scientific insights for the selection of the bioprocessing technology to derive biofuel from SSO. Based on the available literature, *C. phytofermentans* appears to be a candidate for a CBP-type process. The results of this study will aid in the assessment of the suitability of CBP and other biological conversion process configurations for *C. phytofermentans*.

It is known that *C. phytofermentans* is capable of utilizing a wide variety of the sugars present as polymers in plant biomass such as glucose, xylose, arabinose, mannose and galactose (Warnick *et al.* 2002). The first set of experiments, presented in Chapter 4, investigates cell growth, substrate

utilization, and ethanol and hydrogen production on soluble simple sugar substrates to determine maximum product yields expected from *C. phytofermentans*.

In CBP processes, a single type of micro-organism is responsible for the enzymatic hydrolysis of biomass and fermentation of the resulting sugars. Extensive research over several decades into cellulose utilization by cellulolytic bacteria has revealed that cellulose hydrolysis is the rate-limiting factor in substrate degradation (Demain *et al.* 2005, Lynd *et al.* 2002). Cellulose polymers form the primary fermentable component of most lignocellulosic materials, representing between one-third (grasses) and one-half (poplar) of the mass of common biofuels feedstock (Prasad *et al.* 2007). While *C. phytofermentans* is capable of efficiently utilizing a wide variety of biomass sugars, cellulose hydrolysis will determine the rate at which growth and product evolution occurs. The second set of experiments undertaken in this study, presented in Chapter 5, investigates the growth and metabolism of *C. phytofermentans* when cultured on pure cellulose substrates.

crops for energy production include switchgrass, *Miscanthus*, coastal Bermuda grass, hemp and water hyacinth (Monsma 2006, Sun and Cheng 2002, Sierra *et al.* 2008).

Milbrandt (2005) estimated that 92.1 million tons per year of wood residues are available in the United States based on 2002 data: 57 million dry tons from logging, 1.6 million dry tons from primary mills, 2.6 million dry tons from secondary mills and 30.9 million dry tons from urban wood wastes such as construction and packaging wastes.

Municipal solid waste is available wherever there is a human population, but its composition varies tremendously. Recycling programs divert a large fraction of glass, plastics and paper products from this waste stream. In municipalities where the OFMSW is collected separately, it may offer a lignocellulosic feedstock for biofuel. Otherwise, separation technologies must be employed to sort the organic fraction from the unfermentable material prior to use as a feedstock. While the OFMSW may not be an excellent biofuel feedstock due to its complexity, variability and potential contamination, it is very attractive due its negative cost (Wiselogel *et al.* 1996). The city of Toronto collected almost 900,000 tons of residential waste in 2008 (City of Toronto 2009), and the United States Environmental Protection Agency estimated that Americans produced over 250 million tons in 2007 (United States Environmental Protection Agency 2008).

Milbrandt (2005) calculated that there are over 150 million dry tons per year of agricultural residues available after accounting for other uses such as animal bedding, forage and soil protection. Prasad *et al.* (2007) performed a detailed review of lignocellulosic crop residue availability in India. After accounting for other residue uses such as for animal fodder, Prasad estimated that about 450 million tons of crop residues are available annually for ethanol production. Bruce Dale (2006) estimates that 100 million acres – about 6% of the current crop, pasture and forest-use land in the US

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Chapter 2
Creating Biofuels from Lignocellulosic Biomass

Lignocellulosic biomass – or plant biomass – is an abundant renewable resource comprised primarily of cellulose, hemicellulose and lignin. Lignocellulosic materials can be specially cultivated crops, agricultural and forestry residues or municipal and industrial wastes. Cellulose and hemicellulose are polymers of hexose and pentose sugars which can be fermented to ethanol. Ethanol is an attractive renewable fuel because it fits easily within the current infrastructure for distribution and use. It can be blended with gasoline and burned in unmodified gasoline engines. The performance of various lignocellulose-to-ethanol technologies can be compared by considering ethanol yields, environmental benefits and process costs. While there are many possible configurations for producing ethanol from lignocellulosic materials, all of these processes encompass biomass pretreatments, hydrolysis, fermentation of hexose and pentose sugars and product recovery. Enzymatic hydrolysis and fermentation, the two biologically mediated steps in producing ethanol from lignocellulose, can occur separately, simultaneously or be carried out by a single organism in a single-step process. Fermenting organisms include native yeasts and bacteria such as *Saccharomyces cerevisiae*, *Zymomonas mobilis* and some cellulolytic *clostridium* species. Genetically engineered recombinant strains of these micro-organisms have also been developed to increase ethanol yields and ferment a wider array of substrates. Some bacteria, especially clostridia, produce significant amounts of hydrogen gas during fermentation. Biological hydrogen is also a valuable renewable resource.

2.1 Lignocellulosic Biomass

Lignocellulosic biomass is any herbaceous or woody plant biomass containing cellulose, hemicellulose and lignin. Cellulose and hemicellulose polymers are composed primarily of fermentable sugars, so they can serve as a feedstock for biofuel production. Lignocellulosic biomass

is available in many forms: the organic fraction of municipal solid waste (OFMSW), wood industry waste, agricultural residues and cultivated energy crops. However, the structural features of natural lignocellulosic materials make them difficult to degrade. Source-separated organic waste (SSO) is a lignocellulosic material and may be suitable for biofuels production. The city of Toronto collects SSO to divert waste from landfill, but suitable treatment and disposal is problematic.

2.1.1 Sources

Lignocellulosic biomass is the most-produced organic biopolymer on earth (Peters 2006) and one of the most abundant renewable resources available for energy production. Plants capture solar energy and store that energy as biomass. Researchers estimate that about 200×10^9 tons per year of plant biomass are produced globally (Lin and Tanaka 2006), which is primarily lignocellulosic. Any woody or herbaceous plant biomass containing cellulose and lignin is considered to be lignocellulosic. Although the chemical and structural properties of lignocellulosic plant biomass are complex and varied, a large proportion of that biomass is composed of polymers of fermentable sugars. The amount of energy stored in plant biomass and its sheer abundance make it an attractive choice for large-scale energy production.

Lin and Tanaka (2006) outline four categories of lignocellulosic biomass resources: wood, municipal solid waste (MSW), agricultural residues and cultivated energy crops. Wood resources, mostly sawdust and wood chips, are produced as by-products from paper mills, saw mills and furniture manufacturing operations. MSW is complex and variable but contains significant amounts of biomass in the form of paper, food, garden and textile wastes (Li *et al.* 2007). Agricultural residues generally come from inedible portions of food crops including wheat, rice, oat and barley straw, corn stover and sugar cane bagasse (Prasad *et al.* 2007). Cultivated lignocellulosic biomass

crops for energy production include switchgrass, *Miscanthus*, coastal Bermuda grass, hemp and water hyacinth (Monsma 2006, Sun and Cheng 2002, Sierra *et al.* 2008).

Milbrandt (2005) estimated that 92.1 million tons per year of wood residues are available in the United States based on 2002 data: 57 million dry tons from logging, 1.6 million dry tons from primary mills, 2.6 million dry tons from secondary mills and 30.9 million dry tons from urban wood wastes such as construction and packaging wastes.

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Milbrandt (2005) calculated that there are over 150 million dry tons per year of agricultural residues available after accounting for other uses such as animal bedding, forage and soil protection. Prasad *et al.* (2007) performed a detailed review of lignocellulosic crop residue availability in India. After accounting for other residue uses such as for animal fodder, Prasad estimated that about 450 million tons of crop residues are available annually for ethanol production. Bruce Dale (2006) estimates that 100 million acres – about 6% of the current crop, pasture and forest-use land in the US

– could yield enough dedicated lignocellulosic energy crops to produce about 75% of the current US gasoline demand.

2.1.2 Structure and Composition

Lignocellulosic materials include nearly all types of plant biomass. While plant cell structure and organization vary immensely from species to species, the presence of cellulose, hemicellulose and lignin are common to all types lignocellulosic biomass (Lynd *et al.* 2002). Even processed plant biomass, in the form of office paper or newsprint, contains cellulose, hemicellulose and lignin. Table 2–1 lists lignocellulosic materials and the relative proportions of these three key components present in each one. Because there is such a wide variety in the proportions of cellulose, hemicellulose and lignin in different types of lignocellulosic biomass, pretreatment technologies and process configurations need to be tailored to a specific type of biomass.

Cellulose forms the backbone of the plant cell wall structure. The cellulose polymer is composed of subunits of cellobiose joined by β-1,4 glycosidic bonds (Carere *et al.* 2008). Approximately 30 cellulose chains align to form a crystalline structure called an elementary fibril. Many elementary fibrils join to make a microfibril, and groups of microfibrils form cellulose fibres. A schematic of the structure of lignocellulosic biomass is presented in Figure 2–1. The tightly packed crystalline structure of the elementary and microfibrils is maintained by interchain hydrogen bonds and van der Waals forces (Lynd *et al.* 2002). In naturally occurring native plant biomass, cellulose is not purely crystalline, but exists in both crystalline and amorphous forms.

While cellulose is composed of repeating cellobiose molecules, hemicellulose is a more varied polymer. The backbone of the hemicellulose polymer is xylan, or a chain of xylose molecules. Branching side chains contain arabinose, galactose, mannose, fucose, glucuronic acid and acetyl

Table 2–1 Percent dry weight composition of various lignocellulosic materials (Prasad *et al.* 2007, Mosier *et al.* 2005, Lin and Tanaka 2006)

feedstock	cellulose	hemicellulose	lignin
cotton seed hairs	85	20	0
office paper	69	12	11
waste papers from chemical pulps	65	15	8
sorted refuse	60	20	20
poplar	50	17	18
softwood stem	48	30	30
newspaper	48	33	24
pine wood	47	9	29
hardwood stem	45	34	22
corn cobs	45	35	15
rice straw	40	18	6
corn stover	38	22	18
wheat straw	38	21	23
source-separated organic waste[a]	35	27	20
grasses	33	43	20
switch grass	31	21	18
nut shells	28	28	35
coastal bermuda grass	25	36	6
leaves	18	83	0
corn fiber	14	17	8
primary wastewater solids	12	NA	27
swine waste	6	28	0
solid cattle manure	3	2	4

[a] unpublished data from work by Mina Mirzajani, Ryerson University 2009

NA: not available

Values are the mean values of ranges published by Prasad *et al.* (2007) and Mosier *et al.* (2005)

groups. The branched hemicellulose chains are joined by hydrogen bonds to cellulose fibres, lignin and each other (Mosier *et al.* 2005).

Lignin is a complex polymer of phenylpropanoid units (Wright 1988) and is not easily degraded, and presents a major barrier to efficient bioconversion of lignocellulosic biomass. Lignin blocks access to cellulose fibres, and enzymes can become unproductively bound to lignin, decreasing the number of enzymes available for cellulose and hemicellulose hydrolysis. Pretreatments such as AFEX, ARP and lime are able to solubilize and remove lignin, decreasing its inhibitory effects on enzymatic hydrolysis. Cellulose and hemicellulose, which contain fermentable sugars and can be enzymatically degraded, typically occur inside of a lignin sheath. The combination of cellulose, hemicellulose and lignin form a structure that is very strong and resistant to enzymatic attack. There has been extensive research into the chemical and structural features of lignocellulosic materials that contribute to its recalcitrance. Some of the features that affect degradability include: crystallinity and degree of polymerization of cellulose, pore volume, surface area, particle size, lignin content, hemicellulose content, ash content and the degree of acetylation (Chang and Holtzapple 2000). Lynd (2002) noted that in highly crystalline regions of cellulose fibres, pore sizes could be small enough to exclude large particles such as cellulase enzymes.

Chang and Holtzapple (2000) designed an extensive experiment to investigate the effects of various pretreatments on the structural, chemical and digestibility properties of poplar wood. In total, they analyzed 147 types of lignocellulose derived from poplar wood with varying configurations of acetylation, lignin content and crystallinity index. They concluded that the lignin content of the biomass and the crystallinity index of the cellulose are the two major factors affecting enzymatic digestibility. Mathematical relationships between enzymatic digestibility and lignin content of biomass have been established by both Chang and Holtzapple (2000) and Richard (1996).

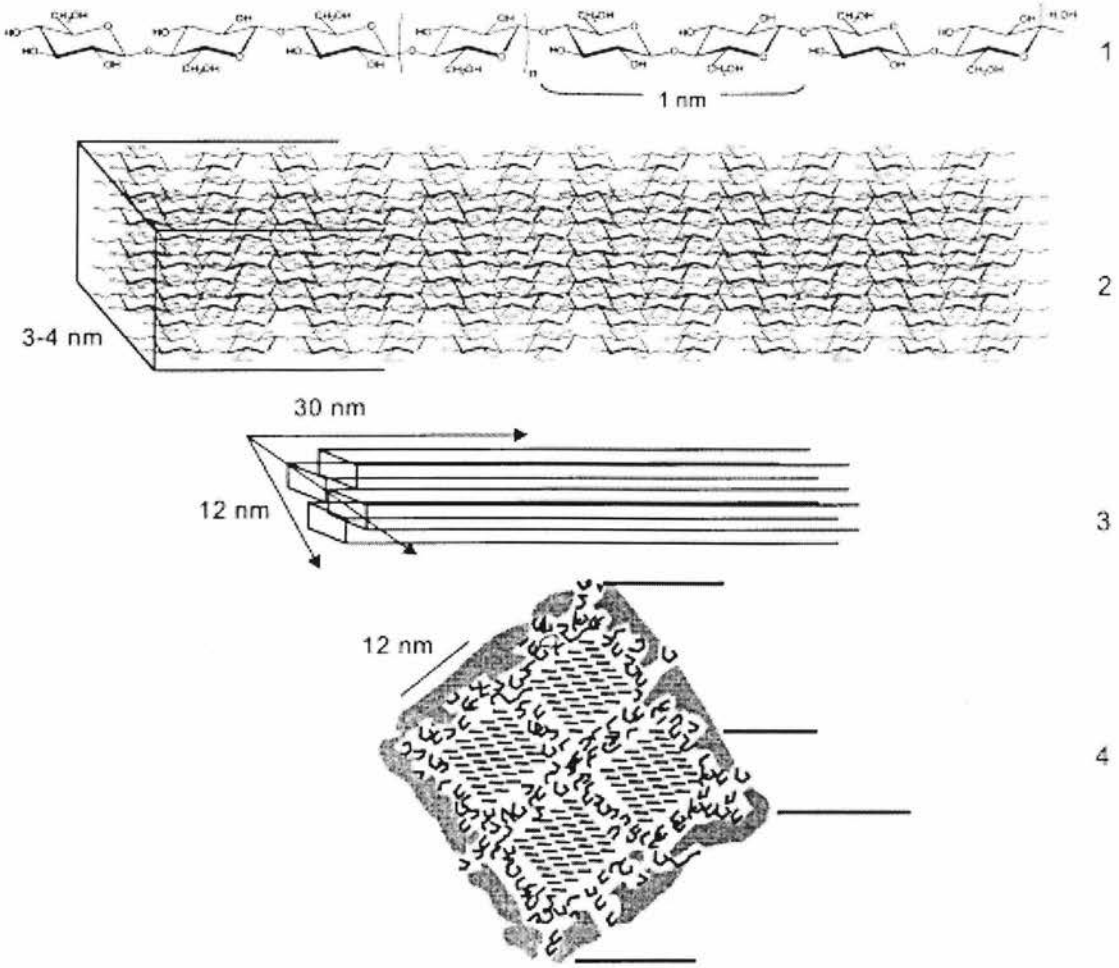

Figure 2–1 Structure of lignocellulosic biomass (Ramos 2003) showing 1: cellulose polymer of glucose sub-unit; 2: cellulose elementary fibril, which is a group of cellulose polymers; 3: cellulose crystallite, which is a bundle of elementary fibrils; and 4: the microfibril cross-section, showing bundles of cellulose crystallite surrounded by hemicellulose and lignin

2.1.3 Source-Separated Organic Waste

Many cities across Canada have rolled out organic waste collection programs, including Toronto, Edmonton, Hamilton, Halifax and Ottawa. In 2004, the total amount of organic waste being diverted from landfill in Canada was 1.7 million tons per year (Elliot 2008), and this quantity is no doubt still on the rise. The city of Toronto collects residential solid waste from approximately 510,000 single-family homes (Butts 2005). Based on a city of Toronto 2000 / 2001 waste audit (Wheatley 2000) those half-million single-family homes generated wastes at a rate of 945 kg / household / year. An average of 299 kg / household / year of compostable materials were produced in these households, meaning that compostable material makes up 32% of total generated wastes. These materials include kitchen wastes, yard wastes and animal wastes (Wheatley 2000).

Work by Mina Mirzajani (2008) has indicated that SSO contains substantial amounts of cellulose, hemicellulose and lignin, making it a candidate lignocellulosic feedstock for biofuel production. While composition of SSO is highly variable, Mirzajani estimates that SSO is approximately 35% cellulose, 27% hemicellulose and 20% lignin.

In 2002 Toronto followed Halifax in implementing a separate collection stream for organic wastes, and in 2005 boasted a 90% participation rate (Butts 2005). Of the estimated 150,000 tons of household organic wastes produced each year in Toronto, about 100,000 tons are collected through the Green Bin program (Butts 2005). An estimated 17,000 tons per year of compostable organics are being put directly into backyard composters (Oates 2000), and the remaining 35,000 tons are either sent to landfill or are misplaced in the recyclables collection stream.

Toronto's decision to collect organic waste was largely motivated by the closure of the Keele Valley Landfill. Keele Valley, located north of Toronto at Major Mackenzie Blvd. and Keele St. was the city's major landfill site from its opening in 1983 until its closure in 2002. In 1998, in anticipation of the closure of the Keele Valley site Toronto began to ship its landfill waste to

Michigan at an average cost of $63 per ton (Estey 2006). During eight years of exporting waste, Toronto has spent upwards of $300 million on hauling trash to Michigan.

The most recent development in Toronto's solid waste management plan has been the purchase of the Green Lane Landfill site near London, Ontario. The fact that Toronto has chosen to send its trash to London at a distance of nearly 200 kilometres demonstrates how difficult it is to find suitable sites for landfills. In 2000 the city of Toronto assembled a group, Task Force 2010, to come up with a waste diversion scheme for the city of Toronto. The report issued by the task force under Mayor Mel Lastman stated clearly the goals of 30% residential waste diversion by 2003, 60% by 2006 and 100% by 2010 (City of Toronto 2001). The report cited "new technologies" as the method of achieving 100% diversion. In 2008, the actual diversion rate was 40% and he most recent update to Toronto's waste diversion goals aims for 70% diversion by 2010 (City of Toronto 2009).

Collecting SSO in a separate stream has diverted hundreds of thousands of tons of waste from landfill, but the treatment and final disposal of the SSO are problematic. At present, the city of Toronto treats SSO by either aerobic composting or anaerobic digestion. Typical outdoor static pile aerobic composting of organic wastes can take up to 18 months (Enviro-Access 2006), and even advanced aerobic composting technologies can only reduce this time to 3 months (Alix *et al.* 2006). Aerobic composting can also cause odour problems, and therefore requires large buffer zones. Facilities are typically sited in remote areas, which increases transportation costs. Anaerobic digestion is much faster than aerobic composting, but is much more energy and capital intensive. About 25,000 tons per year are treated at the Dufferin plant with an additional 18,000 tons per year being treated in Newmarket (Muir 2005). While the Dufferin plant has been successful, the Newmarket plant has been repeatedly shutdown due to odour complaints from the surrounding community. SSO that is not treated at the Dufferin or Newmarket plants is sent to Quebec, which incurs added transportation costs (Muir 2005). The composition, availability and negative cost of

SSO, in addition to current treatment issues for the city of Toronto, make it a potential feedstock for biofuel production.

2.2 Ethanol

Ethanol is an ideal energy currency because it can be obtained from renewable resources and used within the existing transportation fuel infrastructure. The theoretical amount of ethanol that can be fermented from lignocellulosic biomass can be determined if the proportions of cellulose and hemicellulose in the material are known. The theoretical yield provides a tool to evaluate the performance of a lignocellulose-to-ethanol conversion process. Conversion processes may also be able to deliver a fuel that will result in reductions in greenhouse gas emissions on a life-cycle basis when compared to fossil fuels. Economically, lignocellulosic ethanol processes cannot compete with current gasoline prices, but advances will likely make this possible in the near future. Stimulation of rural and agricultural economies is an anticipated benefit of any biomass-based energy initiative.

2.2.1 Ethanol as an Energy Currency

Ethanol is a particularly good energy currency given the current liquid fuel infrastructure. Its potential sources, as well as its physical and chemical properties make it a good fit with the three fundamental energy problems of conversion, utilization and access (Hoffert *et al.* 2002 in Lynd *et al.* 2005).

