Improving the bioconversion yield of carbohydrates and ethanol from lignocellulosic biomass

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Abstract

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Improving the efficiency of lignocellulosic ethanol production is of the utmost importance if cellulosic bioethanol is to be competitive with fossil fuels and first generation bioethanol from starch and sucrose. Improvements in individual processes (pretreatment, saccharification, fermentation) have been ongoing, but few researchers have considered the effect that the incoming raw biomass can have on the process. It is important to understand how biomass can be altered to provide the maximum yield of hydrolysable and fermentable sugars from whatever is available. Since the moisture content is highly variable and easily altered, the effect of drying and rewetting on bioconversion was studied on switchgrass, sugarcane bagasse and hybrid poplar. For switchgrass and sugarcane bagasse, the ethanol yield after simultaneous saccharification and fermentation was improved 18-24% by increasing the moisture content by soaking prior to pretreatment. It was also found that soaking had no effect when the samples were not catalyzed with SO_2, confirming that the effect of moisture content is directly related to

SO_2 uptake and diffusion into the biomass. In hybrid poplar, the results were similar to herbaceous biomass for chips with less than 2% absorbed SO_2. However, when the SO_2 uptake was increased to 3% even the air dried chips exhibited high digestibility, indicating that increased SO_2 uptake can overcome the poor diffusion in dried biomass.

Alongside controlling the biomass moisture content, improving knowledge and control of the processes can also increase efficiency and product yields. By monitoring reactions continuously with accurate, robust, on-line sensors, operators can detect when reactions deviate from the norm, and when they are complete. Avoiding process upsets and contamination could be the difference between an economically viable biorefinery and one that struggles to compete. Real time, continuous Raman spectroscopy was used to continuously monitor both a synthetic glucose and a lignocellulosic hydrolysate fermentation and measure glucose and ethanol. Models developed using offline HPLC validation samples had extremely high correlation between predicted and observed values for ethanol in both fermentations (R^2 = 0.98 and 0.94 for synthetic and hydrolysate, respectively) while glucose proved more difficult to detect in the hydrolysate fermentation (R^2 = 0.92 and 0.51). This work showed that it is possible to monitor the ethanol and glucose in a hydrolysate with a high fluorescent background.

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Preface

Chapter 1. Figures 1-1, 1-2 and 1-3 are used with permission from applicable sources. Portions of the introductory text are also modified from previously written introductory material from my Masters thesis entitled “Bioconversion Of Mountain Pine Beetle-Killed Lodgepole Pine to Ethanol” (2007) completed at the University of British Columbia.

Chapter 2. A version of this material has been published as Ewanick S, Bura R: The effect of biomass moisture content on bioethanol yields from steam pretreated switchgrass and sugarcane bagasse. *Bioresource Technology* 2011, 102:2651–8. I performed all experiments. Renata Bura and I conceived the experiments and I wrote the manuscript for the published paper.

Chapter 3. I performed all experiments. Renata Bura and I conceived the experiments and interpreted the data.

Chapter 4. A portion of this material has been submitted for publication in Biotechnology for Biofuels. I prepared the steam exploded lignocellulosic hydrolysate from switchgrass, and performed fermentation of synthetic sugars and lignocellulosic hydrolysate. Wes Thompson collected Raman spectra and analyzed the Raman data. I collected and ran HPLC samples and analyzed the HPLC data. Wes Thompson, Renata Bura, Brian Marquardt and I conceived experiments and contributed to writing the manuscript for the submitted paper.

Chapter 5. A version of this material has been published as Ewanick SM, Bura R: Hydrothermal pretreatment of lignocellulosic biomass. In *Bioalcohol Production*. edited by Waldron K Oxford UK: Woodhead Publishing; 2010:3–23. Renata Bura and I wrote the manuscript for the review book chapter.

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For Adam, whose love I can always depend on

Chapter 1: Introduction

Lignocellulosic bioethanol, also known as second generation bioethanol, can provide a transition away from first generation bioethanol made from starch and sugar cane. Using lignocellulosic ethanol to meet fuel bioethanol demands will reduce the stress on strained agricultural systems and lead to more sustainable fuel production by using non-food feedstocks. 16 billion gallons of cellulosic ethanol are therefore mandated to be produced by 2020 [1]. Bioconversion of biomass to ethanol is a rapidly growing field of research and a great deal of work has been done in the last 30 years on the effects of biomass composition, pretreatment and fractionation technology, and improvements in saccharification and fermentation. However, there has been less focus on the consequences of the condition of raw materials entering the process, such as the biomass moisture content. Improving the uniformity of feedstocks could improve the efficiency with which they are utilized in the biorefinery, and in the short term improving the efficiency of existing operations will have the greatest affect on overall bioconversion process economics. Another means of increasing process efficiency is to increase the knowledge and understanding of processes through improved analytical sensors. Raman spectroscopy in particular could be used to monitor reactions, leading to increased real-time awareness and enabling instant control over process parameters.