Solar energy stored in plant biomass as polysaccharides can be converted to liquid ethanol through a variety of biological processes in a multitude of process configurations. Plant sugars, starches and lignocellulosic polymers are all ultimately fermentable to ethanol. While many of these conversion technologies are still in their infancy, there are well-established first-generation technologies that convert starches and sugars to ethanol. Newer technologies are capable of converting the fibrous and

Table 2–2 Selected properties of ethanol and gasoline

fuel parameter	ethanol	gasoline
energy density (MJ / kg)	31.1	44.4
H : C	3	2 [a]
octane number	96 – 113	85 – 96

[a] H:C ratio calculated assuming gasoline composition of 30% iso-octane, 30% heptane, 20% cyclopentane and 20% ethyl benzene (Ophardt 2003)

woody portions of plants into ethanol, and major research efforts all over the world are focused on improving these technologies.

The second energy problem identified by Hoffert *et al.* (2002) is that of utilization. Ethanol can be blended with gasoline and used in the internal combustion engines of all gasoline cars without modification. Ethanol-gasoline blends with 10% ethanol (E10) and 85% ethanol (E85) are available in Canada. Any gasoline combustion engine can utilize E10, while engine modifications such as those made to vehicles with the FlexFuel designation can run on E85.

Various physical and chemical properties of liquid fuels affect combustion engine performance: energy density, ratio of reactant to product volume, ratio of hydrogen to carbon in the fuel, heat of vaporization, flame temperature and octane number. While some properties make ethanol less desirable when compared to gasoline, others increase performance. Brent Bailey (1996) states that the three most important factors – energy density, H:C ratio and octane number – will combine to create a 15% efficiency increase in optimized spark ignition engines. Table 2–2 presents a comparison of these three key properties in ethanol and gasoline. In the near term, while E10 is utilized in current gasoline combustion engines, Bailey estimates an efficiency increase in the order of

1 – 2% (Sinor and Bailey 1993). It is important to note that these efficiency increases are calculated as energy efficiencies (i.e. km / J) and not as increases in mileage (i.e. km / l). Because of the lower energy density of ethanol, more volume is required per distance travelled than for gasoline. This factor complicates comparisons, because car efficiencies are typically considered in units of miles per gallon, or litres per 100 km.

While ethanol is a liquid fuel and does not present the same distribution difficulties as gas phase energy currencies such as natural gas, methane or hydrogen, it does suffer from some compatibility issues with the existing fuel distribution system. Gasoline is immiscible with water, while ethanol is fully miscible with water. This means that if ethanol blended gasoline contacts water in a pipeline or underground storage tank, the ethanol can enter into the water phase (Bailey 1996). Ethanol also has corrosive properties, and even at low blends can cause corrosion of some material such as tin, aluminium and zinc. However, commercially available E10 blends are treated with corrosion inhibiting additives such as DCI-11 or NALCO 5403 to solve this problem. In future, if large-scale ethanol production and distribution becomes a reality, special consideration will need to be given to materials choices in the distribution network. This represents a major capital expenditure and could be reflected in increased cost of fuels.

2.2.2 Ethanol Production Process Performance

Commonly accepted metric for comparing ethanol production processes is the percent of the theoretical ethanol yield obtained. The theoretical yield of ethanol is based on the stoichiometry of converting glucose to ethanol according to Equation 2–1.

$$C_6H_{12}O_6 \longrightarrow 2CO_2 + 2C_2H_6O \qquad \text{Equation 2–1}$$

Theoretically, two moles of ethanol are produced per mole of glucose. On a mass basis, this is equivalent to 0.51 g ethanol / g glucose. This ratio is constant for all six-carbon sugars. If fermentable sugars are present in any form other than glucose – as polymeric cellulose or five-carbon sugars such as xylose for example – then they must be converted to glucose equivalents in order to calculate the theoretical ethanol yield.

Recall that elementary fibrils of cellulose are composed of repeating subunits of cellobiose and that a cellobiose molecule is composed of two glucose molecules. When the β-1,4 glycosidic bonds are cleaved by either enzyme or chemical action to decompose cellulose into glucose, one additional water molecule is required to complete the glucose molecule. Therefore, the enzymatic or chemical decomposition of cellulose into glucose will yield 1.111 g glucose / g cellulose. This ratio holds for all six-carbon sugars in polymeric form such as cellulose, mannan and galactan.

Equation 2–2 shows the fermentation reaction stoichiometry for the conversion of five-carbon sugars to ethanol and carbon dioxide (Hamelinck *et al.* 2005).

$$3C_5H_{10}O_5 \longrightarrow 5CO_2 + 5C_2H_6O \qquad \text{Equation 2–2}$$

Fermentation of five-carbon sugars such as xylose and arabinose have the same mass-based theoretical ethanol yield as glucose of 0.51 g / g. When xylose is present in polymeric form – as in xylan in hemicellulose – decomposition will yield 1.136 g xylose / g xylan. This is also true for other five-carbon sugar polymers such as arabinan. Table 2–3 presents glucose equivalents and theoretical ethanol yields on a mass basis for various five- and six-carbon sugars and their polymers.

Table 2–3 Glucose equivalents and theoretical ethanol yields of various sugars and polymers

molecule	glucose equivalents (g / g)	theoretical ethanol yield (g / g)
glucose, mannose and galactose	1.00	0.51
cellulose, mannan and galactan	1.11	0.57
xylose and arabinose	1.00	0.51
xylan and arabinan	1.14	0.58
cellobiose	1.05	0.54

In order to calculate the percent theoretical yield of ethanol obtained from a biomass sample of known composition, all components must be converted to glucose equivalents. Considering the theoretical ethanol yield based on Equation 2–1 and 2–2, and the total glucose equivalents present in the sample, Equation 2–3 is used to calculate the percent theoretical ethanol yield achieved in a particular process (adapted from Dowe and MacMillan 2008).

$$\%Y_{Th} = \frac{[EtOH]_f - [EtOH]_o}{0.51(f[Biomass])} \times 100\% \qquad \text{Equation 2–3}$$

Where $\%Y_{Th}$ is the percent theoretical ethanol yield, $[EtOH]_O$ is the initial ethanol concentration, $[EtOH]_f$ is the final ethanol concentration, $[Biomass]$ is the initial biomass concentration and f is the mass fraction of glucose equivalents in the dry biomass. All concentrations are in units of mass / volume.

2.2.3 Environmental Performance

There is ongoing debate about the environmental benefits of producing and consuming biologically-derived ethanol. Numerous studies have been completed in attempts to address aspects of the life cycle environmental impacts of bioethanol (Hill *et al.* 2007, Kalogo *et al.* 2007, Wang *et al.* 2007, Pimental and Patzek 2005, Spatari *et al.* 2005, Lynd and Wang 2004) and there seems to be no solid consensus on the net environmental benefit gained by a shift towards bioethanol.

While individual estimates vary, there is general agreement among researchers that ethanol derived from lignocellulosic materials has the potential to realize significant greenhouse gas reductions when compared to gasoline. Spatari *et al.* (2005) reported a 60% reduction in life cycle greenhouse gas emissions when comparing E85 with switchgrass- or corn stover-derived ethanol. Some estimates run as high as 90% reduction in greenhouse gas emissions for lignocellulosic ethanol (Brown *et al.* 1998). However, Spatari *et al.* (2005) also found that carbon monoxide, nitrous oxides and non-methane organic compound emissions were higher for the E85 life cycle.

Some studies on the full life cycle of corn ethanol have shown that energy balances, greenhouse gas emissions and criteria air contaminants are not significantly different from those of gasoline (Hill *et al.* 2007, Wang *et al.* 2007). These studies have noted that agricultural practices for producing the feedstock material as well as the energy supply for the bio-refinery are key factors in determining the environmental feasibility of an ethanol production technology. Wang *et al.* (2007) identified the ethanol production plant power source as the major factor in determining greenhouse gas emissions from ethanol production. They showed that lignin recovery and combustion for power generation can lower life cycle greenhouse gas emissions to about 15% of current corn-ethanol life cycle emissions.

2.2.4 Economic Performance

The economic performance of lignocellulosic ethanol varies with the technology and feedstock used. The fact that there are few working lignocellulose-to-ethanol plants in operation, and that most of these plants are pilots is a symptom of the current economic performance of most lignocellulosic ethanol production schemes. Hamelinck *et al.* (2005) quote current ethanol production costs of US $0.60 – 0.79 per litre in Europe and US $0.26 – 0.33 per litre in the United States.

Those who would like to see an economically viable lignocellulosic ethanol production technology come to fruition are optimistic. Cost-competitive projections for future ethanol-from-lignocellulose costs abound: US $0.08 – 0.18 per litre (Lynd *et al.* 1996), US $0.11 – 0.14 per litre (de Boer and den Uil 1997) and US $0.18 – 0.19 per litre (Wooley *et al.* 1999). Assuming mature technologies, Lynd (2005) estimates future wholesale prices of lignocellulosic ethanol produced by SSCF at US $0.33 per litre gasoline equivalent, and by CBP at US $0.27 per litre gasoline equivalent. Lynd compares these to gasoline wholesale prices of $0.30 per litre from 2001 to 2004. However, all of these optimistic cost projections assume major technological breakthroughs in each step of production and the ability to capture and produce value from all of the by-products and residues in the process train.

Some researchers believe that lignocellulosic ethanol can never be cost competitive with conventional fossil fuels on a production basis, but that other factors such as "CO_2 reduction cost-effectiveness and ease of implementation, compared to other biofuels" will allow biologically derived ethanol to develop (Hamelinck *et al.* 2005). Others point to the potential for domestic economic benefit achieved by lignocellulosic ethanol production through stimulation of the rural economy. Susan Leschine refers to the benefits to rural communities through employment and income opportunities. Leschine claims that economic studies have shown "that simply integrating cellulosic biomass crops into the agricultural rotation of existing cultivated acreage could increase the net income of U.S. farmers by 32%, or $23 billion" (Greene 2004 in Leschine 2007). Bruce Dale (2006)

hopes that as the U.S. moves towards cellulosic ethanol, $18 billion per year will be added to the rural economies while capacity is being built, and $70 billion per year will be generated by the plants required to sustain half of U.S. gasoline consumption.

2.3 Unit Processes of Ethanol Production

The production of ethanol from lignocellulosic materials can be accomplished using a wide variety of physical, chemical and biological processes. While many technologies and process configurations can be used to convert lignocellulose to ethanol, there are four major unit processes common to all methods: pretreatment, hydrolysis, fermentation and product recovery. Pretreatment is used to make the biomass more easily degradable by increasing surface area and pore size, decreasing crystallinity, and solubilizing portions of the lignin and hemicellulose. The enzymatic hydrolysis of cellulose and hemicellulose reduces the carbohydrate polymers to fermentable pentose and hexose sugars. These sugars are biologically converted to ethanol by bacteria or yeast in the fermentation step. Ethanol is recovered from the fermentation broth by column distillation and purified by molecular sieve.

2.3.1 Pretreatment of Lignocellulosic Biomass

Decades of research have clearly shown that lignocellulosic biomass cannot be efficiently converted to ethanol without an adequate biomass pretreatment step. Pretreatments can be physical or chemical, and nearly always require some degree of thermal input to increase the reaction rate. According to Mosier *et al.* (2005) an ideal pretreatment technology for lignocellulosic biomass will: eliminate the requirement to reduce biomass particle size, maintain the pentose fraction of sugars from hemicellulose (predominantly xylose) and limit the formation of by-products that will cause negative effects downstream of the pretreatment. Of course, all of these goals should be addressed while minimizing energy and material inputs to incur the lowest possible pretreatment cost.

Table 2–4 Effect of various pretreatment methods on the chemical composition and chemical / physical structure of lignocellulosic biomass (Mosier *et al.* 2005)

	increases accessible surface area	decrystallizes cellulose	removes hemicellulose	removes lignin	alters lignin structure
uncatalyzed steam explosion	■		■		□
liquid hot water	■	ND	■		□
flow through liquid hot water	■	ND	■	□	□
dilute acid	■		■		■
flow-through acid	■		■	□	■
AFEX	■	■	□	■	■
ARP	■	■	□	■	■
lime	■	ND	□	■	■

■: major effect

□: minor effect

ND: not determined

Pretreatment methods change the cell wall structure and increase enzyme-available surface area by a variety of mechanisms: changes in the degree of cellulose crystallinity, solubilization of lignin, solubilization of hemicellulose and lignin disruption.

While physical pretreatments such as communition and milling are sometimes necessary to facilitate material handling and chemical pretreatments, they are rarely sufficient on their own. Ball milling is the only physical pretreatment that has been shown to significantly increase enzyme digestibility, but it is not a cost-effective option for large scale biomass processing. Fukazawa *et al.* (1982) demonstrated a clear link between the extent of ball-milling aspen wood and it's susceptibility to degradation by cellulase enzymes. Similarly, Fields *et al.* (2000) discovered that a cellulolytic bacterium, *Fibrobacter succinogenes* S85 degraded ball-milled cellulose at the same rate as it

degraded cellobiose, until accessible surface area became a limiting factor. Ball-milled cellulose has also been employed in cellulolytic bacteria work for its ease of digestibility (Leschine and Canale-Parola 1983, Warnick *et al.* 2002).

Mosier *et al.* (2005) present an in depth overview of lignocellulosic pretreatment methods that show promise for the future. Several pretreatment methods were identified, but many were ruled out as not being cost effective due to the cost of chemical inputs. These expensive pretreatments include concentrated sulphuric acid, concentrated sodium hydroxide, ozonation, and the use of solvents such as glycerol, dioxane and phenol. The pretreatments identified as being potentially cost-effective and which were reviewed by Mosier *et al.* (2005) are presented in Table 2–4. A brief explanation of each of the reviewed pretreatments in Table 2–4 is provided below, and has been compiled from Prasad *et al.* (2007), Mosier *et al.* (2005) and Sun and Cheng (2002).

Uncatalyzed steam explosion is a process whereby biomass is heated by steam, held at high temperature, and then rapidly decompressed. The high temperature steam has the effect of breaking down and removing hemicellulose, while the explosive decompression causes a physical change in the biomass structure. Mosier *et al.* (2005) conclude that increased digestibility of biomass treated in this way is due primarily to hemicellulose removal, while the physical change induced has only a minor effect on digestibility.

Liquid hot water and flow through liquid hot water treatments are essentially batch and continuous versions of the same treatment. Water is heated under pressure to maintain it in a liquid phase at temperatures above 200°C and biomass is brought into contact with the water for durations of up to 15 minutes. This pretreatment type can dissolve from 40 – 60% of the total biomass, with almost all hemicellulose removed, and up to 20% of the cellulose and 60% of the lignin solubilized. Lignin

removal rates for the flow through hot water process are slightly higher than those for the static process, as some lignin solids are also removed by the flow.

Dilute acid pretreatments typically employ sulphuric acid at concentrations up to 3%, however nitric and hydrochloric acids have also been investigated. In this pretreatment biomass is exposed to liquid or vapour phase dilute acids at elevated temperatures for durations on the scale of minutes to seconds. Acid pretreatments tend to completely solubilize hemicellulose into its component sugars, and can also hydrolyze cellulose to glucose. Drawbacks of acid pretreatment are the requirement to neutralize the treated liquid prior to fermentation which results in substantial solids in the process stream, and the potential for degrading hexoses and pentoses to furfurals, which are inhibitory to microbial fermentation and reduce the available sugars for fermentation to ethanol.

Flow through acid pretreatment employs acids at lower concentrations of 0.07% and a reactor configuration with high and low temperature zones. Heated dilute acid and biomass flow through the reactor from the high temperature to the low. Almost all of the hemicellulose is removed, a quarter to a half of the lignin is solubilized, and the cellulose remains intact but is highly degradable after the pretreatment. Up to 90% glucose recovery has been reported using flow through acid pretreatment and enzymatic hydrolysis. The reported drawback of this particular configuration is high water demand and expensive reactor configuration.

Ammonia fibre expansion (AFEX) and ammonia recycle percolation (ARP) processes both use 15% aqueous ammonia solutions to solubilize lignin and break the cellulose-lignin bonds, leaving cellulose and hemicellulose relatively in tact. The AFEX process involves an explosive decompression step similar to that in the steam explosion process. Both AFEX and ARP have demonstrated changes in the degree of cellulose crystallinity and subsequent high cellulose

digestibility after the treatment. While these ammonia-based processes are both very effective pretreatments, they suffer from high costs due to complex reactor configurations.

Lime pretreatment uses lower temperatures and pressures than the other pretreatments reviewed by Mosier *et al.* (2005) but typically take much longer to complete. Lime pretreatment involves spraying biomass with aqueous lime slurry, and allowing it to react for several hours to several days at elevated temperatures. The results of lime pretreatment are primarily lignin solubilization, with a minor effect on hemicellulose. This results in the preservation of much of the cellulose and hemicellulose in their polymeric form, with lignin removed, and reported increases in digestibility of up to 80%. Other lime pretreatment studies (Chang *et al.* 1998, Chang *et al.* 1997) have shown that high temperatures drastically shorten the treatment time required to achieve high sugar yields. For example: for a given lime and water loading, the same results are achieved with a 24-hour pretreatment at 50°C as with a one-hour pretreatment at 125°C (Chang *et al.* 1998).

2.3.2 Enzymatic Hydrolysis

The hydrolysis of plant polymers is the bottleneck of any process converting lignocellulosic materials into ethanol. The goal of this process step is to convert cellulose and xylan into fermentable sugars. There are two approaches to breaking down pretreated plant polymers into fermentable sugars: acid hydrolysis and enzymatic hydrolysis. Historically, acid hydrolysis has been preferred, but current research is focused primarily on the use of enzymes to hydrolyze biomass because enzymatic hydrolysis offers several advantages over acid hydrolysis.

Dilute acid hydrolysis typically employs sulphuric acid at concentrations of up to 3% (Mosier *et al.* 2005) due to its low cost over nitric and hydrochloric acids. Steam treatment at temperatures up to 260°C (Schell and Duff 1996) of acid-saturated biomass results in solubilization of cellulose and hemicellulose. Advantages of dilute acid hydrolysis include the relatively low cost, short reaction

cellulose decomposition	*cellulose → glucose → HMF → tars*
xylan decomposition	*xylan → xylose → furfural → tars*

Figure 2–2 Generalized decomposition of cellulose and xylan (Schell and Duff 1996)

time and absence of product inhibition during hydrolysis as observed in enzymatic hydrolysis (Taherzadeh and Karimi 2007). However, acid hydrolysis creates a waste stream that is difficult to treat and the neutralization reactions create large volumes of gypsum to be landfilled (Hamelinck *et al.* 2005).

There is general agreement among those searching for a cost effective lignocellulosic biomass-to-ethanol technology that both hexose and pentose sugars present in the biomass must be captured and converted. While this presents the challenge of choosing appropriate organisms for fermentation, it also presents major challenges in the hydrolysis technology employed. Dilute acid hydrolysis is capable of reducing cellulose to glucose and xylan to xylose, but the reactions do not take place at the same rate. According to Schell and Duff (1996), at the higher temperatures required to acid-hydrolyze cellulose to glucose – 180°C to 260°C – xylan quickly breaks down to furfural and tars. The major drawback of dilute acid hydrolysis is that degradation reactions continue after the cellulose and hemicelluloses have been broken down into monosaccharides. As shown in Figure 2–2, glucosebreaks down to hydroxymethyl furfural (HMF) and xylose decomposes to furfural. If the reactions continue, both will eventually become tars. Furfural, HMF and tars are inhibitory to most fermentation processes, and are therefore not desired in the process stream.

Enzymatic hydrolysis offers an attractive option over acid hydrolysis in that the reaction between enzyme and substrate results in saccharification without degradation products (i.e. furfurals and tars),

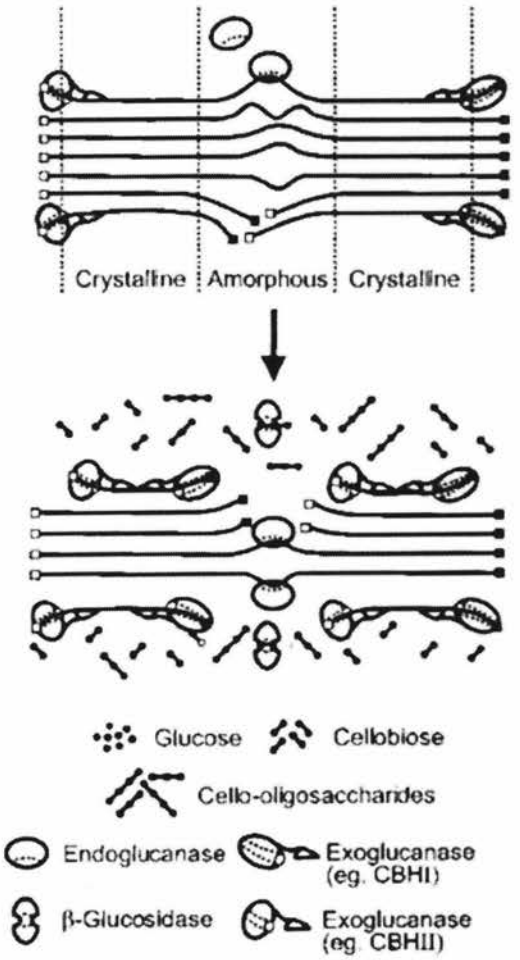

Figure 2–3 Schematic of enzymatic hydrolysis of cellulose by endoglucanase, exoglucanase and β-glucosidase (Lynd *et al.* 2002)

and it does not require extreme temperatures or pH conditions and the associated equipment and energy costs. These advantages are tempered by the long reaction times – on the order of 3 days – and the high cost of manufacturing or purchasing enzymes. It has been estimated that in a lignocellulosic biomass conversion process, the cost of enzymes can run as high as US $0.30 per gallon of ethanol produced, but improvements are continuous and apparently forthcoming. In April 2004 Novozymes Biotech and Genecor had both decreased cellulase production costs to around US $0.20 per gallon, with promises to reach US $0.10 per gallon in the near future (Greer 2005). This is particularlyimpressive, as several years earlier, costs were in the prohibitive range of about US $5.00 per gallon.

Within the last two decades, investigations into the degradation of cellulose by micro-organisms have made significant progress toward understanding the complex biochemical mechanisms employed by micro-organisms to degrade lignocellulosic materials. Enzymes are a key component in nature's approach to degrading these materials and liberating molecules that are useable as food for micro-organisms. Researchers have identified over 30 hemicellulases and cellulases, originating from more than 60 species of fungi and a host of aerobic and anaerobic bacteria (Brigham *et al.* 1996).

In practice, the term cellulase refers to a mix of enzymes from a few major groups of enzymes, which tend to have both cellulolytic and hemicellulolytic activity. Brigham *et al.* (1996) note that while several individual enzymes have been characterized, many are functionally redundant and cellulolytic organisms produce several enzymes that essentially accomplish the same task. Philippidis (1996) identifies the three major groups of cellulase enzymes as endoglucanases, exoglucanases and β-glucosidases. Endoglucanases and exoglucanases function by attaching to insoluble cellulose substrate, while β-glucosidases are soluble and participate in the breakdown of soluble oligomers. A schematic of the breakdown of cellulose by cellulase enzymes is presented in Figure 2–3.

The bulk of commercially manufactured cellulase enzymes are produced by the aerobic fungus *Trichoderma reesei* (Philippidis 1996) and sold under brand names such as Celluclast 1.5 L and Speyzyme CP. Cellulase enzymes perform optimally under slightly acidic conditions at pH 4 – 5 and temperatures of 40 – 50°C (Taherdazeh and Karimi 2007).

One of the drawbacks of cellulase enzyme systems is that they are affected by end-product inhibition. As the exoglucanases cleave cellobiose units from the ends of cellulose chains, the increase in cellobiose concentration inhibits the action of the enzymes, meaning that the reaction slows down. Two solutions have been adopted to the end-product inhibition problem. One solution is to add an excess loading of β-glucosidase, which converts cellobiose to glucose, thereby preventing cellobiose build-up and subsequent inhibition (Taherdazeh and Karimi 2007). Excess β-glucosidase is typically produced by *Aspergillus* species and sold in commercial preparations such as Novozyme 188. This combination of cellulase enzymes supplemented with β-glucosidase has been adopted in many studies (Chang *et al.* 1997, 1998, 2001) and is recommended by the National Renewable Energy Laboratory (NREL) in its protocols for the enzymatic saccharification of biomass (Selig *et al.* 2008). The alternate solution is to utilize the end product on a continuous basis as it is produced. This idea led to SSF and SSCF process configurations, where fermentative organisms continuously consume cellobiose and glucose as they are made available by enzymes. While SSF solves the product inhibition problem, it creates another one: optimal enzyme conditions are not the same as optimal micro-organism conditions. Therefore, SSF process parameters are always sub-optimal for both the enzyme and fermentative organism. Vásquez *et al.* (2007) present a method of determining optimal SSF conditions using response surface methodology, but optimal conditions will always depend on feedstock type, feedstock concentration and pretreatment technology.

Under optimal conditions and with high enzyme loadings, enzymatic hydrolysis yields of close to 100% have been demonstrated (Taherzadeh and Karimi 2007). However this is rarely economical in

practice. Ideally, a balance must be struck to maintain low enzyme costs with good conversion rates. Chang *et al.* (1997) showed that the three-day sugar yield from lime-pretreated switchgrass improved steeply up to an enzyme loading of 10 FPU / g dry biomass, while only marginal improvement was observed from loadings of 10 up to 100 FPU g / dry biomass.

2.3.3 Fermentation

Dominik Antoni *et al.* (2007) explain that anaerobic microbial processes for ethanol production tend to preserve significant amounts of the original substrate's energy in the final ethanol product. Anaerobic metabolism of sugars provides much less energy to the organism than aerobic metabolism. This is a boon for biotechnology because an anaerobic organism will need to consume far more substrate to obtain the same amount of energy as an aerobic organism, and in so doing produces far more ethanol as a product of metabolism. Antoni *et al.* (2007) estimate that for a given quantity of substrate an anaerobic organism will glean only 2 – 3 ATP from the metabolism of glucose into CO_2 and ethanol, while an aerobic organism may gain 26 – 38 ATP from the same transformation.

Once the sugars have been liberated by the hydrolysis of cellulose and hemicellulose, microbes can metabolize them for energy. Recall Equation 2–1, the fermentation reaction stoichiometry for six-carbon sugars such glucose, which gives a molar yield of 2 mol ethanol / mol hexose. On a mass basis, the yield is 0.51 g ethanol / g hexose. Similarly Equation 2–2 shows that the molar ethanol yield for five-carbon sugars is 5 mol ethanol / 3 mol pentose with a mass yield of 0.51 g ethanol / g pentose.

Depending on the process configuration, fermentation may occur separately from (SHF) or simultaneously with (SSF, SSCF and CBP) hydrolysis. The organism used for ethanol production will determine the maximum possible yields of ethanol from the available sugars. The theoretical yield of ethanol from glucose of 0.51 g / g is not achievable in practice, but some micro-organisms

can come remarkably close. The bacterium *Zymomonas mobilis* has been shown to be capable of producing 0.49 g ethanol / g glucose (Miyamoto 1997) and some strains of *Saccharomyces cerevisiae* can produce up to 0.46 g ethanol / g glucose (Vallet *et al.* 1996). The incredible efficiency of these two organisms at converting glucose into ethanol is the reason that they are both widely employed in biofuel production and extensively researched.

Z. mobilis and *S. cerevisiae* are both efficient glucose fermenters, and they are employed whenever biomass has been pre-hydrolyzed by either acid or enzymes. For CBP processes, micro-organisms must be able to produce enzymes as well as ferment carbohydrates to ethanol. At the present time, most of the organisms identified as potential candidates for CBP are *clostridium* species. Many of these species have ethanol yields of less than 50% of theoretical, and typically produce other by-products like acetate, formate, butyrate and lactate in significant quantities. *Clostridium thermocellum* has demonstrated conversion efficiencies of 43% of theoretical (Lynd *et al.* 1989) and *Clostridium cellulolyticum* has been shown to produce up to 33% of the theoretical yield (Desvaux *et al.* 2000).

Because xylose makes up such a large fraction of most lignocellulosic biomass, and because it is a fermentable sugar, xylose fermentation should be included in any lignocellulose-to-ethanol process to increase yields and economic potential. The challenge with xylose fermentation is that the most efficient and well characterized glucose fermenters – such as *S. cervisiae* and *Z. mobilis* – are unable to convert xylose to ethanol in significant quantities. One approach has been to add a separate xylose-fermenting step to the process after glucose fermentation. However, this adds expense as one more fermentation vessel is required and another micro-organism will be employed in the process. The alternative is to identify or genetically modify an organism to convert both glucose and xylose to ethanol.

2.3.4 Product Recovery

In most applications where ethanol is produced from lignocellulosic materials, product recovery occurs downstream of the pretreatment, hydrolysis and fermentation processes, regardless of their configuration. Ethanol has a boiling point of 78°C, and can therefore be recovered efficiently from aqueous mixtures by distillation. When ethanol-water mixtures are distilled, the resulting azeotrope is 95.57% (w/w) ethanol and 4.43% (w/w) water. This mixture is directly combustible and can also be mixed with gasoline without any further purification (Antoni *et al.* 2007). Further purification can be accomplished using molecular sieve adsorption to remove water and produce a final product of 99.5% ethanol (Aden *et al.* 2002).

The NREL produced a document in 2002 detailing the engineering design considerations and process economics of an SSF configured lignocellulose-to-ethanol conversion plant (Aden *et al.* 2002). In their configuration, the product recovery consists of a double distillation column installation, followed by a vapour phase molecular sieve. The first column receives the effluent from the fermentation, and removes about 90% of the water producing a 40% ethanol stream. This stream is fed to a second distillation column, which further concentrates the ethanol to over 90%, which is approaching the azeotropic concentration. Finally, this high ethanol mixture is superheated and fed through a molecular sieve, to which most of the remaining water is adsorbed, with a final product stream that is 99.5% ethanol.

The notable exception to this type of downstream ethanol recovery is simultaneous hydrolysis, fermentation and product recovery that is possible when higher process temperatures are employed. Weilnhammer and Blass (1994) performed this type of extractive fermentation using the thermophile *Clostridium thermocellum*. Ethanol is typically an inhibitory end product, so the continuous removal of ethanol means that production rates remain high throughout the fermentation process.

2.4 Ethanol Production Process Configurations

The various lignocellulosic biomass conversion technologies vary mainly in their treatment of the two biologically mediated processes involved in ethanol production: hydrolysis and fermentation. Pretreatments generally occur upstream of and separate from hydrolysis and fermentation, and product recovery usually occurs downstream of fermentation as a final step. In the SHF configuration, a chemical or enzymatic hydrolysis step occurs prior to a biological fermentation, producing a carbohydrate rich liquid to be fermented. The SSF configuration combines enzymatic saccharification with micro-organism fermentation to relieve cellulase enzyme end-product inhibition, and eliminates a reaction vessel from the process. The yet-to-be-proven CBP configuration would eliminate the need to produce cellulase enzyme separately by employing a single organism to both produce cellulase enzyme and ferment the resulting sugars to ethanol.

2.4.1 Separate Hydrolysis and Fermentation (SHF)

The first incarnation of cellulose-to-ethanol technology was the SHF configuration. In a separate first step, cellulosic biomass is converted to fermentable sugars. This sugar-rich liquid is then fed into a typical ethanol fermentation process. Figure 2–4 shows an SHF process schematics incorporating glucose and xylose fermentation. In SHF, each process step is isolated, and can therefore be carefully controlled and optimized. SHF allows enzymatic saccharification to be carried out at an enzyme loading, temperature, pH and biomass concentration that provides optimum sugar yields, whereas in SSF or CBP configurations, conditions must suit both enzyme activity and the fermenting micro-organism. The disadvantage of operating a separate enzymatic hydrolysis step is that cellulase activity is subject to end-product inhibition even at relatively low concentrations, and increasing cellobiose and glucose concentrations will decrease the effectiveness of the cellulase (Taherdazeh and Karimi 2007). This means that higher cellulase loadings and supplemental β-glucosidase are required to achieve efficient hydrolysis resulting in high enzyme costs (Hahn-Hägerdal *et al.* 2006).

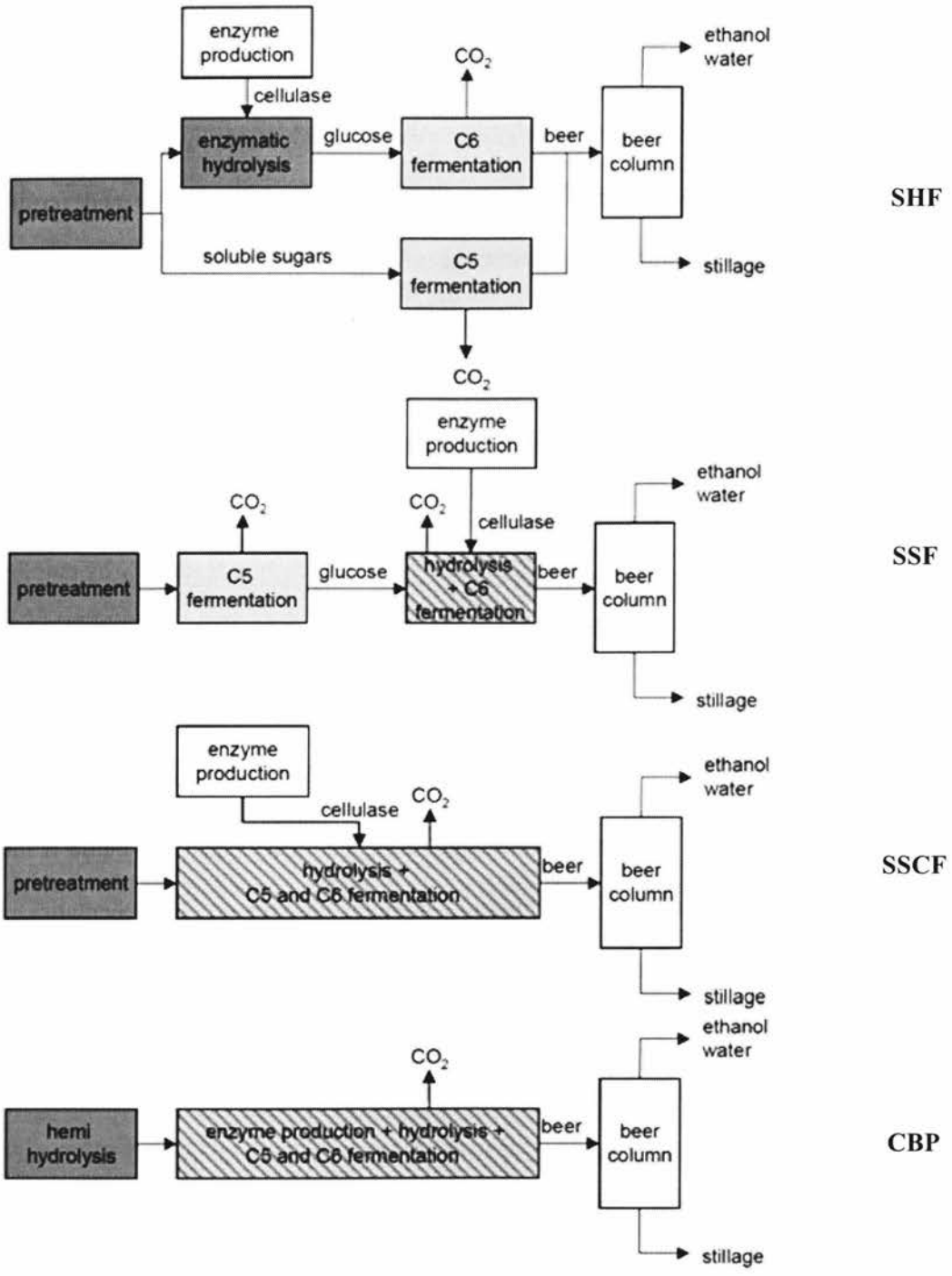

Figure 2–4 Schematic of SHF, SSF, SSCF and CBP lignocellulosic ethanol production processes (Hamelinck *et al.* 2005)

While the SHF process configuration produces high sugar and ethanol yields by optimizing each unit process' operating conditions, it suffers from the disadvantage of long process time and high capital cost. Comparison of SHF and SSF conversion of steam-exploded wheat straw (Alfani *et al.* 2000) showed that the SHF process produced 81% theoretical ethanol yield while the SSF produced only 68% of the theoretical yield. However, the SHF process required 96 hours of total reaction time, while the SSF process required only 30 hours. Therefore the SHF process had a much lower volumetric productivity of 0.313 g / l / h than the SSF process at 0.837 g / l / h, giving the SSF configuration greater economic potential.

2.4.2 Simultaneous Saccharification and Fermentation (SSF)

In the SSF configuration enzymes are applied to the pretreated biomass in the presence of a fermenting micro-organism. This solves the enzyme inhibition problem by immediately utilizing the sugars released during hydrolysis, and also reduces capital costs by integrating enzymatic hydrolysis and fermentation into a single process vessel. While overall conversion efficiencies may be lower than for SHF processes, SSF has proven to be more economical. This is due to capital cost reductions, the higher volumetric ethanol productivity achieved, the more efficient use of enzymes, lower requirement for sterility as sugar concentrations are low and ethanol is produced, and a significantly shorter process time (Sun and Cheng 2002). The SSF process configuration is currently the preferred method of the NREL for commercialization (Aden *et al.* 2002).

SSF technology has been well developed, and SSF processes are used extensively in the literature to evaluate the effectiveness of feedstock and pretreatments combinations (Chang *et al.* 2001, Eklund and Zacchi 1995, Georgieva *et al.* 2008, Vásquez *et al.* 2007, Kádár *et al.* 2004). Because neither the enzymes nor the fermenting micro-organisms are able to operate at their optimal conditions during SSF, researchers have worked to identify process optima for SSF (Vásquez *et al.* 2007).

Xylose fermentation in the SSF configuration can occur either separately from or simultaneously with glucose fermentation. In order to capture the ethanol potential of the xylose in the SSF configuration, the following three options are available: add a separate xylose fermentation step before or after the SSF vessel; co-ferment the glucose and xylose in the SSF vessel with two exclusive glucose and xylose-fermenting organisms; or, ferment glucose and xylose simultaneously in the SSF vessel using a single organism capable of both glucose and xylose bioconversion. The SSF variant where enzymes, a glucose fermenting organism, and a xylose fermenting organism are all simultaneously applied to the pretreated biomass is called simultaneous saccharification and cofermentation (SSCF) (Lynd *et al.* 2002). Figure 2–4 shows process schematics for SSF and SSCF processes.

2.4.3 Consolidated Bio-Processing (CBP)

Lynd *et al.* (2005) define CBP as a process "featuring cellulase production, cellulose hydrolysis and fermentation in one step." CBP is an unproven technology: It is still under investigation, and researchers disagree as to the likelihood of CBP being commercialized.

The promise of CBP lies in the potential capital cost savings realized from reducing a multi-step biological process into a single step where all processes are occurring simultaneously. Figure 2–4 shows a CBP process schematic in comparison to SHF, SSF and SSCF processes. According to Diane Greer (2005), by 2005 the biotechnology company Genecor had reduced the cost of enzyme production from several dollars to US $0.10 – 0.20 per US gallon of ethanol produced. Even with this remarkable cost reduction, enzymes costs comprise a significant portion of lignocellulosic ethanol costs. With ethanol prices near US $1.60 / US gallon in January of 2009, the enzyme production cost is significant at 6% – 12% of the total price. Lynd *et al.* (2005) estimated that CBP has the potential

to reduce ethanol production costs by as much as 25% per gallon of ethanol when compared with an SSCF process.

Lynd *et al.* (2005) suggest that "micro-organisms with the combination of substrate-utilization and product formation properties required for CBP are not currently available, but could probably be developed given sufficient effort." While there are some native bacteria that synthesize cellulase and produce ethanol as a primary metabolic product, none of these have proven sufficient to run a CBP process. It is more likely that genetic engineering will provide the as yet undiscovered CBP organism through modification of a well-understood ethanol producer such as *S. cerevisiae* or *Z. mobilis* to allow enzyme production, or modification of robust cellulose degraders such as *T. reseii* to produce and tolerate ethanol.

2.5 Micro-Organisms for Ethanol Production

The ethanologenic, or ethanol-producing, micro-organisms used in ethanol production must be able to produce significant amounts of ethanol and be able to tolerate relatively high ethanol concentrations without inhibition. While the historical focus has been on the bio-conversion of glucose to ethanol, the ideal ethanologenic micro-organism would efficiently ferment both the hexose and pentose fractions of lignocellulosic biomass to ethanol. Many native yeasts and bacteria are high-level ethanol producers, and the most common species used are the yeast *S. cerevisiae* and the bacteria *Z. mobilis*. The bacteria *E. coli* and *K. oxytoca* are native pentose utilizers, but do not naturally produce high ethanol yields. CBP organisms must be both ethanologenic and produce cellulase enzymes. There are some native species that are CBP candidates, and recombinant micro-organisms have been developed towards achieving a viable CBP process. *C. phytofermentans* is a novel species of bacteria, and a native CBP candidate. It is the subject of this study.

2.5.1 Native Ethanol Producers

Lin and Tanaka (2006) present data on over a dozen strains of *Saccharomyces cerevisiae* that have been studied for ethanol production. In these studies, fermentations of various hexose sugars were carried out by the yeasts at pH 4.5 – 6.5 and temperatures of 24 – 30°C. Ethanol concentrations near 10% were achieved and almost all strains produced over 80% of the theoretical ethanol yield. Ethanol tolerance of *S. cerevisiae* is quite high: 50% inhibition at concentrations of 25% ethanol (Baskaran *et* al. 1995). *S. cerevisiae* is commonly employed in laboratory biofuel studies to ferment hydrolyzed lignocellulosic biomass. However, this yeast's major shortcoming is its inability to convert the pentose fraction of biomass. In fact, "no naturally occurring yeast can ferment all these [plant] sugars to ethanol" (Saha 2004).