1.1 Benefits of bioethanol

Global warming is widely recognized as being at least partially accelerated by increasing emissions of CO_2, largely from the burning of fossil fuels [2]. Oil reserves around the world are being depleted, and as result of this, as well as political instability, prices at the time of writing are close to $90/barrel. The price of oil is expected to continue to rise as it becomes increasingly

difficult to extract enough oil to meet the demands of a rapidly industrializing world [3]. Use of fuels produced from plant biomass (biofuels) can help to reduce reliance on oil and other fossil fuels like coal and natural gas. Bioethanol is the most prevalent biofuel, and is currently produced primarily from starch and sucrose. However, the use of food crops to produce fuel and the amount of energy required to grow them have led to concerns [4]. With corn in particular, concerns over the whether or not there was a net reduction in greenhouse gas emissions after the energy required for fertilizer, pesticides, transportation and the water usage and soil degradation to grow corn [4]. In Brazil, the ease of growing sugar cane as well as the simplified process to convert it to ethanol have made it a major success story; however, sugarcane can only be grown in certain tropical climates [5]. Producing bioethanol from lignocellulosic materials instead will allow greater volumes to be produced in temperate climates without the concerns associated with use of agricultural products [6].

1.3 billion tons of biomass are available annually in the U.S. from forestland and agricultural land, enough to produce sufficient biofuels to displace a third of the fossil fuel consumption in the US [7]. Making use of existing infrastructure, ethanol can be blended with gasoline and used in conventional engines at up to 10% ethanol, or in modified flex-fuel engines at blends of up to 85% ethanol [8, 9]. Ethanol is also an effective replacement for methyl tertiary butyl ether (MTBE) as a fuel oxygenant to prevent engine knock and provide cleaner combustion [10].

As a fuel, ethanol provides considerable benefits over gasoline. Combustion of ethanol in fuel results in up to a 12% reduction in energy-specific CO_2 emissions (g CO_2 per BTU) [11]. In addition, emission of the toxic compounds formaldehyde, benzene and 1,3-butadiene is reduced, although acetaldehyde emissions are increased [11]. However, since modern catalytic converters

can remove these compounds [12], there is a net reduction in emissions of harmful compounds when ethanol is used as a fuel. In terms of energy, ethanol has only two thirds of the volumetric energy content of gasoline. However, it is about 15% more efficient than gasoline in optimized engines, meaning that it is possible to travel 75-80% as far on a given volume of pure ethanol compared to the same volume of gasoline [13, 14].

Since domestic feedstocks can be used to produce bioethanol, the price fluctuations and political instability associated with petroleum are removed. For instance, the country of Brazil independently produces enough bioethanol, 21 billion liters in 2011, to provide 50% of its driving fuel [15]. Combined with the US, which produced 52.6 billion liters in 2011, they make up 87% of global ethanol production [15]. Currently all ethanol produced commercially worldwide is from either starch or sucrose, with cellulosic ethanol being produced only on the pilot or demonstration scale. In order to increase ethanol production, cellulosic ethanol must become more economical to produce by making efficient use of locally available feedstocks.

1.2 Bioconversion of lignocellulosic biomass

1.2.1 Biomass

The use of lignocellulosic biomass as a feedstock is the defining characteristic of second generation bioethanol. Unlike the starch and sucrose that make up the majority of first generation feedstocks, lignocellulosic biomass is primarily composed of a matrix of cellulose, lignin and hemicellulose. Many types of lignocellulosic biomass can be utilized to produce bioethanol, including hardwoods, softwoods and herbaceous materials like energy crops and agricultural residues. Of particular interest is residual biomass available as a by-product as agriculture, logging or saw milling operations [16, 17]. These materials are often inexpensive and widely

available, although more and more processes utilizing biomass for energy production such as combustion, gasification, pyrolysis, and anaerobic digestion are creating competition for any available biomass [18].

Biomass composition and structure

Each component of lignocellulose is made up of smaller subunits; carbohydrates in the case of cellulose and hemicellulose, and phenolic groups in lignin. Plant cell walls of all types of biomass share the same structure, with cellulose forming the "skeleton" of the cell, surrounded by a matrix of hemicellulose and encrusted with lignin [19]. The ratio of lignin:cellulose:hemicellulose defines each type of feedstock (Table 1-1).

Table 1-1. Composition of various lignocellulosic feedstocks [10, 20].

	Composition (%)		
	Lignin	Cellulose	Hemicellulose
Softwood	25-35	45-50	25-35
Hardwood	18-25	40-55	24-40
Agricultural residues	10-30	25-45	10-40

Cellulose is a homopolysaccharide composed of chains of glucose units linked together by β-(1-4) glycosidic bonds [19]. The number of glucose units in each chain or molecule of cellulose is known as the degree of polymerization (DP), although it has been shown that the basic structural unit is actually cellobiose [21]. Cellulose chains can aggregate and connect via inter-molecular hydrogen bonds to form units known as elementary fibrils that contain ordered, crystalline, regions as well as disordered, amorphous regions [19]. Crystalline regions are generally more resistant to enzymatic attack, while amorphous regions present more accessible sites [22].

Hemicellulose shows much more chemical variability between different types of biomass than cellulose, with different carbohydrate composition, degree of branching and functional groups such as acetate and methoxyl [23]. Acetate groups are of particular interest as they can form organic acids during pretreatment, aiding in the breakdown of hemicellulose [24]. However, remaining acetate groups on insoluble hemicellulose can hinder hydrolytic enzymes [25]. Hardwoods and herbaceous crops contain highly acetylated glucuronoxylan and small amounts of glucomannan, while softwoods are made up of galactoglucomannans and partly acetylated arabinoglucuronoxylans [19]. The pentan-rich hemicellulose found in herbaceous residues and hardwoods is thus more susceptible to acid autohydrolysis than hexan-rich softwood hemicellulose [26, 27].

Hardwoods have less lignin overall, with their lignin made up of both guaiacyl and syringyl subunits [28]. Softwood lignin is composed of primarily guaiacyl subunits, which is known to restrict fibre swelling and enzyme permeability more than syringyl lignin due to reduced methoxylation [29]. Guaiacyl lignin is also known to condense during steam pretreatment, reducing the accessibility for subsequent enzymatic hydrolysis [30]. These differences in lignin composition as well as differences in distribution in and between cells likely account for the increased recalcitrance of softwoods compared to hardwoods. In herbaceous material, lignin contains significant amounts of p-hydroxyphenyl groups in addition to both guaiacyl and syringyl [31]. The composition and structure of different species varies considerably, necessitating specific fractionation conditions for each herbaceous crop [31, 32].

In addition to carbohydrates and lignin, lignocellulosics contain a variety of extractives including phenols such as tannins, terpene alcohols, ketones, and resin components such as fatty acids,

alcohols, resin acids, and phytosterols [19, 33].

1.2.2 Pretreatment

Due to the recalcitrant nature of lignocellulosic biomass it is necessary to apply some form of physical, chemical and/or biological treatment in order to fractionate it and improve the accessibility of the cellulose to cellulytic enzymes by removing or modifying the surrounding hemicellulose and lignin [34]. Pretreatments are numerous and are classed as physical, chemical or biological in nature.

Physical methods include mechanical processes such as ball milling, attrition, and wet disk refining [35], hydrothermolysis, pyrolysis [36], irradiation using electron beams and other high-energy radiation [33] as well as microwave heating [37]. Uncatalyzed steam explosion is possible for some feedstocks, and is known as autohydrolysis [38]; the resulting breakdown of glycosidic linkages is dependent on acids formed within the biomass itself [38].

Chemical pretreatments include ammonia [39], solvent [40], wet oxidation [41], alkali [42], acid [43] and others. Chemicals can also be used to catalyze physical pretreatments, as in the case of pH-controlled hydrothermolysis [44] and acid-catalyzed steam explosion [45, 46].

Biological treatments make use of the agents of wood decay in nature; bacteria, fungi, and soil microflora [20]. However, for effective pretreatment for bioconversion purposes, it is important that minimal sugars be consumed and only the lignin affected. The primary organisms that degrade wood are white rot, brown rot, and soft rot fungi. Of these, the most useful for pretreatment is the white rot variety, typically of the group Basidiomycota, which oxidizes and breaks down lignin [47]. While there is no energy input required during this type of pretreatment,

it takes days to weeks and is considered too slow to be technically and economically feasible.