Efforts to give *S. cerevisiae* the ability to ferment xylose have been relatively successful. Chu and Lee (2007) present a review of this topic, outlining non-invasive adaptive methods for strain development, as well as the creation of recombinant strains. This has mainly been accomplished by giving it the capacity to produce the xylose isomerase enzyme, which breaks xylose down to xylulose – a substrate that many yeast species can ferment (Saha 2004). *S. cerevisiae* has received genes from *Pichia stipitis* (Kotter and Ciriacy 1993), *Thermus thermophilis* (Walfridsson *et al.* 1996) and fungal *Pyromyces* species (Kuyper *et al.* 2003) to allow it to ferment xylose as well as glucose. While many successes have been achieved in creating xylose-fermenting strains of *S. cerevisiae*, Karhumaa *et al.* (2007) demonstrated that some of these strains are incapable of producing ethanol in undetoxified lignocellulosic hydrolyzates, giving them limited industrial relevance.

Z. mobilis is a bacterium that can ferment only glucose, sucrose and fructose (Antoni *et al.* 2007), and it has been applied in both SHF and SSF (Kádár *et al.* 2004) configurations. Like *S. cerevisiae*, *Z. mobilis* can ferment hexose sugars to produce ethanol at efficiencies close to the theoretical maximum (Miyamoto 1997). In fact, *Z. mobilis* can typically produce 5 – 10% more ethanol per

gram substrate than *S. cerevisiae*. *Z. mobilis* also exhibits a high tolerance for ethanol in its environment, with 50% inhibition occurring at 28% ethanol (Baskaran *et al.* 1995). Because of its higher ethanol tolerance and fermentation rate, *Z. mobilis* has a specific ethanol productivity that is more than double that of *S. cerevisiae* (Lin and Tanaka 2006). *Z. mobilis* has been the source of genes for the genetic engineering of many other bacteria (Guedon *et al.* 2002, Dien *et al.* 2000, Doran and Ingram 1993).

Z. mobilis suffers from the same limitation as *S. cerevisiae* when applied to the fermentation of lignocellulosic hydrolyzates: It can not ferment the pentose sugars from the hemicellulose fraction. To overcome this limitation, several strains of *Z. mobilis* have been engineered to ferment pentose sugars by introducing genes from *E. coli* (Hahn-Hägerdal *et al.* 2006). One such strain, engineered with *E. coli* genes, was able to produce 86% of the theoretical ethanol yield when cultured on xylose alone (Zhang *et al.* 1995). Zhang *et al.* (1998) went on to engineer another *Z. mobilis* strain capable of fermenting xylose and arabinose in a multiple sugar mixture to 82 – 84% of the theoretical yield.

2.5.2 Native Hexose and Pentose Utilizers

Klebsiella species are generally efficient utilizers of mixed sugar substrates, including most of the hexose and pentose sugars present in lignocellulosic hydrolyzates. These soil organisms are abundant in cellulose-rich pulp and paper waste (Doran and Ingram 1993). *K. oxytoca* is not an immediately obvious choice for ethanol production, because native strains do not actually produce any ethanol. However, introduction of genes from ethanologenic bacteria like *Z. mobilis* can allow *K. oxytoca* to convert pyruvate, which it does produce, into ethanol. The most common modified strain of *K. oxytoca* is strain M5A1, which contains the ethanol-producing pathway from *Z. mobilis* strain P2 (Antoni *et al.* 2007).

Table 2–5 Native and modified organisms with potential for deployment in CBP processes

micro-organism	substrate and concentration	ethanol yield [a] (g / g substrate)	major by-products	reference
native				
Clostridium phytofermentans	ball-milled cellulose at 6 g / l	NR	acetate	Warnick *et al.* 2002
Clostridium thermocellum	Avicel at 2.5 g / l	0.22	acetate	Lynd *et al.* 1989
Clostridium cellulolyticum	Avicel at 6.7 g / l	0.165	acetate	Desvaux *et al.* 2000
modified				
Klebsiella oxytoca SZ21	acid-swollen cellulose at 6.85 g / l	0.39	NR	Zhou and Ingram 2001
Saccharomyces cerevisiae	Avicel at 10 g / l	0.45	NR	Fujita *et al.* 2004

[a] yields as determined in batch culture

NR: not reported

Escherichia coli is also a very efficient carbohydrate utilizer, and it can metabolize both hexose and pentose sugars. However, it must be modified in order to produce ethanol, as this is not a major metabolic product in native strains. *E. coli* strain KO11 has been reported to produce 96% of the theoretical ethanol yield and possess a moderate specific ethanol productivity (Hahn-Hägerdal *et al.* 2006).

2.5.3 Organisms for Consolidated Bioprocessing

Consolidated bioprocessing requires an organism that produces ethanol from carbohydrates to be able to produce the cellulase enzymes required to hydrolyze plant polymers. Lynd *et al.* (2005) suggest

two strategies for developing these organisms: the native strategy, and the recombinant one. The native strategy makes use of existing cellulose-degraders by improving their capacity to produce ethanol. The recombinant strategy would seek to give high-volume ethanol producers the ability to produce saccharolytic enzymes.

The vast majority of organisms that have been identified as potential candidates for CBP via the native strategy are anaerobic bacterial species. These include *C. thermocellum*, *C. phytofermentans*, *C. cellulolyticum*, *Thermoanaerobacterium thermosaccharolyticum* and *Thermoanaerobacterium saccharolyticum* (Leschine 2007, Lynd *et al.* 2005), some of which are shown in Table 2–5. These organisms generally produce ethanol, acetic acid, and a mix of other organic acids in addition to carbon dioxide and hydrogen gas. They meet the crucial requirements of being both ethanologenic and cellulolytic, but to date none have been proven adequate for CBP. Ethanol yields and tolerances are too low while cellulose degradation rates are also slow. Demain *et al.* (2005) note that maximumethanol tolerances of only 5% have been reported for strains of *C. thermocellum*, and Susan Leschine (2007) in her patent application for biofuels production technology notes that *Clostridium phytofermentans* can be adapted to tolerate ethanol concentrations above 7%. By the standard of industrial workhorses *S. cerevisiae* and *Z. mobilis*, this is very low. There is potential to adapt or engineer these native strains to improve their ethanol yields, ethanol tolerances and cellulose degradation rates. For example, *C. cellulolyticum* has been modified with *Z. mobilis* genes to increase cellulose consumption and result in 93% more ethanol and 75% more hydrogen yield than native *C. cellulolyticum* strains (Guedon *et al.* 2002).

The recombinant strategy involves inserting cellulase genes into ethanologenic organisms to allow them to breakdown cellulose and ferment it with high yields to high final concentrations. According to Lynd (2005) high-yield strains of *S. cerevisiae*, *Z. mobilis*, *E. coli* and *K. oxytoca* have all been modified with some degree of success to manufacture cellulase, and available data are shown in Table

2–5. Ito *et al.* (2004) have engineered a strain of the yeast that expresses the genes from *T. reesei* required for cellulose binding, but cellulose hydrolysis has not been demonstrated. Similarly, *K. oxytoca* modified for cellulase production could not grow without the addition of cellulase enzyme (Zhou and Ingram 2001). Fujita *et al.* (2004) realized some success in expressing cellulolytic enzymes from the fungus *T. reesei* in the bacterium *Z. mobilis*, and have demonstrated ethanol yields by direct fermentation of cellulose using the engineered strain.

2.5.4 *Clostridium phytofermentans* ISDg^T

Relatively little is written about this bacterium with potential for CBP. In 2002 Warnick *et al.* published a paper proposing the novel species *Clostridium phytofermentans* ISDg^T. The name – *phyto* meaning plant and *fermentans* meaning fermentor – refers to the bacterium's ability to break down cellulosic plant materials and metabolise the resulting sugars. The bacterium was isolated from forest soil near Massachusetts' Quabbin Reservoir. The native environment of this species is in soils where decaying plant material has created an anaerobic environment rich in cellulose.

C. phytofermentans was isolated from forest soil in Massachusetts. It is able to metabolise a variety of mono- and polysaccharides including: arabinose, cellobiose, fructose, galactose, glucose, lactose, maltose, mannose, pectin, ribose, starch, xylan and xylose. Siezen and Wilson (2008) identified *C. phytofermentans* as a microbe of special relevance to biofuel production in a review of recently sequenced genomes, citing its ability to "anaerobically ferment a vast array of plant sugars, starches and cellulose to produce economically substantial amounts of ethanol and acetate." Siezen and Wilson further observed, "the genome of *Clostridium phytofermentans* contains over 100 ABC-type transport systems and 52 of these appear to be dedicated to transporting carbohydrates into cells. Some of these are monosaccharide transporters but others are involved in the transport of disaccharides (e.g cellobiose), tri- and tetrasaccharides." According to Leschine (2007) *C.*

phytofermentans is a cellulolytic bacterium that does not produce cellulosome complexes, but rather produces cellulolytic enzymes that are released into the surrounding environment to bind to cellulose. This makes it substantially different from other native CBP organisms like *C. thermocellum* that degrade cellulose via cellulosomes. Cellulosomes are external but remain attached to the bacterial cell walls during cellulose hydrolysis.

Warnick *et al.* (2002) named ethanol, acetate, lactate, formate, carbon dioxide and hydrogen as the metabolic by-products of *C. phytofermentans*. However, in a more recent study by Harvey *et al.* (2008) hydrogen was not detected by the native strain. According to Warnick and Leschine (2007) there may be other fermentation products expected such as n-propanol, isopropanol and n-butanol. Warnick *et al.* (2002) characterized the bacterium as a mesophile growing optimally at 37°C, with no growth observed above 45°C or below 5°C. They also observed that maximum growth rate occurred at pH 8 and the maximum population density occurred at pH 8.5: very poor growth was observed above pH 9 and below pH 6.

Both Warnick *et al.* (2002) and Harvey *et al.* (2008) recognized that *C. phytofermentans* had specific requirements for nitrogen uptake. Warnick *et al.* (2002) observed that the bacterium preferred nitrogen in the form of amino acids or peptides and that nitrogen added in the form of urea or ammonium chloride did not support growth. Harvey *et al.* (2008) discovered that the bacterium is able to fix atmospheric nitrogen by culturing it in a fermentor with nitrogen sparging as the sole nitrogen source. They concluded that *C. phytofermentans* accomplished nitrogen fixation through the production of the nitrogenase enzyme – an ability shared by only about 100 species of bacteria (Harvey *et al.* 2008). In their study, they isolated the strain *C. phytofermentans* CPNIT1, selected for high nitrogenase activity, and subsequent increases in hydrogen yield. Ren *et al.* (2007) made a comparative study of six *clostridium* species including *C. phytofermentans.* In this study, bacteria were cultured in a modified CM3 medium (Weimer and Zeikus 1977) and ethanol and hydrogen were

both detected from *C. phytofermentans* when cultured on cellobiose, micro-crystalline cellulose and amorphous cellulose.

The bioenergy potential of a bacterium that can produce both ethanol and hydrogen from cellulose is obvious. Thomas Warnick and Susan Leschine – both authors of the original publication on *C. phytofermentans* – have jointly submitted a patent application for a CBP technology using *C. phytofermentans* for bioethanol production (Leschine and Warnick 2007). Susan Leschine is the founder and chief scientist of Qteros (formerly SunEthanol), which plans to employ the bacterium – which they refer to as "Q-microbe" – for the production of lignocellulosic ethanol (Qteros 2009).

2.6 Biological Hydrogen

The subject of this study, *Clostridium phytofermentans*, produces hydrogen gas as a metabolic by-product during fermentation. Hydrogen is a valuable biofuel and with applications in combustion engines and fuels cells. Lignocellulosic ethanol technologies are closely related to biological hydrogen technologies because they share feedstock and some micro-organisms. Biological hydrogen can be produced from carbohydrates, such as those present in the polymeric cellulose and hemicellulose in plant biomass. Many clostridium species that ferment carbohydrates to ethanol also produce hydrogen gas as a by-product of the fermentation. Hydrogen gas is an excellent energy currency because its end use does not produce any harmful emissions. However, its transportation and storage present some problems. While hydrogen produced by conventional methods is a good energy currency, it does not eliminate dependence on fossil or nuclear energy. Biologically produced hydrogen offers an opportunity to produce hydrogen gas from renewable resources such as plant biomass. Biological systems for hydrogen production include biophotolysis of water by algae and cyanobacteria, photodecomposition of organics by photosynthetic bacteria and bacterial fermentation. This section will focus on hydrogen production via bacterial fermentaion, as it is closely related to

biological ethanol production. The maximum theoretical yield of biological hydrogen from carbohydrates is 4 mol H_2 / mol hexose equivalent.

2.6.1 Hydrogen as an Energy Currency

Hydrogen gas can be combusted in engines to produce mechanical and thermal energy, or can be employed in fuel cells to create electricity. It is considered a clean fuel, because its end use in engines or fuel cells does not directly produce any particulate or greenhouse gas emissions. Hydrogen gas has an energy density of 122 MJ / kg (Carere *et al.* 2008), which is significantly higher than gasoline at 44.4 MJ / kg.

Despite these advantages, a number of factors are keeping hydrogen from finding widespread use as a transportation fuel. Hydrogen does not fit easily into the existing infrastructure: modified engines, fuel storage facilities, fuel distribution lines and fuel dispensing are all required for hydrogen gas to be used in transportation. Vehicle mileage also remains a key obstacle: on-board hydrogen storage is as yet unable to deliver the 500 km range that drivers demand (Chalk and Miller 2006).

2.6.2 Feedstock for Hydrogen Production

A major criticism of hydrogen as an energy currency is centred on the fact that large amounts of energy are required to generate hydrogen gas via electrolysis, coal gasification or steam reformation of natural gas (Carere *et al.* 2008) and this energy typically comes from non-renewable resources. Biologically derived hydrogen offers an advantage over thermally or chemically generated hydrogen by requiring far less energy in production (Antoni *et al.* 2007). As in the case for ethanol production, hydrogen can be biologically derived from a wide variety of renewable resources and waste streams.

Lignocellulosic biomass has been identified as a substrate for hydrogen production (Harvey *et al.* 2008, Liu *et al.* 2008). As in ethanol production, the polysaccharide plant polymers present in all lignocellulosic biomass could be converted to hydrogen directly by cellulolytic micro-organisms, or a

sugar-rich hydrolyzate could be converted to hydrogen after a chemical or enzymatic hydrolysis process.

2.6.3 Organisms for Biological Hydrogen Production

Two of three major biological hydrogen production processes are carried out with photosynthetic micro-organisms as bio-catalysts. The process used by algae and cyanobacteria to produce carbon-containing plant biomass can be adapted to produce hydrogen gas via the photolysis of water by hydrogenase enzymes. By creating an anaerobic environment and conditions of temporary darkness, these micro-organisms will synthesize and activate hydrogenase enzymes, producing up to 1 mol H_2 / mol H_2O. Alternatively, photosynthetic bacteria such as *Rhodobater sphaeroides* and *R. capsulatas* can use organic wastes such as sewage sludge, the OFMSW and some industrial effluents to produce hydrogen gas (Das and Veziroglu 2001). Fermentative bacteria are the biocatalysts for the third major method of biological hydrogen production. Many species of *enterobacter* and *clostridium* as well as *E. coli* produce high hydrogen yields while they ferment carbohydrates to organics compounds such as acetate, butyrate, formate and ethanol.

Biologically derived hydrogen from either pure cellulose or lignocellulosic materials has been investigated using both microbial communities and pure cultures. A common method of increasing a microbial community's hydrogen production is to heat-shock samples of soil, wastewater sludge or cow dung before inoculating media (Lin and Hung 2008, Chen *et al.* 2005, Logan *et al.* 2002, Lay 2001, Chen *et al.* 2001). Hydrogen yields from cellulose by these communities is generally low. Logan *et al.* (2002) reported a yield of 0.35 mol H_2 / mol hexose equivalent for soil-inoculated cellulose media.

Hydrogen yields for pure bacterial cultures grown on pure cellulose have been reported for *clostridium* species *C. cellulolyticum*, *C. cellobioparum*, *C. celerecrescens*, *C. populeti*, *C.*

phytofermentans (Ren *et al.* 2007, Desvaux *et al.* 2000) and *Ruminoccocus albus* (Miller and Wolin 1995). Ren *et al.* (2007) reported a hydrogen yield of 1.00 mol H_2 / mol hexose for *C. cellulolyticum* cultured on MN301 cellulose in modified CM3 medium. Desvaux *et al.* (2000) reported a maximum molar hydrogen yield of 1.66 mol H_2 / mol hexose for *C. cellulolyticum* cultured on cellulose, but found that the hydrogen yield was inversely proportional and highly dependent on the initial cellulose concentration. Harvey *et al.* (2008) reported near-theoretical hydrogen yields by *C. phytofermentans* cultured on glucose when nitrogen gas was provided as the sole nitrogen source. They attributed the high hydrogen production to reactions catalyzed by the nitrogenase enzyme produced by *C. phytofermentans*.

2.6.4 Basis for Comparison of Process Performance

The maximum theoretical hydrogen yield is 4 mol H_2 / mol hexose (Ren *et al.* 2007). However, according to Ren *et al.* (2007), this yield cannot actually be achieved in practice. Energy is used to produce bacterial biomass, as well as to produce a variety of organic acids as metabolic by-products. In mixed microbial communities hydrogen yields can be further compromised as hydrogen may be taken up by other community members (Hallenbeck and Benneman 2002 in Ren *et al.* 2007). When working with glucose as the substrate under ideal conditions, some bacteria are capable of producing near-theoretical hydrogen yields (Harvey *et al.* 2008), but hydrogen yields from cellulose-fed cultures rarely exceed 2 mol H_2 / mol hexose equivalent.

Chapter 3
Experimental Plan

3.1 Methodology

This study was carried out in two parts. First, the growth and metabolism of *C. phytofermentans* was investigated by culturing the bacteria on a variety of substrates, including glucose, cellobiose, xylose and a mixed media containing glucose, xylose, arabinose, mannose and galactose. Glucose is the fundamental structural unit of cellulose, and the most abundant fermentable sugar in most lignocellulosic biomass. Cellobiose is a repeating sub-unit of cellulose, and is composed of two β-1,4-linked glucose molecules. When cellulose is enzymatically degraded, endoglucanases cleave cellobiose units from the ends of cellulose chains, and these are either utilized directly, or are further broken down by β-glucosidase enzymes. Xylose is the predominant fermentable sugar present in most hemicelluloses, and xylose fermentation has been deemed necessary for any economical lignocellulosic ethanol production process. The mixed carbohydrate media contained hexose and pentose sugars in proportions similar to those found in corn stover. This first set of experiments was designed to provide scarcely available information on the ethanol yields, substrate preferences and growth characteristics of *C. phytofermentans*.

In the second set of experiments, *C. phytofermentans* was cultured on pure forms of cellulose. In native and most pretreated lignocellulosic biomass, cellulose is present as long, insoluble polymeric chains. For micro-organisms to utilize cellulose, they must produce cellulase enzymes that reduce the cellulose polymer to cellobiose and glucose. *C. phytofermentans* has been reported by Warnick *et al.* (2002) and Ren *et al.* (2007) as a cellulolytic bacteria, but little quantitative data is available. This set of experiments was designed to investigate the extent of growth of *C. phytofermentans* on insoluble cellulose, where enzymatic activity is required. Sigmacell microcrystalline cellulose and ball-milled

cellulose were chosen as substrates to provide cellulose sources with a range of degradability. The effect of stirring was investigated on ball-milled cellulose-fed cultures. The effect of the addition of supplemental cellulase enzyme, to complement enzyme production by *C. phytofermentans*, was also investigated in this set of experiments.

3.2 Materials and Methods

3.2.1 Media and Culture Conditions

C. phytofermentans strain ISDg^T (ATCC 700394) was obtained as a lyophilized culture from the American Type Culture Collection. The lyophilized cell pellet was revived in a carbohydrate meat broth (ATCC Medium 1015) under anaerobic conditions in a Coy Laboratories anaerobic chamber. After the initial revival, *C. phytofermentans* was cultured on GS-2 media with cellobiose, and after 48 hours was removed from incubation and stored at 4°C. This stock was examined for active growth on cellobiose and incubated aerobically on solid ATCC 1015 media to detect contamination by facultative anaerobes. Experimental inocula were prepared by adding 0.5 ml of the refrigerated stock to fresh GS-2 media with cellobiose, and incubating at 37°C for 48 hours. All trials were inoculated with 0.5 ml of this actively growing culture.