Steam explosion

Steam explosion pretreatment is capable of fractionating a wide variety of biomass types, from softwood to hardwood to herbaceous residues, making it a versatile method [48–51]. Biomass can be added to the reactor in either a wet or dry state at a variety of particle sizes while the reaction conditions remain the same with minimal extra time required to heat.

An acid catalyst is required for effective steam explosion of many feedstocks. Acetylated hemicellulose groups release acetic acid during pretreatment, leading to autohydrolysis, but addition of acid enables shorter residence times and lower temperatures to be employed [38]. The most commonly used acid catalysts are liquid sulphuric acid and gaseous sulphur dioxide. Both require the feedstock to be impregnated for a period of time with the acid prior to pretreatment, and their effectiveness depends on the feedstock and conditions used. However, in general, SO_2 is easier and faster to introduce, and also results in reduced steam consumption [52]. Studies comparing SO_2 to H_2SO_4 have found that impregnation with SO_2 provides approximately the same sugar yields after pretreatment, but the resulting substrate is more readily fermented due to the presence of fewer fermentation inhibitors [45, 53]. For these reasons, many researchers prefer SO_2-catalyzed steam explosion.

The extent of pretreatment during SO_2-catalyzed steam explosion is determined by three factors: residence time in the reactor, temperature, and concentration of SO_2. The levels of each of these three factors determine how severe the pretreatment is. Severity can be quantified by calculating the severity factor R_o (Equation 1.1), where t is time in seconds and T is temperature in degrees Celsius [54].

$$R_o = te^{(T-100)/14.75} \qquad \text{[Equation 1.1]}$$

Since this factor takes into account only time and temperature and not acid concentration, there is not always a direct correlation between the degree of pretreatment and R_o. The combined severity (CS, Equation 2.1) factors in the acid concentration, and is more suitable for comparison of acidic pretreatments to other pretreatments [55]. It is used to approximate the severity of acid-catalyzed reactions by incorporating the measured pH of the pretreated hydrolysate [56].

$$CS = \log R_o - pH \qquad \text{[Equation 2.1]}$$

As pretreatment severity on a given substrate increases, hemicellulosic sugars are the first to be solubilized due to their low degree of polymerization and amorphous structure [19]. As severity increases further, cellulose begins to break down. One method used to increase sugar recovery is to separate pretreatment into two steps of increasing severity. The first step, at relatively mild severity, allows recovery of most hemicellulosic sugars. Following removal of soluble sugars, the higher severity conditions used for the second step allow for degradation and partial hydrolysis of cellulose, reducing the amount of enzyme required during subsequent enzymatic hydrolysis [57, 58]. The drawback to this method is that there is dilution of the sugars [59], reducing the potential concentration of ethanol after fermentation, as well as the increased production of fermentation inhibitors during the second, higher severity step [53]. As such, one step steam explosion necessitates finding conditions that form a compromise between high sugar recovery and low inhibitor formation (low severity) and easily hydrolysed solids (high severity) [38].

1.2.3 Hydrolysis and fermentation

Enzymatic hydrolysis

In nature, many different microorganisms produce extracellular enzymes that degrade cellulose. However, few of these organisms are able to digest the highly crystalline, hemicellulose-and-lignin-encrusted cellulose present in pretreated softwoods. Organisms that have been investigated include the fungi *Trichoderma*, *Penicillium*, and *Aspergillus* [22]. While these organisms produce a host of different glycolytic enzymes, there are three primary activities necessary to efficiently hydrolyse cellulose to glucose monomers. Endo-1,4-β-glucanases randomly cleave β-1-4 glycosidic linkages over the length of the chain. Exo-1,4-β-glucanases cleave off cellobiose from reducing and non-reducing ends, and cellobiose is subsequently hydrolysed to glucose by 1,4-β-glucosidases [22]. Since cellobiose is highly inhibitory to cellulase enzymes, supplemental 1,4-β-glucosidase is often added to compensate for low levels of this enzyme in the native cellulase complex [60]. Other inhibitors of cellulase enzymes include glucose [60] and, to a lesser extent, ethanol [61]. Inhibition by glucose and cellobiose is known as end product inhibition and prevents hydrolysis of cellulose at high soluble sugar concentrations, leading to the development of simultaneous saccharification and fermentation.