The ATCC Medium 1015 was prepared by boiling a mixture of 500 g / l lean ground beef in water with 25 ml / l 1 N NaOH. The mixture was cooled, skimmed of fat and filtered. The filtrate was restored to original volume and supplemented with 30.0 g / l peptone, 5.0 g / l yeast extract, 4.0 g / l glucose, 1.0 g / l cellobiose, 1.0 g / l starch, 1.0 g / l maltose, 5.0 g / l K_2HPO_4, 0.001 g / l resazurin and 0.5 g / l L-cysteine HCl × H_2O. The media was boiled, cooled, and the pH was adjusted to 7 using 1 N NaOH. Media was dispensed under nitrogen over meat particles (one part meat particles to five parts media), capped and autoclaved at 121°C for 15 minutes. Media was reduced after autoclaving with concentrated sterile L-cysteine HCl × H_2O. For solid media, 15 g / l agar was added

prior to autoclaving, and media was dispensed while still hot into Petri dishes without the addition of L-cysteine HCl × H_2O.

All experimental trials were carried out in GS-2 medium (Warnick *et al.* 2002, Johnson *et al.* 1981) with various substrate additions. GS-2 contained 6.0 g / l yeast extract, 2.1 g / l urea, 2.9 g / l K_2HPO_4, 1.5 g / l KH_2PO_4, 10.0 g / l MOPS, 3.0 g / l $Na_2C_6H_5O_7 \times 2H_2O$, 2.0 g / l L-cysteine HCl × H_2O and 0.001 g / l resazurin. Concentrated stocks of soluble substrates were added to the media after autoclaving as 0.2 μm filter-sterilized solutions.

Final concentrations of substrates were selected so that all trials had an identical theoretical ethanol yield. For the growth and metabolism studies presented in Chapter 4, the following concentrations were employed: 6 g / l glucose; 6 g / l xylose; 5.7 g / l cellobiose; and mixed media contained 3.5 g / l glucose, 1.8 g / l xylose, 0.3 g / l arabinose, 0.2 g / l galactose and 0.2 g / l mannose. After autoclaving, media was reduced with concentrated L-cysteine HCl × H_2O and pH was adjusted to 7 using 1 N NaOH.

For cellulose-fed and SSO-fed cultures, substrate was added prior to autoclaving. Microcrystalline cellulose was added to bottles prior to dispensing media, and ball-milled cellulose slurry was diluted with concentrated GS-2 stock prior to dispensing to a final concentration of 5.4 g / l. Thermal screw-pretreated SSO was weighed directly into bottles prior to dispensing media and diluted with concentrated GS-2 stock to a final concentration of 20.0 g / l.

All cultures were grown in batch, in 125 ml Wheaton glass serum bottles, sealed with solid blue butyl rubber stoppers (Bellco) and with an initial media volume of 60 ml. Headspace was made anaerobic before sterilization by continuous purging with nitrogen gas. Cultures were incubated at 37°C and 90 rpm on an orbital shaker table, except for trials where a magnetic stirrer was employed

as noted. Gas and liquid samples were made concurrently at 24-hour intervals. Each set of substrate and culture conditions were run in triplicate.

3.2.2 Enzymes

Celluclast 1.5 L, from *T. reseei*, was used in this study. The cellulase activity was determined to be 48.8 FPU / ml by the filter paper assay (Ghose 1987) using DNS reagent (Miller 1959) according to published NREL procedures (Adney and Baker 2008). Enzymes were diluted in 0.05 M sodium citrate buffer, added to a tube containing 50 mg Whatman No. 1 filter paper and incubated at 50°C for 60 minutes. 3 ml DNS reagent was added, and the mixture was boiled for five minutes. Absorbance was read by spectrophotometer at 540 nm and calibrated against a four-point glucose standard. Where employed, enzymes were applied at a loading of 60 FPU / g substrate.

Enzymatic saccharification of SSO was accomplished according to NREL procedures (Dowe and McMillan 2008). Thermal screw-pretreated SSO was added to 0.05 M sodium citrate buffer to a final concentration of 20 g / l in a 500 ml Erlenmeyer flask with a 200 ml working volume. The entire contents were autoclaved, the pH was adjusted to 5.0 with sterile 1 N HCl, and enzymes were added at a loading of 60 FPU / g SSO. Flasks were incubated at 50°C on an orbital shaker at 130 rpm.

3.2.3 Microscopic Examination

Cultures samples were examined by confocal laser scanning microscopy (CLSM) and light microscopy. Acridine Orange was used to stain culture samples prior to examination by light microscopy. For CLSM, an RNA-binding stain and a carbohydrate-binding stain were added to 10 ml cultures at a concentration 0.025 mg/l. After 10 minutes, supernatant was removed, and solids were carefully transferred to a tube. Solids were re-suspended in 1.5% agarose, and cooled on ice to solidify. Thin slices were cut and slide-mounted for microscopic examination.

3.2.4 Ball-Milled Cellulose

Cellulose (Whatman No. 1 Filter Paper) was wet ball-milled at a concentration of 50 g / l for 48 hours (Leschine and Canale-Parola 1983) in a 4-litre stainless steel ball mill with ½ inch stainless steel ball bearings as the grinding media. The entire slurry was recovered from the ball mill by washing the vessel and grinding media with water. The total solids concentration of the resultant slurry was determined gravimetrically by drying 10 ml aliquots of well-mixed slurry at 105°C to a constant weight, and the slurry was added directly to the media prior to dispensing to achieve the desired substrate concentration

3.3 Product Formation

3.3.1 Cell Growth

Cell growth was measured as protein using a modification of the Bradford dye method (Bradford 1976) according to Pavlostathis *et al.* (1988) and calibrated using a five-point curve with bovine serum albumen as the standard (BioRad Kit #500-0202). A syringe was used to anaerobically and aseptically withdraw 1 ml samples from well-mixed cultures. Samples were centrifuged at 16,000 × g for 15 minutes. The supernatant was reserved for carbohydrate, alcohol and organic acid analyses, and the cell pellet was stored frozen prior to performing the protein assay. Cell pellets were thawed, re-suspended in 1 ml 1 N NaOH, heated in a boiling water bath for 10 minutes, cooled and centrifuged again. 60 µl of the supernatant was added to 3 ml Bradford dye reagent and absorbance was read in a 5 ml square cuvette at 595 nm.

3.3.2 Ethanol and Organic Acids

Liquid phase fermentation products including ethanol, acetic acid, formic acid and lactic acid were measured according to NREL procedures (Sluiter *et al.* 2008). Supernatant from 1 ml samples centrifuged at 16,000 × g for 15 minutes was filtered through 0.2 µm PTFE membrane syringe filters

into 1.8 ml autosampler vials and stored frozen prior to analysis. Thawed samples were analysed by HPLC (PE Series 200) with an Aminex HPX-87H column at 60°C (Eppendorf CH-30), refractive index detector (HP 1047A) at 50°C and a 0.005 M H_2SO_4 mobile phase at 0.6 ml / minute. An injection volume of 40 µl and refractive index detector sensitivity of 1 / 64 × 10^{-5} RIU / FS was selected to provide good peak resolution and low detection limits. Chromatogram peak areas were calibrated using a five-point curve, and standards were analyzed at the beginning and end of each sample run to ensure consistency.

3.3.3 Sugars

Quantification of residual sugars in liquid samples was accomplished by HPLC (PE Series 200) with either an Aminex HPX-87H column as above, or with an Aminex HPX-87P column. The 87P column was operated at 85°C (Eppendorf CH-30) with a refractive index detector (HP 1047A) at 50°C and water as the mobile phase at 0.6 ml / minute. An injection volume of 10 µl and refractive index detector sensitivity of 1 / 64 × 10^{-5} RIU / FS was selected to provide good peak resolution and low detection limits. The HPLC was calibrated using a five-point standard curve, and standards were run before and after each sample run.

3.3.4 Biogas

Biogas volume was measured using an intermittent gas-release method (Ren *et al.* 2007, Logan *et al.* 2002) with water-lubricated glass syringes (Owen *et al.* 1979). Sterile water-lubricated syringes and needles were flushed with sterile L-cysteine HCl × H_2O to prevent oxygen contamination. The serum bottle, needle and syringe were held horizontally as the stopper was pierced. Gas volumes were read at atmospheric pressure and room temperature.

Hydrogen gas in headspace samples was measured by gas chromatography (Perkin Elmer XL Series) using 100 µl injections onto a Supelco Carboxen PLOT 1010 column (30 m × 0.53 mm), and

a thermal conductivity detector (Kramer and Bagley 2005) with helium as the carrier gas at 10 ml / minute and reference gas at 15 ml / minute. Manual injector and detector temperatures were both 230°C. Oven temperature was programmed at 60°C for 1.7 minutes, and ramped to 200°C at 24°C / minute to purge water impurities from the column and stabilize the baseline for subsequent injections. The cumulative volume of hydrogen gas produced was calculated by mass balance as proposed by Logan *et al.* (2002)

$$V_{H_i} = V_{H_{i-1}} + C_{H_i}\left(V_{G_i} - V_{G_{i-1}}\right) + V_{HS}\left(C_{H_i} - C_{H_{i-1}}\right) \qquad \text{Equation 3–1}$$

where V_H is the cumulative volume of hydrogen gas produced, V_G is the cumulative total biogas volume, V_{HS} is the reactor headspace volume and C_H is the fraction of hydrogen in the headspace as determined by gas chromatography.

Chapter 4
Growth and Metabolism of *Clostridium phytofermentans*

4.1 Chapter Abstract

C. phytofermentans was cultured in triplicate batch on GS-2 medium at pH 7 on glucose, xylose, cellobiose and a mixed substrate containing glucose, xylose, arabinose, mannose and galactose. Daily monitoring and sampling was carried out to measure ethanol, organic acids, hydrogen production and cell growth. Maximum growth and product evolution was observed when *C. phytofermentans* was cultured on cellobiose, with a maximum protein density of 251 mg / l, an ethanol yield of 73% of the theoretical maximum and 0.29 mol H_2 / mol hexose. Cultures grown on glucose and the mixed medium performed similarly to each other, with the mixed medium cultures slightly outperforming those on glucose. Marginal growth was observed on xylose. Apparent diauxic behaviour was observed for the mixed media containing both glucose and xylose. The experimental results generally concurred with available published data.

4.2 Description

As lignocellulosic ethanol technology matures, there is an increasing research focus on micro-organisms that are able to ferment both hexose and pentose sugars to ethanol. While cellulose is the primary component of most lignocellulosic plant biomass, hemicellulose is often present in high proportions as well. In many grass species hemicellulose is more abundant than cellulose. Therefore, there are significant gains in efficiency made possible by the fermentation of both the glucose the and xylose fractions of lignocellulosic biomass. The commonly employed *S. cerevisiae* and *Z. mobilis* are strong glucose fermenters, but native strains lack the ability to metabolize pentoses. Research has clearly established that *C. phytofermentans* is capable of utilizing a wide variety of the sugars present as polymers in plant biomass (Leschine and Warnick 2007, Warnick *et al.* 2002). However, there is

little information about the extent or efficiency with which *C. phytofermentans* metabolizes these substrates, and few quantitative data are available to support these observations.

In this investigation *C. phytofermentans* was cultured on GS-2 medium containing the carbohydrates cellobiose, glucose, xylose, mannose, arabinose and galactose. Four substrates were examined in triplicate, with substrate concentrations selected to produce a maximum theoretical ethanol concentration of 3.1 g / l across all trials as follows: Trial 1, 5.7 g / l cellobiose; Trial 2, 6 g / l glucose; Trial 3, 6 g / l xylose; and Trial 4, 3.5 g / l glucose, 1.8 g / l xylose, 0.3 g / l arabinose, 0.2 g / l galactose and 0.2 g / l mannose. Measurements were made as described in Chapter 3 to determine cell growth, substrate utilization, ethanol and organic acid production, and hydrogen evolution on each substrate.

4.3 Results and Discussion

C. phytofermentans cultured in batch on GS-2 medium exhibited similar growth patterns on all examined substrates. As shown in Figure 4–1, all cultures exhibited a one-day lag phase and grew rapidly to two days, after which population density declined. The highest cell density of 251 mg / l was achieved when *C. phytofermentans* was cultured on cellobiose. These cultures became slightly turbid within one day with apparent uniform dispersal of organisms throughout the media. Turbidity increased to two days, with non-uniform dispersal of the bacteria. While some cellulolytic bacteria such as *C. thermocellum* produce a yellow-orange colour change by producing carbohydrate binding molecules, no colour changes were observed other than the increasing turbidity.

Media blanks, with GS-2 containing no carbohydrates other than the trace amounts present in the yeast extract, were run in triplicate under identical experimental conditions. No gas production or by-product formation was detected.

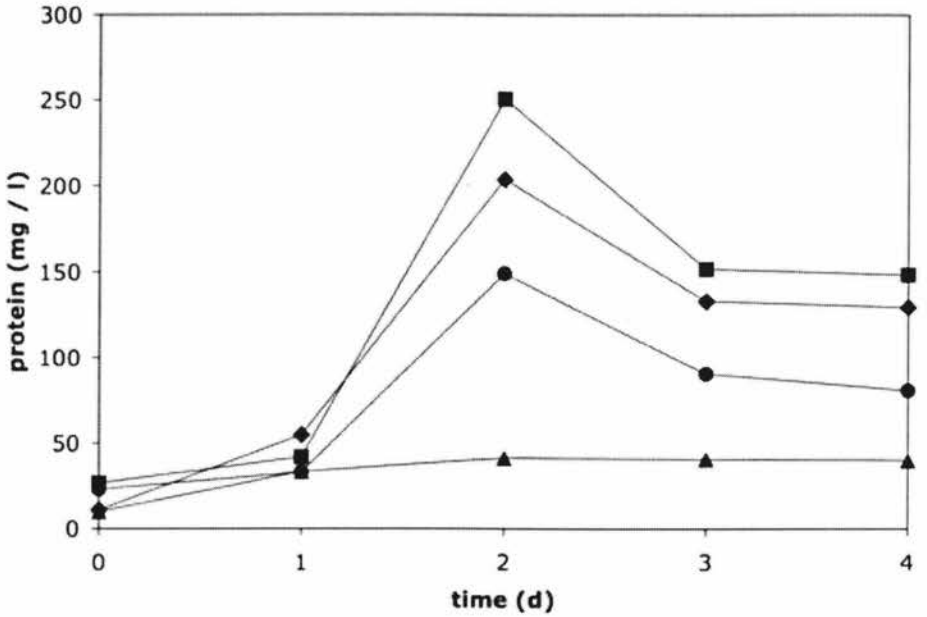

Figure 4–1 Cell growth of *C. phytofermentans* cultured on cellobiose (■), glucose (●), xylose (▲) and mixed soluble carbohydrate media (◆)

Macroscopic examination showed that the bacteria were interconnected, forming a mass as opposed to being uniformly dispersed throughout the media. The bacteria heavily colonized any insoluble cellulose or meat particles present in the media that were carried over from inoculant. In one instance, attachment of the mass of cells to the bottom surface of the serum bottle was observed, but this is likely due to the presence of contaminants on the glass surface, as *C. phytofermentans* does not generally colonize non-nutritive surfaces (Alonso 2007). Microscopic examination of liquid cultures showed cells that were slightly curved rods, approximately 10 μm long as previously described (Warnick *et al.* 2002). Terminal spores were evident in many bacteria. The presence of long chains of bacteria was also observed in several samples. These observations were found to be in accordance with available observations from the literature (Warnick *et al.* 2002, Ren *et al.* 2007).

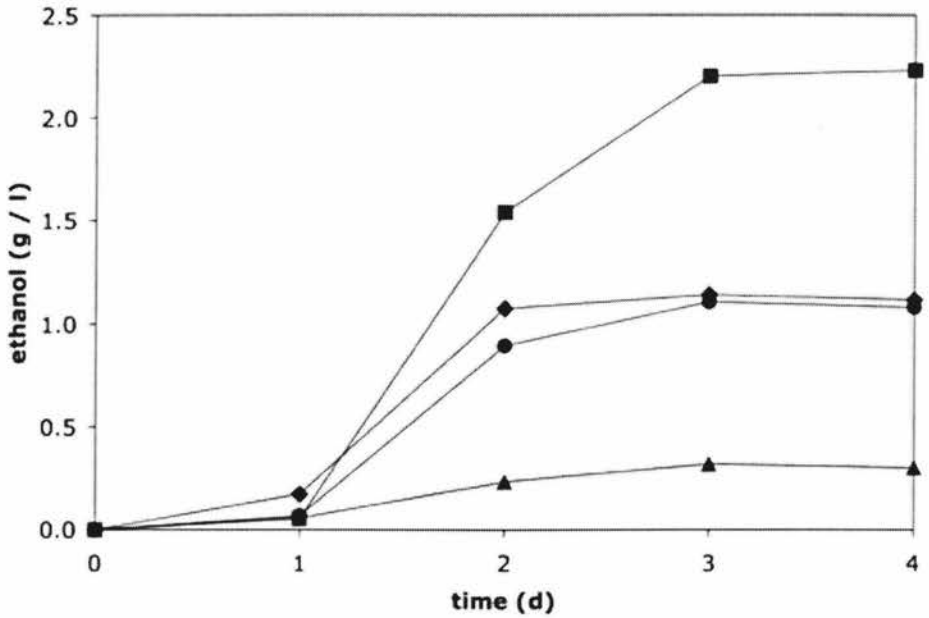

Figure 4–2 Ethanol production by *C. phytofermentans* cultured on cellobiose (■), glucose (●), xylose (▲) and mixed soluble carbohydrate media (◆)

Table 4–1 presents a summary of the experimental results, including standard deviation statistics which were excluded from the figures for clarity. Growth patterns were similar on all substrates and are presented in Figure 4–1. All cultures showed an approximate one-day lag phase, followed by high-rate growth to maximum cell density at two days, and declining density thereafter. Figure 4–2 shows that ethanol production ceased at approximately three days, indicating that the death phase occurred from day two to day three. Cultures on cellobiose achieved a maximum protein concentration of 251 mg / l, followed by protein concentrations of 204, 149, and 41 mg / l for cultures on mixed carbohydrates, glucose and xylose. Figure 4–2 shows the evolution of ethanol in the fermentation broth for each of the substrates studied. Maximum ethanol production was achieved when cultured on cellobiose with a final ethanol concentration of 2.2 g / l which equates to 73% of the theoretical maximum ethanol production. It

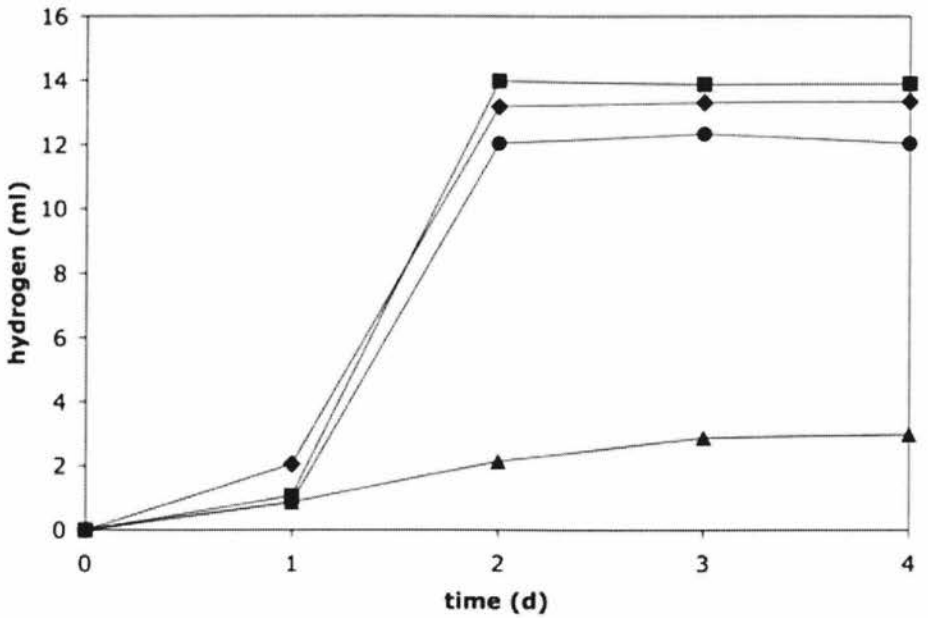

Figure 4–3 Hydrogen production by *C. phytofermentans* cultured on cellobiose (■), glucose (●), xylose (▲) and mixed soluble carbohydrate media (◆)

has been suggested by many researchers that any organism under consideration for ethanol production should produce at least 90% of the theoretical yield. All four tested substrates exhibited similar ethanol production patterns, with continuously increasing ethanol concentration up to a maximum at three days. Comparison of Figure 4–1 and Figure 4–2 shows the highest ethanol production rate occurred during high growth from one to two days, and that some ethanol was still being produced during the death phase from day two to day three.