Structurally, a number of factors are thought to limit accessibility of enzymes to cellulose, preventing effective hydrolysis. As crystallinity and degree of polymerization increase, the cellulose contains fewer reducing ends, limiting the number of sites that can be acted on by endoglucanases [10]. Despite this, many researchers have found that increased DP does not correlate to reduced hydrolysis yield [62]. Increased crystallinity has been shown to decrease the initial rate of hydrolysis but not the overall conversion after an extended period of time [63].

Acetyl groups on hemicellulose are thought to sterically hinder the access of cellulases [25]. Deacetylation has been shown to increase swellability and enzymatic digestibility in both poplar and wheat straw [26, 27]. Lignin can also affect hydrolysis by physically blocking enzyme binding sites on cellulose and irreversibly binding to cellulases [64, 65].

Fermentation

A number of microorganisms are capable of fermentation of carbohydrates to ethanol. Anaerobic thermophilic bacteria and filamentous fungi have been shown to convert cellulose to ethanol [16]. However, this process is generally slow (3-12 days) and provides low ethanol yields, likely due to the inability of these organisms to survive in increasingly ethanol-rich environments. In addition, undesirable by-products such as acetic acid and lactic acid are often generated [16]. In order to circumvent these shortcomings, yeast are often utilized for fermentation. Pentose fermenting organisms such as *Pichia stipitis*, *Pachysolen tannophilus*, *Candida shehatae* and *Candida guilliermondii* are well suited for hardwoods and agricultural residues, since these feedstocks typically contain high concentrations of xylose [66, 67]. Endophytes found in the tissues of plants such as a strain of *Rhodotorula mucilaginosa* isolated from hybrid poplar have recently been shown to effectively utilize both pentoses and xyloses, producing ethanol and xylitol [67, 68]. While many of these organisms are capable of utilizing pentoses and hexoses, they are often sensitive to inhibitory compounds, require extensive nutrient and gas supplementation, or are difficult to culture and maintain. For these reasons, *Saccharomyces cerevisiae* continues to be extensively utilized.

The ethanologenic properties of *S. cerevisiae* have been known for thousands of years, and it continues to be one of the preferred yeast species due in large part to its hardiness at high ethanol

concentrations [69]. The main limitations of this organism are its low tolerance to some inhibitory compounds generated during pretreatment, and its inability to ferment pentoses. To reduce sensitivity to inhibitors such as furfural, 5-hydroxymethyl furfural (HMF) and acetic acid, yeast can be adapted to these compounds by growing them on the water-soluble fraction prior to fermentation [70]. Certain strains of *S. cerevisiae* can be selected which ferment alternative sugars such as galactose [71] or grow particularly well on pretreatment hydrolysates or pulping liquor [70, 72]. Alternatively, strains can be selected which thrive at higher temperatures, allowing simultaneous saccharification and fermentation without a compromise in temperature [73]. Genetic modification is another means of improving the yield or rate of ethanol production by engineering strains of *S. cervisiae* that effectively ferment pentoses [74], produce glycolytic enzymes [75, 76], or metabolize inhibitors [77].

Hydrolysis and fermentation can be carried out separately or simultaneously, and there are advantages and disadvantages for each method. The primary differences between the processes of fermentation and hydrolysis are the optimum temperature and, to a lesser extent, pH, for the enzyme and fermenting organism. Separate hydrolysis and fermentation (SHF) allows each process to run at the optimum temperature and pH and avoids inhibition of enzymes by ethanol [61]. However, end product inhibition by cellobiose and to a lesser extent, glucose, can reduce the rate and extent of hydrolysis. Simultaneous saccharification and fermentation (SSF) solves the problem of end product inhibition by glucose since sugars are fermented as soon as they are produced. A compromise in temperature is required since *S. cerevisiae* requires that the temperature is kept below 40 °C for efficient fermentation [78], while cellulases from *T. reesei* are most effective at 50 °C, but the lack of glucose inhibition can more than make up for the reduced hydrolysis temperature. It has been observed that cellulose hydrolysis rates can be

increased by 13-30% when using SSF rather than SHF [79], reducing the amount of enzyme required. Other benefits of SSF include shorter process times, reduced risk of contamination since glucose is removed and immediately converted to ethanol, and reduced capital costs due to the need for only one vessel [80]. In order to further improve the hydrolytic performance, a pre-hydrolysis step at the enzyme optimum temperature can be added for a period of time prior to adding the yeast and reducing the temperature. This process is known as hybrid hydrolysis and fermentation (HHF) or "non-isothermal SSF" [81, 82].