Figure 4–3 tracks the evolution of hydrogen gas over the four-day trials. As with ethanol production, the highest rates of hydrogen evolution were observed during the highest rates of growth. Cellobiose cultures produced the highest hydrogen yields while xylose cultures produced very low yields. In this case, the cellobiose culture produced 13.9 ml hydrogen which is equivalent to 0.29 mol H_2 / mol hexose or 7% of the maximum theoretical yield. Hydrogen production for all four substrates

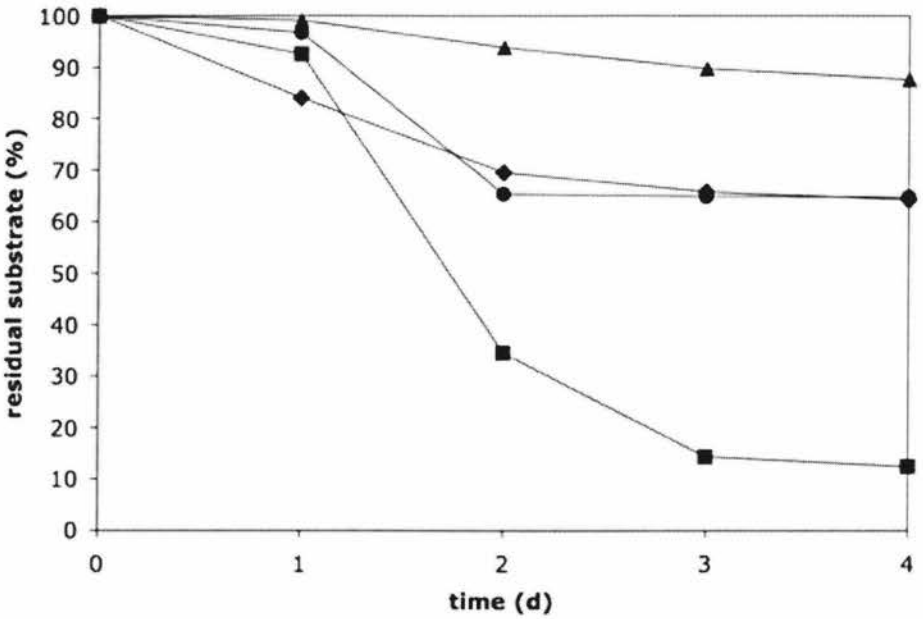

Figure 4–4 Substrate consumption by *C. phytofermentans* cultured on cellobiose (■), glucose (●), xylose (▲) and mixed soluble carbohydrate media (◆)

showed a similar pattern of low production up to day one and then high production up to a maximum at day two. It is evident that for cultures grown on cellobiose, glucose and mixed carbohydrate media, there was no significant hydrogen production after day two, indicating that hydrogen was not produced during the death phase.

It is very interesting to note that hydrogen production on cellobiose, glucose and mixed carbohydrate media differ very little. If cellobiose and glucose cultures are compared, it is seen that while cell density was 68% higher and ethanol production was 106% higher, hydrogen production was only 13% higher. The close grouping of the cellobiose, glucose and mixed carbohydrate hydrogen production curves in Figure 4–3 suggests that hydrogen production was not limited by

growth, but by another factor common to all of the cultures. Possible limiters include by-product toxicity, substrate inhibition or nutrient availability, but the direct cause is not clear at present.

Figure 4–4 confirms the growth patterns established in Figure 4–1 and Figure 4–2. The highest rate of substrate consumption occurred between day one and day two, and substrate degradation had essentially ceased after three days. *C. phytofermentans* made very effective use of cellobiose, consuming 87% of the available substrate in 3 days. In contrast, only 36%, 35% and 12% of available substrate were consumed in the mixed carbohydrate, glucose and xylose cultures. The reason for such limited glucose utilization is unclear.

C. phytofermentans achieved the highest protein density and produced the most ethanol and hydrogen when cultured on cellobiose. This indicates a strong preference for cellobiose over glucose. The ability to directly utilize cellobiose is an advantage in most biofuels production processes. Enzymatic hydrolysis of cellulose by cellulase results in the production of cellodextrins, cellobiose and glucose. Endo-β-1,4-glucanases act on amorphous cellulose regions (Carere *et al.* 2008) creating cellodextrins and new sites for enzymatic attack, exo-β-1,4-glucanases attack the ends of cellulose chains and cellodextrins resulting in the release of units of cellobiose (Aiudan *et al.* 2007) and β-glucosidase hydrolyzes cellobiose to monomeric units of glucose (Demain *et al.* 2005). Cellulolytic preparations, most commonly produced from cultures of the fungus *T. reseii*, have low β-glucosidase activity and are inhibited by cellobiose (Lynd *et al.* 2002). When ethanologenic organisms unable to efficeintly utilize cellobiose are employed, the addition of cellulase from *Aspergillus* species with high β-glucosidase activity is often required to achieve efficient high-yield hydrolysis to glucose (Lynd *et al.* 2002). This is why efforts have been made to engineer strains of high-ethanol yield yeasts and bacteria with direct cellobiose utilization pathways (Doran and Ingram 1993). Any

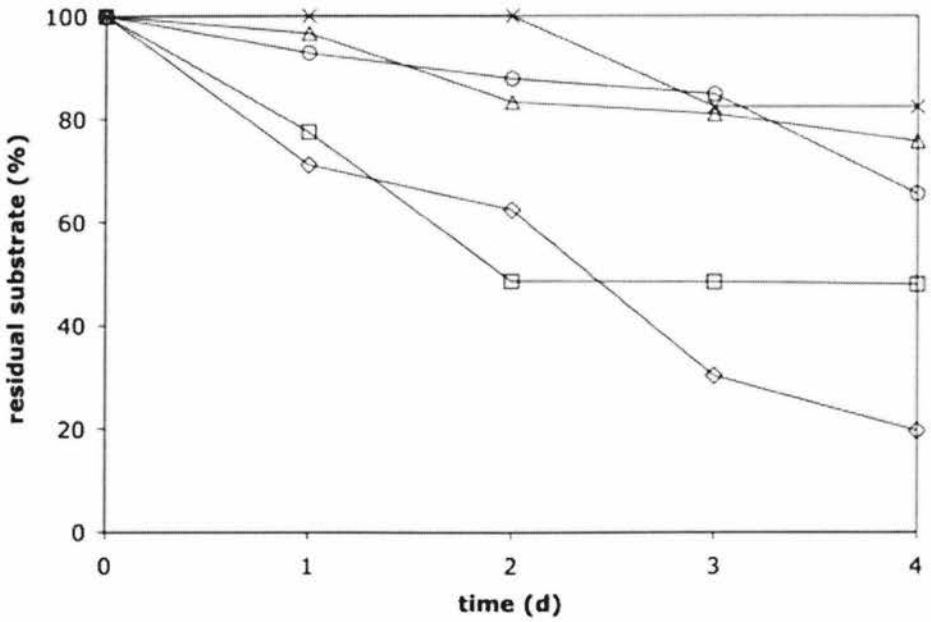

Figure 4–5 Substrate consumption by *C. phytofermentans* cultured on mixed soluble carbohydrate media containing glucose (□), xylose (○), arabinose (△), galactose (◇) and mannose (×) in a 23:13:2:1:1 mass ratio

potential biofuels production process utilizing *C. phytofermentans* would need to take the observed cellobiose preference into account in the selection of process parameters and pretreatment technologies.

By all measures, growth of *C. phytofermentans* on xylose was very poor, although examination of Figures 4–1, 4–2, 4–3 and 4–4 reveals that growth and product evolution on xylose may continue slowly beyond day four. Substrate degradation on that long a time scale would not be commercially appropriate. However, the small amounts of ethanol, hydrogen and other by-products produced clearly show that *C. phytofermentans* does possess the ability to ferment xylose.

Biomass growth, and ethanol and hydrogen production on the mixed carbohydrate medium containing glucose, xylose, arabinose, mannose and galactose was superior to growth on glucose

Table 4–1 Summary of growth and metabolism experimental results

	cellobiose	mixed	glucose	xylose
biomass (mg BSA / l)	251 ± 17	204 ± 28	149 ± 23	41 ± 5
ethanol (g / l)	2.23 ± 0.04	1.14 ± 0.25	1.10 ± 0.08	0.32 ± 0.03
hydrogen (ml)	13.9 ± 0.4	13.3 ± 1.8	12.3 ± 0.5	3.0 ± 0.1
biogas (ml)	68.2 ± 0.5	60.5 ± 3.1	47.0 ± 2.8	20.4 ± 0.6
acetic acid (g / l)	0.80 ± 0.02	0.56 ± 0.06	0.74 ± 0.23	0.21 ± 0.01
formic acid (g / l)	0.64 ± 0.05	0.23 ± 0.05	0.39 ± 0.04	0.09 ± 0.01
% theoretical yield	72.9 ± 1.3	37.3 ± 8.2	35.9 ± 2.6	10.5 ± 1.0

All values are reported as MEAN ± STANDARD DEVIATION of triplicates

alone. This behaviour could indicate that *C. phytofermentans* is sensitive to and inhibited by glucose as the glucose concentration in the mixed carbohydrate media was only 3.5 g / l, compared to 6 g / l in the glucose medium. Alternatively, the results could indicate a preference of *C. phytofermentans* for more complex substrates as suggested by Susan Leschine (2009a).

Figure 4–5 shows the percentage remaining of each of the components of the mixed carbohydrate media as it decreases with time. Evidently glucose utilization dominated in these cultures, reinforcing that *C. phytofermentans* prefers hexose to pentose sugars. The amount of glucose consumed in the

mixed carbohydrate media trials was approximately 2 g / l, with an initial glucose concentration of 3.5 g / l. In the glucose-only cultures with initial glucose concentrations of 6 g / l, approximately the same amount of glucose was consumed. Arabinose, mannose and galactose concentrations were near the detection limits, but show a slight decreasing trend. Referring to Figure 4–5, it can be seen that glucose consumption is complete after two days. Xylose utilization proceeds very slowly up to day three and then seems to increase significantly. This may be an example of diauxic behaviour, which is the inability to effectively utilize two carbon sources simultaneously (Kovárová-Kovar and Egli 1998).

While there is relatively little published quantitative data that describes the growth and metabolic by-products of *C. phytofermentans*, there are two studies that can provide some basis for comparison and validation of the experimental results. Relevant data from these previous studies and the current study are presented in Table 4–2. All three studies employed cellobiose as a carbon source at concentrations of 5 or 6 g / l, and all measured final ethanol, acetic acid and formic acid concentrations. The measured ethanol production was highest in this study, and lowest in the study by Ren *et al.* (2007). The large difference between these two measurements could be attributed to a different growth media formulation and the lower pH used in the Ren *et al.* study. *C. phytofermentans* grows optimally in the pH 8.0 – 8.5 range (Warnick *et al.* 2002) and while growth is possible at pH 6.5, it is less optimal than pH 7. The extent of cellobiose consumption by *C. phytofermentans* in this study agrees with that reported by Ren *et al.* (2007), with values of 87 and 88% respectively. Hydrogen production was observed by Warnick *et al.* (2002) but no quantities were given. Hydrogen production in this study was significantly lower than that reported by Ren *et al.* (2007).

Table 4–2 Comparison of behaviour of *C. phytofermentans* cultured on cellobiose in 3 studies

parameter	Warnick *et al.* (2002)	Ren *et al.* (2007)	current study
media	MI	CM3	GS-2
pH	7.0	6.5	7.0
cellobiose (g / l)	6.0	5.0	5.7
cellobiose consumed (%)	NR	88	87
ethanol (mM)	38.8	13.6	49.3
acetic acid (mM)	16.1	13.2	13.3
formic acid (mM)	2.2	< 0.2	5.4
hydrogen (mM)	NR	35.6	9.6

NR: not reported

Chapter 5
Cellulose Fermentation by *Clostridium phytofermentans*

5.1 Chapter Abstract

Clostridium phytofermentans was cultured in triplicate batch on GS-2 medium at pH 7 on Sigmacell microcrystalline cellulose and ball-milled cellulose. Ball-milled cellulose cultures were also examined with magnetic stirring and with the addition of supplemental cellulase enzyme. Daily monitoring and sampling was carried out to measure ethanol, organic acids, hydrogen production and cell growth. Growth of *C. phytofermentans* on cellulose was marginal. Growth was improved on ball-milled cellulose (19% theoretical ethanol yield) relative to microcrystalline cellulose (3% theoretical ethanol yield). An extensive extra-cellular polymeric substance (EPS) coating was observed on cellulose particles, but the introduction of shear by magnetic stirring did not improve growth. Cellulase (Celluclast 1.5 L) was added at an enzyme loading of 60 FPU / g cellulose, resulting in an improved ethanol yield (55% theoretical ethanol yield) and a nearly four-fold increase in hydrogen production. *C. phytofermentans* was characterized as a weakly cellulolytic species with insufficient enzyme activity for consideration in a CBP process.

5.2 Description

It is known that *C. phytofermentans* is a cellulose-degrader and that it can manufactures cellulase enzymes. However, in their initial characterization Warnick *et al.* (2002) did not publish any quantitative data describing cellulose hydrolysis and fermentation by this species. Before considering application to a more complex lignocellulosic material, it is essential to know how well and to what extent *C. phytofermentans* degrades cellulose.

C. phytofermentans was cultured in GS-2 medium on pure two cellulose substrates: microcrystalline cellulose and ball-milled cellulose filter paper. Bacterial growth and product yields were measured as described in Chapter 3. The results of a series of four experiments with pure cellulose substrate are presented in this chapter. In the first experiment, *C. phytofermentans* was cultured on Sigmacell microcrystalline cellulose. In the second experiment, *C. phytofermentans* was cultured on ball-milled cellulose. In the third experiment, magnetic stirring was introduced in to a ball-milled cellulose culture. Finally, in the fourth experiment, supplemental cellulase enzyme (Celluclast 1.5 L) was introduced to a ball-milled cellulose culture at an enzyme loading of 60 FPU / g cellulose.

5.3 Results and Discussion

When *C. phytofermentans* was provided with microcrystalline cellulose as the sole carbon source in GS-2 medium, very limited growth was observed. The production of biogas, hydrogen, ethanol, organic acids and cell protein were all detectable but extremely low. No change in colour or turbidity of liquid cultures was observed. However, macroscopic observation of serum bottle cultures showed evidence of biofilm formation after approximately 48 hours. Media blanks, with GS-2 containing no cellulose, were run in triplicate under identical experimental conditions. No gas production or by-product formation was detected.

Prior to inoculation, the microcrystalline cellulose in GS-2 media appeared as a white powdery solid that settled on the bottom of the serum bottles. The cellulose powder could be easily re-suspended with brief agitation, and would re-disperse evenly throughout the media. Approximately 48 hours after inoculation with *C. phytofermentans*, the settled microcrystalline cellulose appeared to be bound together, and could not be re-suspended or re-dispersed throughout the media. If agitated,

the microcrystalline cellulose powder would behave as a single agglomerate mass. Microscopic examination of samples from these cultures showed evidence of coating on the cellulose crystals.

Figure 5–1 shows individual microcrystalline cellulose crystals taken from a *C. phytofermentans* culture at 72 hours. Prior to inoculation the cellulose crystals appear as smooth, regular and sometimes patterned surfaces, which are very light in colour due to the high reflectivity of the crystal surface. The bright white regions in Figure 5–1 are typical of crystals prior to inoculation with *C. phytofermentans*. No cells were observed attached to the cellulose crystals. Because this coating is produced by *C. phytofermentans*, it absorbs the stain to some degree, and it is not made up of cells, it is likely an EPS. Figure 5–2 shows images captured by CLSM of *C. phytofermentans* cultures on ball-milled cellulose that have been immobilized in agarose gel. In Figure 5–2A, the red colour highlights the cellulose fibres. The green colour in Figure 5–2B highlights both cells and the EPS, and Figure 5–2C is a composite image of A and B. Cell attachment was not observed in any samples. Attached growth is typical behaviour for most cellulolytic bacteria, therefore either *C. phytofermentans* does not exhibit this type of attachment behaviour, or cell densities were so low that attached cells were not sampled. Whether or not *C. phytofermentans* cell grow as attached cultures, there was evidence of an EPS coating as shown in Figure 5–1 on all of the samples observed under the microscope.

When microcrystalline cellulose was replaced with ball-milled cellulose in GS-2 medium, *C. phytofermentans* showed improved growth. For both substrates the maximum protein density occurred after four days as shown in Figure 5–3 , with 17% higher protein density in ball-milled cellulose cultures. Ethanol production is shown in Figure 5–4. The Sigmacell cultures produced very small quantities of ethanol with the concentration peaking after four days, and the ball-milled cellulose cultures produced almost six times as much ethanol with the maximum concentration also occurring after four days.

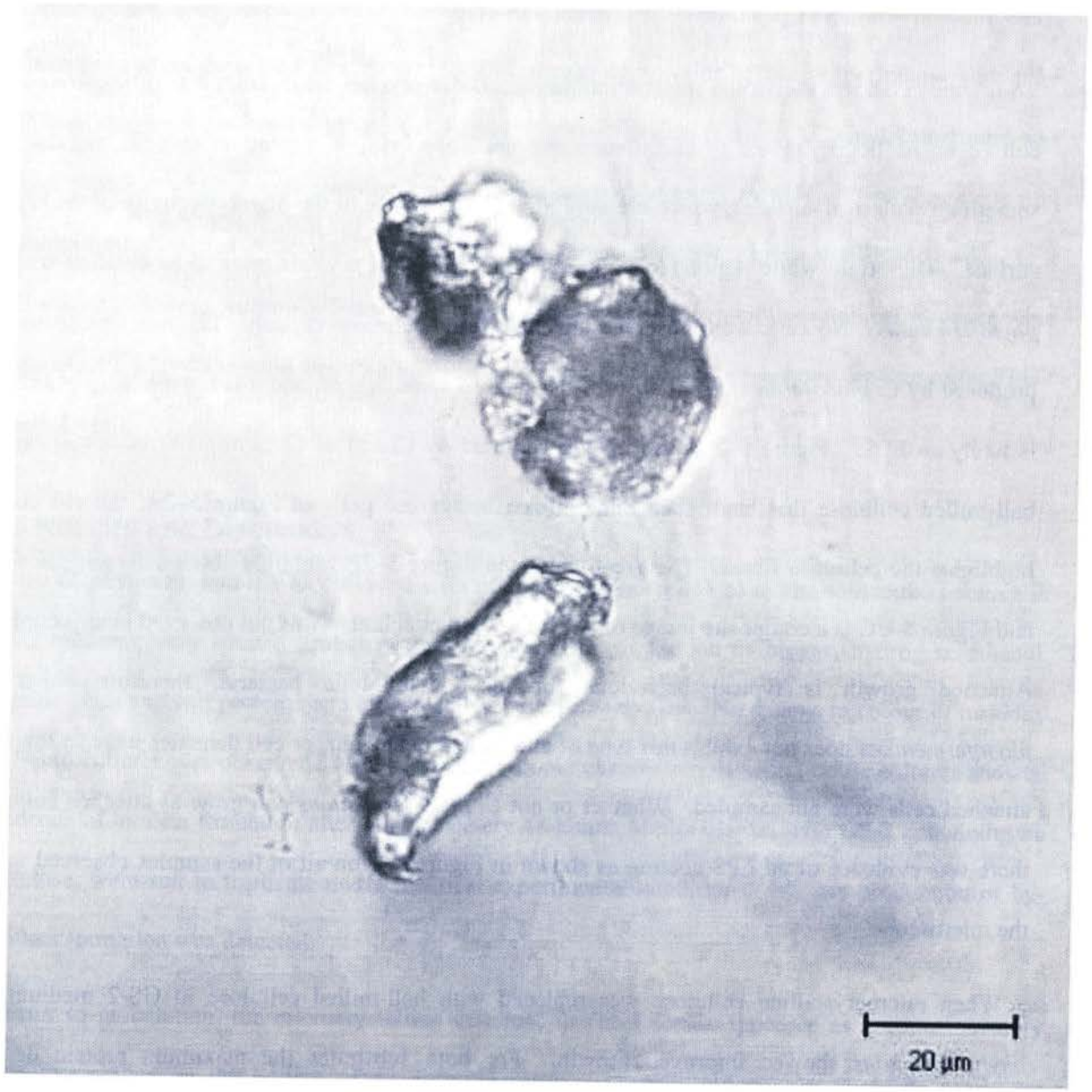

Figure 5–1 Sigmacell Type 20 cellulose crystals removed from *C. phytofermentans* broth culture at approximately 72 hours. Light regions on crystal surfaces are clear, and dark regions show deposits of extra-cellular proteins

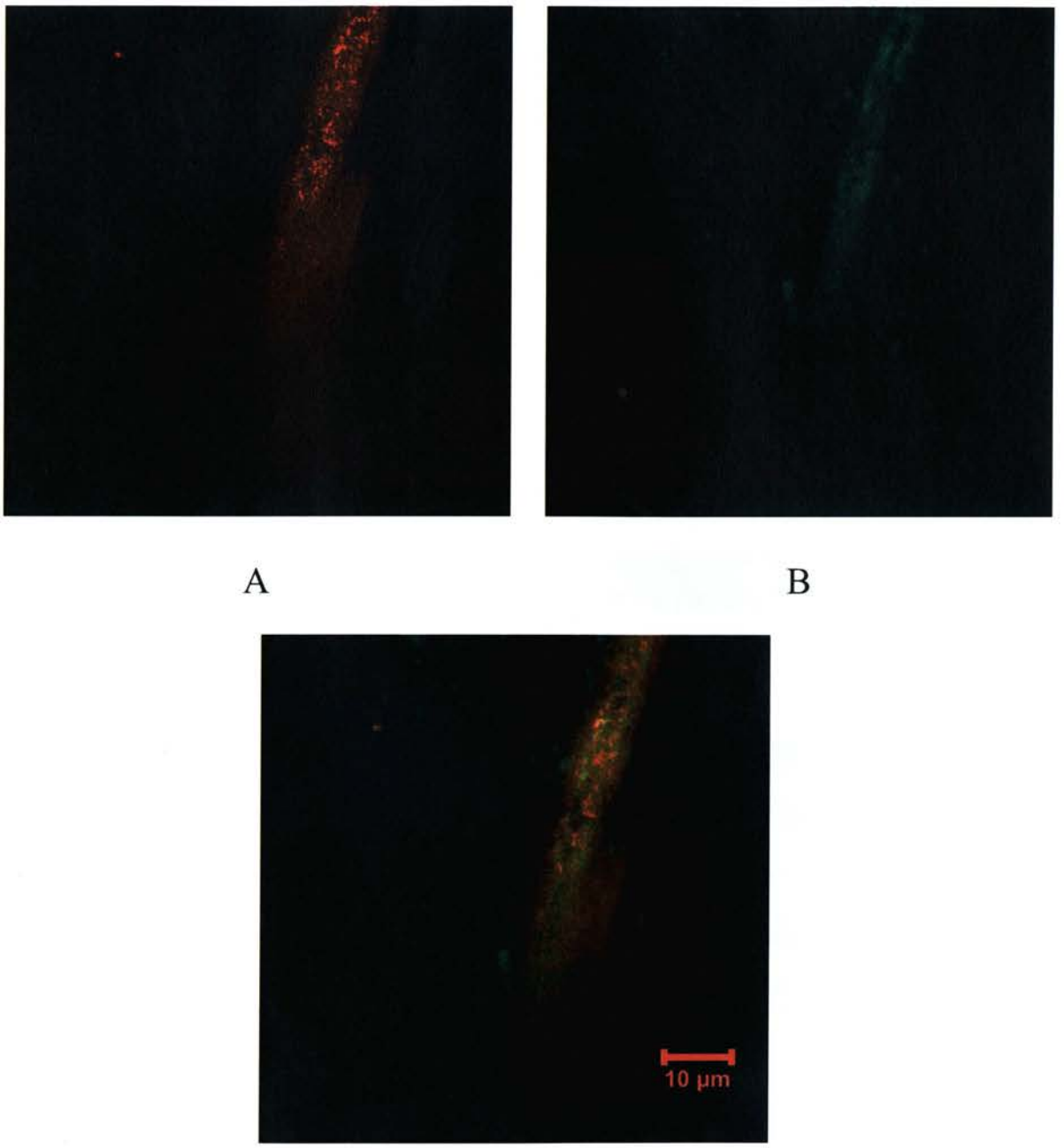

Figure 5–2 *C. phytofermentans* cultured on ball-milled cellulose. (**A**) cellulose fibre, (**B**) cells and EPS and (**C**) composite image

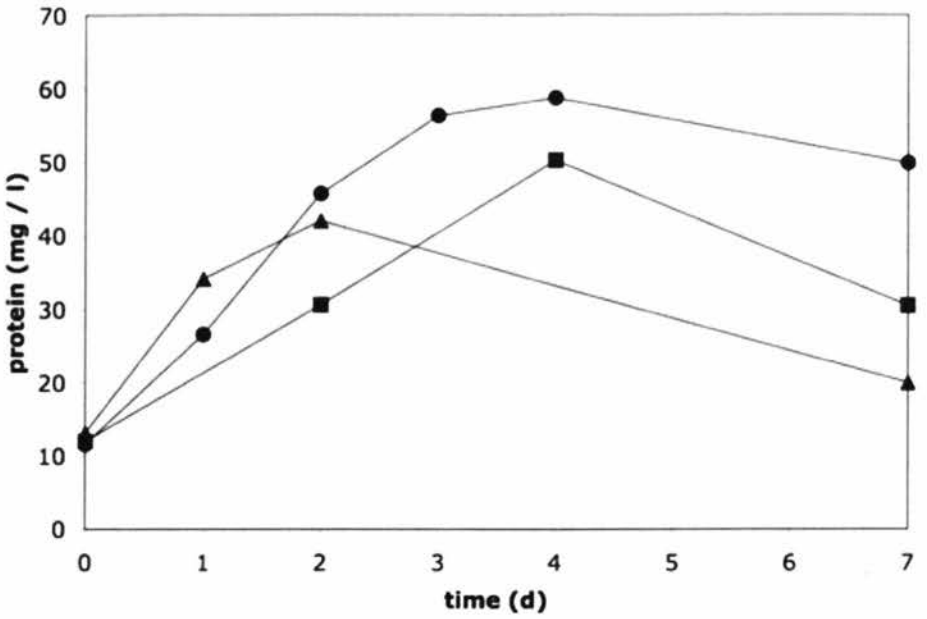

Figure 5–3 Cell growth of *C. phytofermentans* cultured on microcrystalline cellulose (■), ball-milled cellulose (●) and ball-milled cellulose with stirring (▲)

Hydrogen production followed a similar trend as shown in Figure 5–5. Hydrogen evolution ceased after four days, and the ball-milled cellulose cultures produced 50% more hydrogen than the Sigmacell cultures. All of the data shown in Figures 5 – 3 through 5 – 6 are presented along with their standard deviations in Table 5 – 1.

There was a consistent increase in all of the measured parameters when Sigmacell was replaced with ball-milled cellulose. Ball-milling results in an increase in the number of amorphous regions and the decrystallization of cellulose (Maier *et al.* 2005). A low degree of crystallinity has been shown to dramatically improve the degree of enzymatic digestibility of native celluloses (Chang and Holtzapple 2000). Amorphous cellulose regions provide ideal binding sites for endoglucanase (Lynd

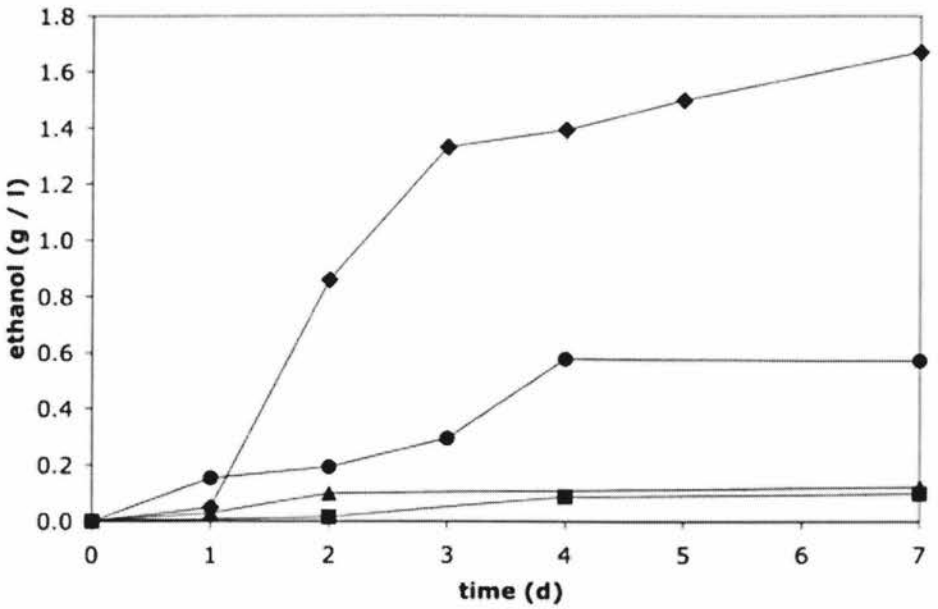

Figure 5–4 Ethanol production by *C. phytofermentans* cultured on microcrystalline cellulose (■), ball-milled cellulose (●), ball-milled cellulose with stirring (▲) and ball-milled cellulose with cellulase (◆)

et al. 2002). It follows that the extent of growth on the ball-milled cellulose was greater than on the highly crystalline Sigmacell. However, when compared to growth on cellobiose, *C. phytofermentans* grew very poorly on both types of cellulose. When cultured on ball-milled cellulose, *C. phytofermentans* produced only 26% of the ethanol yield and 16% of the hydrogen yield observed in cellobiose cultures. *C. phytofermentans* is cellulolytic and therefore produces the enzymes necessary to utilize cellulose directly, as reported by Warnick *et al.* (2002) and Ren *et al.* (2007). Warnick *et al.* (2002) did not report any kinetic or quantitative data on the extent of cellulose utilization, so it is not possible to make a comparison other than to confirm that cellulose was degraded. Ren *et al.* (2007) cultured *C. phytofermentans* on Avicel, which is very similar to Sigmacell, and found an ethanol yield of 68 mg ethanol / g cellulose. In this study, the yield was much lower with only 19 mg ethanol / g cellulose observed.

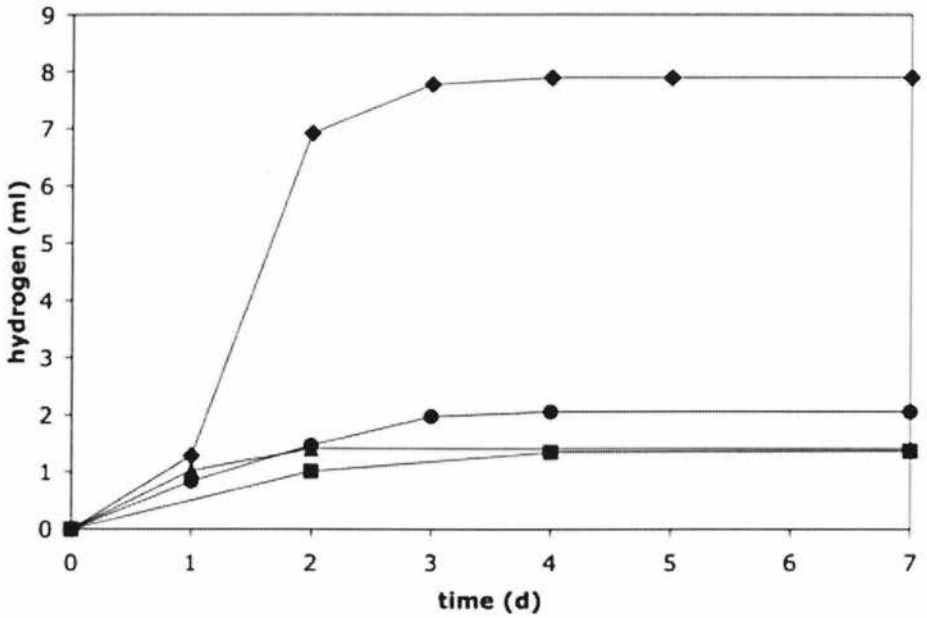

Figure 5–5 Hydrogen production by *C. phytofermentans* cultured on microcrystalline cellulose (■), ball-milled cellulose (●), ball-milled cellulose with stirring (▲) and ball-milled cellulose with cellulase (◆)

Microscopic examination of the ball-milled cellulose cultures showed an EPS coating similar to that observed in the Sigmacell cultures, and no attached growth of cells was observed. Based on the observation of severely limited growth and the extensive coating of cellulose particles, it was hypothesized that the EPS coating and agglomeration of cellulose particles was limiting the growth of *C. phytofermentans*. Therefore, another set of ball-milled cellulose cultures was run under identical environmental conditions, with high-speed agitation introduced by a magnetic stirrer. In these experiments, the stirring speed was selected to ensure that solids did not settle, but remained continuously suspended in the fermentation broth. The results of these experiments are presented in Figures 5–3, 5–4 and 5–5. It was evident that under stirred conditions *C. phytofermentans* was less able to utilize the ball-milled cellulose than under conditions of gentle agitation. This suggests that

important physical microbe-substrate and enzyme-substrate interactions were not able to take place under the high-shear mixing conditions.

The relatively poor performance of *C. phytofermentans* when cultured on pure cellulose suggests that it may not be a good candidate for biofuel production from lignocellulosic materials using the CBP approach. However, the detectable development of cell protein, biogas and liquid phase by-products demonstrates that *C. phytofermentans* is indeed cellulolytic and therefore able to manufacture the enzymes necessary for the breakdown of cellulose. Alonzo (2007) examined the biofilm forming behaviour of *C. phytofermentans* on cellulose using dialysis membranes (regenerated cellulose) as the cellulose source. In these studies *C. phytofermentans* degraded the cellulose very slowly, with complete degradation requiring more than 60 days. Correspondence with researchers and authors Susan Leschine (2009a), John Regan (2009) and Almaris Alonzo (2009) confirmed that other laboratories have characterized *C. phytofermentans* as a weakly cellulolytic species when cultured on pure celluloses.

In the fourth set of triplicates *C. phytofermentans* cultures were grown on ball-milled cellulose with the addition of supplemental cellulase enzyme. Based on the observation that pure cultures of *C. phytofermentans* cultivated on pure cellulose grew slowly and in a limited way, it was hypothesized that the enzymes necessary for cellulose decomposition were not abundant enough for rapid and complete cellulose utilization. This experiment therefore moves away from the CBP approach and examines an assisted SSF scenario, where the micro-organism produces cellulolytic enzymes and the process is further supplemented with an external source of enzymes.

Data from this experiment are presented in Figures 5–4 and 5–5. Note that protein measurements are not presented due to interference from the supplemental enzyme addition. When *C. phytofermentans* was cultured on ball-milled cellulose with an additional 60 FPU / g cellulose of

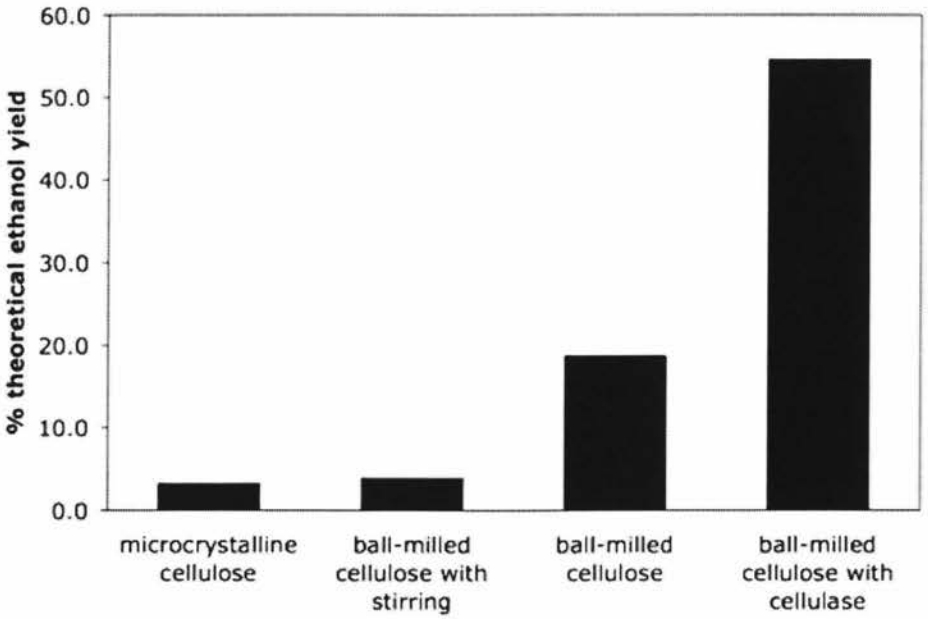

Figure 5–6 Percent of theoretical ethanol yield by *C. phytofermentans* cultured on microcrystalline and ball-milled cellulose

cellulase enzyme, growth and conversion rates increased dramatically. The seven-day ethanol concentration was 1.7 g / l, which is 55% of the theoretical ethanol yield. Therefore the addition of cellulase enzyme contributed to a nearly three-fold increase in the amount of ethanol produced after seven days over cultures without the addition of cellulase. Figure 5–6 shows the percentages of the theoretical ethanol yield achieved in each of the four experiments. An increasing trend can be seen in the data in Figure 5–4, suggesting that cellulose degradation and conversion to ethanol could continue beyond day seven. Hydrogen production was similarly increased with the addition of cellulase, with a total of 7.9 ml hydrogen, meaning that enzyme addition contributed to nearly a four-fold increase in hydrogen produced.

Table 5–1 Summary of cellulose fermentation experimental results

	Sigmacell	ball-milled	ball-milled + stirred	ball-milled + cellulase
biomass (mg BSA / l)	50 ± 12	59 ± 6	42 ± 1	N/A
ethanol (g / l)	0.13 ± 0.03	0.60 ± 0.08	0.11 ± 0.01	1.39 ± 0.11
hydrogen (ml)	1.4 ± 0.1	2.1 ± 0.1	0.8 ± 0.0	8.4 ± 0.5
biogas (ml)	7.7 ± 0.1	18.9 ± 0.4	16.8 ± 0.6	51.4 ± 1.5
acetic acid (g / l)	0.16 ± 0.03	0.23 ± 0.02	0.16 ± 0.01	0.57 ± 0.03
formic acid (g / l)	0.09 ± 0.01	0.10 ± 0.5	0.11 ± 0.01	0 .20± 0.02
% theoretical yield	3.3 ± 0.8	18.8 ± 2.5	3.9 ± 0.4	54.6 ± 4.3

All values are reported as MEAN ± STANDARD DEVIATION of triplicates

The selected enzyme loading of 60 FPU / g represents an enzyme overload condition. Generally for SSF processes, optimal enzyme loadings are close to 10 FPU / g (Sun and Cheng 2002). However, enzyme loadings have been employed between 5 – 35 FPU / g (Taherzadeh and Karimi 2007). Based on the published results of studies of enzyme dose optimization (Chang *et al.* 2001, Chang *et al.* 1997) it would be reasonable to expect that the conversion levels obtained at this high enzyme loading could also be achieved at a significantly lower optimized loading.

The results of the experiments where *C. phytofermentans* was cultured on pure cellulose substrates demonstrate that *C. phytofermentans* is weakly cellulolytic: It can produce enzymes to catalyze the enzymatic breakdown of cellulose, but it accomplishes this task very slowly with the result that cellulose degradation is incomplete. This finding has been confirmed by correspondence with other researchers and authors studying *C. phytofermentans*. Susan Leschine (2009a,b) has suggested that *C. phytofermentans* produces a wide range of cellulase and hemicellulase enzymes, but that it is not a specialist in any particular type of enzyme. Therefore, the inability of *C. phytofermentans* to degrade microcrystalline, regenerated or ball-milled cellulose efficiently may be due to the low activity of particular enzymes such as avicelase. Other cellulosic materials, including pretreated biomass could be more completely utilized by this bacterium. According to recent work by Bin Yang (2009), native plant biomass is significantly more structurally diverse and less crystalline than prepared celluloses. Therefore *C. phytofermentans* may be more appropriate for the fermentation of pretreated native plant biomass than for purified celluloses.

Chapter 6
Conclusions and Recommendations

6.1 Summary

This study has been successful in addressing two of the three core project research questions: "Is *Clostridium phytofermentans* an appropriate micro-organism to accomplish the direct conversion of SSO into biofuels?" and "What is a feasible process configuration for the bioconversion of SSO to ethanol?" The experimental investigations presented in Chapters 4 and 5 were primarily designed to answer the second question, and they also provide valuable data to guide process configuration and technology selection. The results of all fermentation experiments are presented in Table 6–1.

Chapter 4 presented the results of experiments where *C. phytofermentans* was cultured in triplicate batch on GS-2 medium at pH 7 on glucose, xylose, cellobiose and a mixed substrate containing glucose, xylose, arabinose, mannose and galactose. The highest levels of ethanol and hydrogen production were observed when cultured on cellobiose, with an ethanol yield of 72.9% of the theoretical maximum and 0.29 mol H_2 / mol hexose. *C. phytofermentans* cultured on glucose and on mixed carbohydrate medium performed similarly to one another. Mixed medium and glucose-fed cultures yielded 37.3% and 35.9% of the theoretical ethanol yields respectively. Marginal growth was observed in xylose-fed cultures with only 10.5% of the theoretical ethanol yield observed. Apparent diauxic behaviour was observed for the mixed media containing both glucose and xylose, and the experimental results generally concurred with available published data.

In Chapter 5, *Clostridium phytofermentans* was cultured on pure cellulose in triplicate batch on GS-2 medium at pH 7. Sigmacell microcrystalline cellulose and ball-milled cellulose filter paper were employed as substrates. Ball-milled cellulose cultures were also tested with magnetic stirring

and with the addition of supplemental cellulase enzyme. Growth of *C. phytofermentans* on cellulose was marginal on all types of cellulose. Growth was improved on ball-milled cellulose (19% theoretical ethanol yield) relative to microcrystalline cellulose (3% theoretical ethanol yield). Microscopic examination of cellulose cultures by light microscopy and CLSM revealed an extensive EPS coating on cellulose particles, but the introduction of shear by magnetic stirring did not improve growth. Cellulase (Celluclast 1.5 L) was added at an enzyme loading of 60 FPU / g cellulose, resulting in an improved ethanol yield (55% theoretical ethanol yield) and a nearly four-fold increase in hydrogen production. This investigation concluded that C. *phytofermentans* was a weakly cellulolytic species with insufficient enzyme activity for consideration in a CBP process.

6.2 Conclusions

The results showed that *C. phytofermentans* was capable of growth on a variety of the sugars present in plant biomass. *C. phytofermentans* produced significant ethanol yields when cultured on cellobiose, but growth rates and ethanol yields on other substrates such as glucose and xylose were at insufficient levels for commercial biofuel production. Hydrogen was produced concurrently with ethanol. Hydrogen yields by *C. phytofermentans* were low but not insignificant and could be captured as a valuable by-product of ethanol fermentation.

C. phytofermentans was shown to be cellulolytic and to produce the enzymes required to degrade cellulose. When cultured on cellulose *C. phytofermentans* produced ethanol and hydrogen. However, cellulose breakdown by *C. phytofermentans* was very slow and required longer than would be acceptable for commercial biofuel production. The rate and extent of cellulose degradation by *C. phytofermentans* was increased by decrystallizing the cellulose substrate, and by the addition of supplemental cellulase enzyme, but stirring did not increase cellulose degradation. It was shown to be possible to supplement *C. phytofermentans* with additional cellulase enzyme to increase reaction

rates and product yields. When cultured on pure cellulose, *C. phytofermentans* formed an EPS coating on nutritive surfaces, but did not necessarily grow as an attached culture. It was concluded that *C. phytofermentans* was not ideal for CBP because it was found to be weakly cellulolytic. Of the process configurations outlined in Figure 2–4, *C. phytofermentans* may be more applicable the SSF or SSCF scenarios than the CBP configuration. With supplemental enzymes, *C. phytofermentans* has the potential to produce ethanol from both the C5 and C6 sugars present in the pretreated biomass.

6.3 Recommendations

High ethanol yield, high ethanol tolerance, lack of substrate inhibition and the ability to ferment both five- and six-carbon sugars simultaneously are important characteristics of any organism to be used for biofuels production. While it appears that the native strain of *C. phytofermentans* is not appropriate for CBP processes, it may have potential in other process configurations if it can be demonstrated to possess other desirable characteristic89

Based on experimental results and discussions with researchers, it seems likely that *C. phytofermentans* exhibits a strong preference for oligomeric sugars over monomeric sugars: The ethanol yield on cellobiose was double the yield on glucose. It is recommended that studies be undertaken to examine this preference. In particular, the growth of *C. phytofermentans* on di-, tri- and tetra-saccharides of glucose and xylose should be evaluated. Substrate inhibition, as well as ethanol tolerance and tolerance for other by-products should be investigated by culturing *C. phytofermentans* at increasing substrate concentrations. Ethanol tolerance could also be investigated to determine the extent of inhibition at various ethanol concentrations and find the maximum ethanol concentration for adequate performance.

In the investigation presented in Chapter 4, diauxic behaviour was apparent when culture broth contained both glucose and xylose. *C. phytofermentans* should be evaluated for its ability to utilize

five- and six-carbon sugars simultaneously. If it is confirmed that *C. phytofermentans* prefers oligomeric sugars, then any lignocellulosic ethanol process utilizing this microbe should consider pretreatments, enzyme loadings and process configurations that will maximize soluble oligomers and minimize monomeric sugar concentrations.

SSO has potential as a lignocellulosic feedstock independent of *C. phytofermentans*. Studies are already underway to quantify the spatial and temporal variations in the cellulose, hemicellulose and lignin content of SSO and determine if it is stable enough for use as a lignocellulosic ethanol feedstock. As with all lignocellulosic feedstocks such as hardwood, softwood, switchgrass, corn stover and wheat straw, it is necessary to determine a pretreatment technology and pretreatment parameters to optimize the enzymatic digestibility of the material. A variety of pretreatments should be selected and used to treat the SSO. Evaluations should include enzymatic digestibility and screening for potential toxic by-products caused by the pretreatment.

Appendix A
Cell Growth

Cell growth was measured as protein using a modification of the Bradford dye method (Bradford 1976) according to Pavlostathis *et al.* (1988) and calibrated using a five-point curve with bovine serum albumen as the standard (BioRad Kit #500-0202).

A.1 Protein Standard Curve

A five-point standard curve was established each time a set of samples was prepared and analyzed for protein content. Figure A–1 shows a sample standard curve.

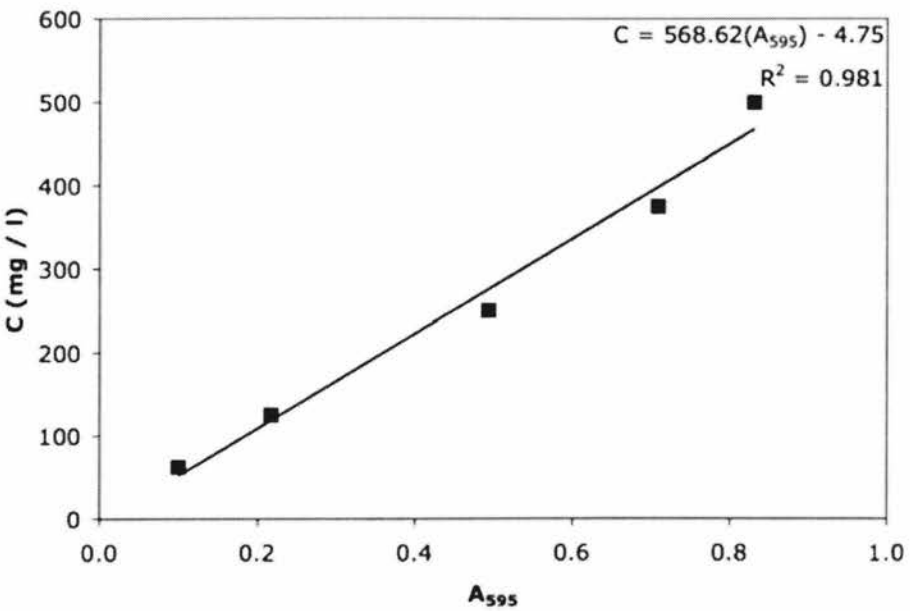

Figure A–1 Bradford dye method standard curve

A.2 Protein Concentration Calculations

Protein concentration in liquid samples after solids separation and solubilization in 1 N NaOH was calculated using Equation A–1.

$$C = 568.62(A_{595}) - 4.75 \qquad \text{Equation A–1}$$

where C is the protein concentration in mg / l *and* A_{595} is the absorbance read at 595 nm 10 minutes after addition of Bradford dye reagent. Table A–1 shows data and calculated protein concentrations for cellobiose-fed cultures at t = 3 d.

Table A–1 Sample protein calculations for cellobiose-fed cultures at t = 3 d

bottle	A_{595}	C (mg / l)
1001	0.294	162.4
1002	0.259	142.5
1003	0.281	155.0
	average:	153.3
	standard deviation:	10.1

Appendix B
Hydrogen Gas

Biogas volume was measured using an intermittent gas-release method (Ren *et al.* 2007, Logan *et al.* 2002) with water-lubricated glass syringes (Owen *et al.* 1979). Gas volumes were read at atmospheric pressure and room temperature. Biogas was analyzed for hydrogen content by GC-TCD on a Supelco 1010-PLOT capillary column.

B.1 Hydrogen Standard Curve

The standard curve for hydrogen gas was established prior to experimental analysis. At the outset of each set of injections on the GC-TCD, a hydrogen gas standard of known concentration was injected to confirm the validity of the calibration shown in Figure B–1.

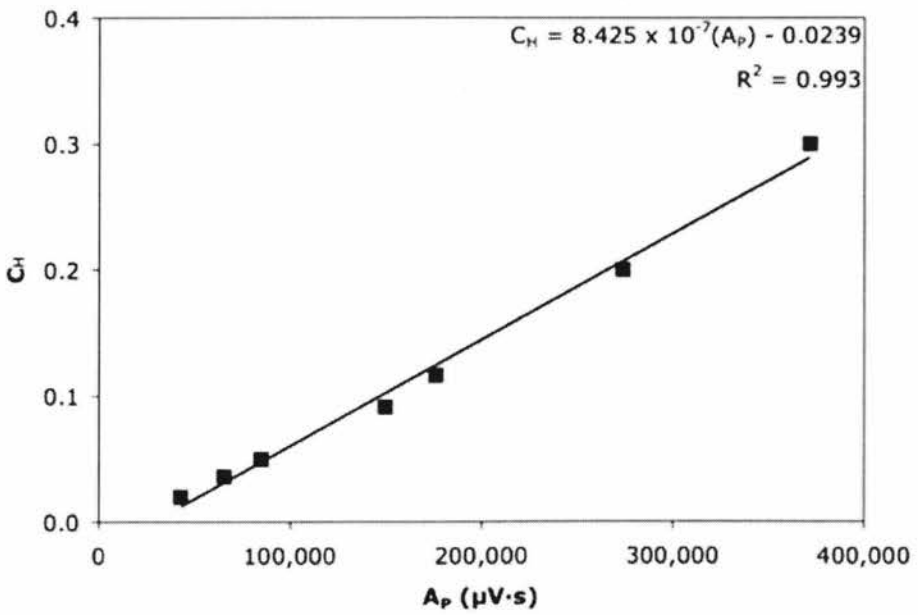

Figure B–1 GC-TCD standard curve for hydrogen

B.2 Hydrogen Content Calculations

Biogas volumes were measured daily, prior to the removal of any other liquid or gas samples. After biogas volume was measured, gas was discarded leaving the bottle headspace at atmospheric pressure. 1 ml liquid samples were then removed for analysis and 100 µl gas samples were removed and injected directly onto the GC column. Therefore, the following gas measurement was adjusted by 1.1 ml using Equation B–1.

$$V_G = V_M + 1.1 \qquad \text{Equation B–1}$$

where V_G is the gas volume in ml produced by the culture since the last measurement and V_M is the gas volume in ml measured with the lubricated glass syringe. Similarly, each time a sample was removed from the culture, the volume of the headspace was altered. The headspace volume V_{HS} was initially 65.0 ml for all cultures, and increased after each sampling interval according to Equation B–2.

$$V_{HS_i} = V_{HS_{i-1}} + 1.1 \qquad \text{Equation B–2}$$

The GC-TCD peak area A_P was used to calculate the fraction of hydrogen in the headspace gas C_H according to Equation B–3.

$$C_H = 8.425 \times 10^{-7}(A_P) - 0.0239 \qquad \text{Equation B–3}$$

Table B–1 shows sample calculations for biogas volumes and hydrogen production in a cellobiose-fed culture.

Table B–1 Sample biogas and hydrogen calculations for a cellobiose-fed culture

time (d)	V_M (ml)	V_G (ml)	V_{TOT} (ml)	A_P (μV·s)	C_H (v/v)	V_{HS} (ml)	V_H (ml)
0	0	0	0	0	0	65	0
1	10.5	11.5	11.5	38332.88	0.0129	66.1	1
2	43	44.1	55.6	196451.31	0.1058	67.2	11.9
3	12	13.1	68.7	189024.33	0.0989	68.3	14.2
4	1	2.1	70.8	201036.67	0.1093	69.4	15.2

Appendix C
Ethanol, Organic Acids and Sugars

Ethanol, acetic acid, lactic acid, formic acid, glucose, cellobiose, xylose, arabinose, mannose and galactose in the liquid fraction of culture samples were all measured by HPLC. After centrifuging 1 ml samples, supernatant was filtered an stored frozen prior to analysis. The standard curves and calculations for ethanol and the organic acids and sugars listed above are all very similar. Data and calculations for ethanol will serve to illustrate the procedure for all components.

C.1 Ethanol Standard Curve

Figure C–1 shows a sample five-point standard curve for ethanol measurement by HPLC.

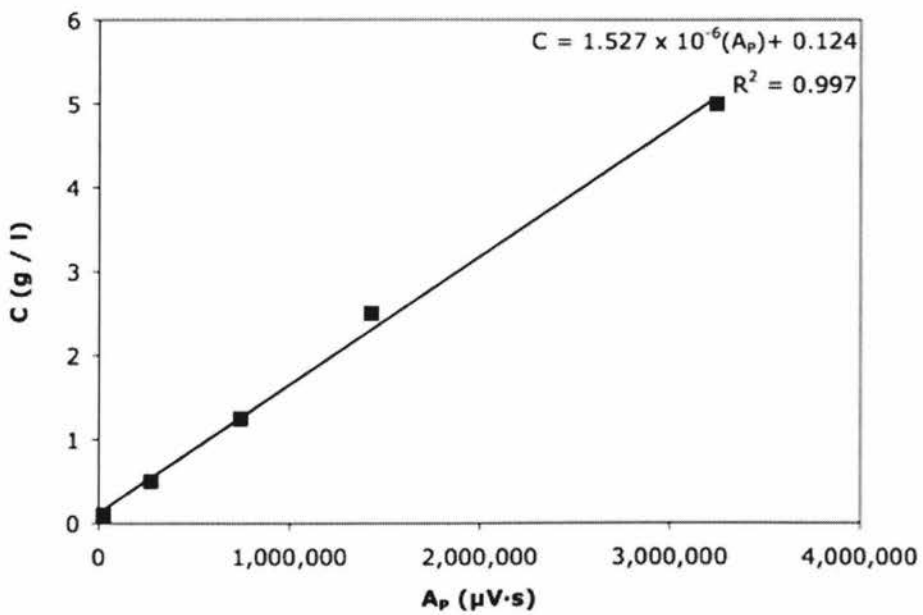

Figure C–1 HPLC standard curve for ethanol

C.2 Ethanol Concentration Calculations

Ethanol concentration in liquid samples after solids separation filtration was calculated using Equation C–1.

$$C = 1.527 \times 10^{-6}(A_P) + 0.124 \qquad \text{Equation C–1}$$

where C is the ethanol concentration in g / l and A_P is the peak area as determined by HPLC. Table C–1 shows data and calculated ethanol concentrations for cellobiose-fed cultures at t = 3 d.

Table C–1 Sample ethanol calculations for cellobiose-fed cultures at t = 3 d

bottle	A_P (μV·s)	C (g / l)
1001	1261246.00	2.05
1002	1210002.00	1.97
1003	1292875.60	2.10
	average:	2.04
	standard deviation:	0.06

Appendix D
Enzyme Activity

Cellulase enzyme (Celluclast 1.5 L) was employed in several experiments. In all cases, enzymes were applied at 60 FPU / g substrate. The enzyme activity of the Celluclast 1.5 L was measured using the Filter Paper Assay for Saccharifying Cellulase (Ghose 1987) and reported as filter paper units (FPU) per ml.

D.1 Glucose Standard Curve

Figure D–1 shows a linear glucose standard curve constructed by plotting absolute amounts of glucose (mg glucose / 0.5 ml) liberated during the procedure against the absorbance read at 540 nm after completion of the DNS reaction.

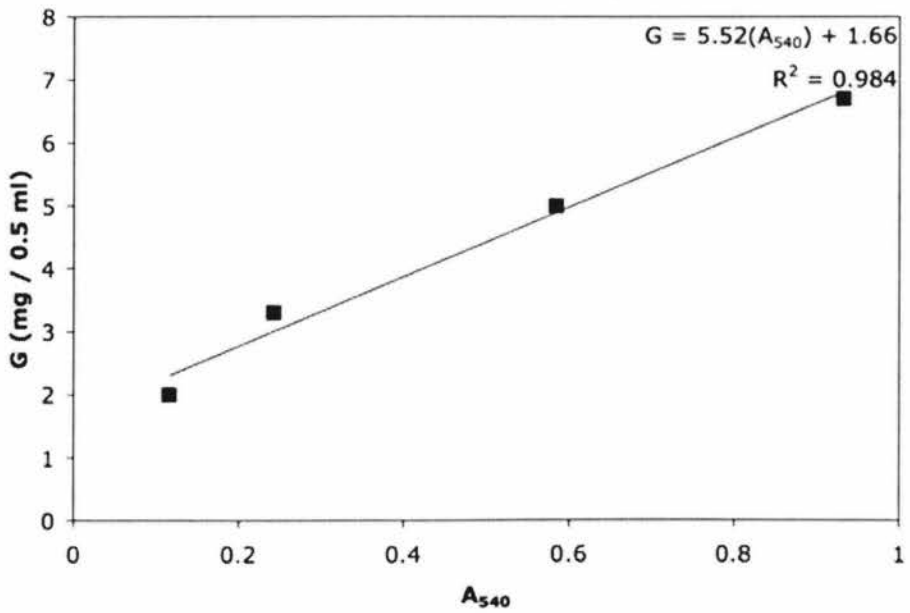

Figure D–1 Linear glucose standard

D.2 Enzyme Activity Calculations

The absorbance read from sample dilutions was converted into absolute amounts of glucose using Equation D–1

$$G = 5.52(A_{540}) + 1.66 \qquad \text{Equation D–1}$$

where G is the absolute amount of glucose released during the assay in mg glucose / 0.5 ml, and A_{540} is the absorbance of the sample read at 540 nm. Enzyme dilutions were translated into enzyme concentrations using Equation D–2

$$C = \frac{1}{D} \qquad \text{Equation D–2}$$

where C is the enzyme concentration and D is the enzyme dilution. Table D–1 shows data and calculations for five enzyme dilutions.

Table D–1 Sample data and calculations for five dilutions of Celluclast 1.5 L

D	C	ln (C)	A_{540}	G (mg / 0.5 ml)
10	1.258	-2.303	1.258	8.612
20	0.881	-3.996	0.881	6.529
80	0.273	-4.382	0.273	3.170
100	0.160	-4.605	0.16	2.546
150	0.050	-5.011	0.05	1.938

In Figure D–2, the natural logarithm of the enzyme concentration was plotted against the absolute amount of glucose released during the assay at that dilution.

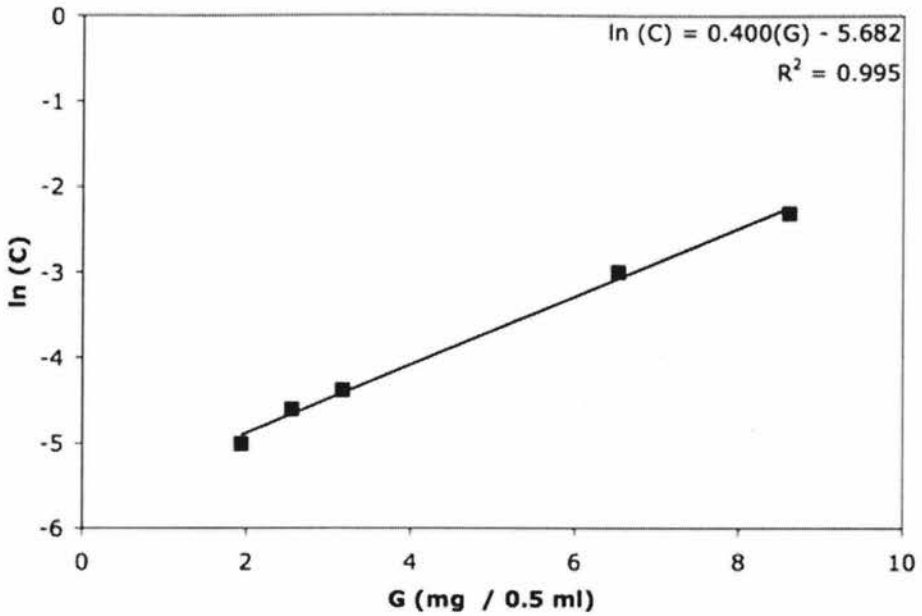

Figure D–2 Semilog plot of concentration *vs.* glucose liberated in the FPU assay

The linear trend in Figure D–2 is described by Equation D–3.

$$\ln(C) = 0.400G - 5.682 \qquad \text{Equation D–3}$$

Equation D–3 can then be used to estimate the critical enzyme concentration C_C required to release 2 mg of glucose during the assay i.e. G = 2 mg / 0.5 ml. The result is a critical enzyme concentration of C_C = 0.00758. Using Equation D–4, this concentration is used to calculate the activity of the enzyme in FPU / ml.

$$FPU = \frac{0.37}{C_C}$$ Equation D–4

The result is a calculated enzyme activity of 48.8 FPU / ml Celluclast 1.5 L.

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