Inhibitory compounds

Steam pretreatment of biomass often leads to the formation of compounds which are inhibitory to yeast, leading to a reduction in productivity or product formation [83]. These inhibitors can be categorized as (1) compounds released during pretreatment, (2) sugar degradation products, (3) lignin degradation products, (4) fermentation products and (5) compounds released from equipment [66].

Compounds released during pretreatment include extractives and acetic acid. Released extractives might include terpenes, alcohols, phenolics and aromatics like tannins [66]. Acetyl groups associated with the hemicellulose can be released and form acetic acid, which can be inhibitory to *S. cerevisiae* in concentrations of above 5 g/L or less, depending on the pH [84]. Because the pKa of acetic acid is 4.76, at lower pH more acetic acid is present than acetate. Since acetic acid is able to penetrate the cell wall, the intracellular pH thus decreases and weakens the microorganism [66]. At low levels acetic acid can actually be beneficial, as it suppresses cell growth in favour of ethanol production [84].

Sugar degradation products are important inhibitors of *S. cerevisiae*. Furans such as furfural from

pentoses and 5-hydroxymethyl furfural (HMF) from hexoses are the products of dehydration reactions during pretreatment under severe acidic conditions (Figure 1-1) [83, 85]. Furan inhibition can often be overcome by assimilation by the yeast; furfural is normally metabolized by *S. cerevisiae* much faster than HMF, which can take as long as 24 hours [86].

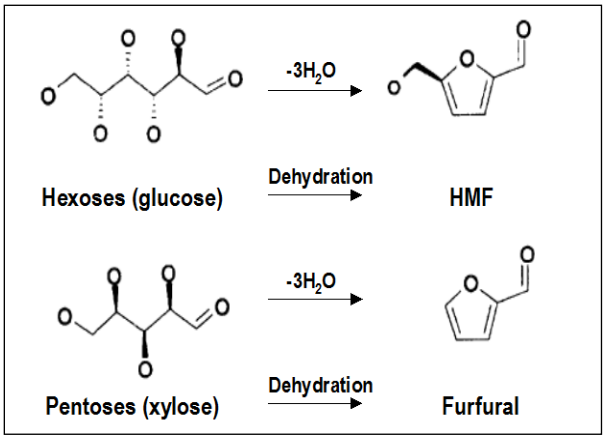

Figure 1-1. Formation of 5-hydroxymethyl furfural from hexose (above) and furfural from pentose (below). Adapted from Taherzadeh, et al. [87].

Degradation products of lignin include furaldehyde, acetaldehyde, hydroxymethylfuraldehyde, syringaldehyde, hydroxybenzaldehyde, and vanillin [86]. Some of these compounds can be metabolized by *S. cerevisiae*, particularly vanillin and furaldehyde, reducing their inhibitory effects [86].

Inhibitory metal compounds such as chromium, copper, iron and nickel can be liberated from equipment during pretreatment [66]. Compounds generated from introduced SO_2 can also have an effect on pretreated hydrolysate pH and composition. These include sulphites, which have been shown to have a mild inhibitory effect on the growth of *S. cerevisiae* [88].

For *S. cerevisiae*, a theoretical maximum of 0.51g of ethanol are produced for every gram of hexose consumed [89] as a large portion of the consumed sugar is lost as CO_2. Anywhere from 5-12% of the assimilated carbohydrate is used for cell growth and maintenance, so in practice, the maximum ethanol yield is rarely more than 0.47 g ethanol/g sugar consumed [90]. During fermentation, yeast can produce other potentially inhibitory compounds, such as acetaldehyde, glycerol, formic, lactic, and acetic acids, 1-propanol, 2-methyl-1-butanol, and 2,3-butanediol [91].

1.3 Biomass procurement and assembly

Improved biomass pre-processing such as size reduction and drying of feedstock for use in a lignocellulosic biorefinery has the potential to improve the overall efficiency of the supply chain. However, there has been substantially less focus on the impact of such pre-processing on overall biofuels yields. Similarly, much work has been done on the effects of biomass composition, pretreatment, enzymatic hydrolysis and fermentation on the overall ethanol yield, but there has been less focus on the conditions of raw materials entering the process, for example, particle size, bark content, and moisture content. Indeed, researchers often alter the biomass to a point that is far from industrially relevant to address fundamental questions. Consideration of the properties of the actual biomass available in a given geographic area is an important consideration when planning a future biorefinery.

Many pretreatments are optimized to utilize fresh biomass, while in reality herbaceous biomass is typically dried before or immediately after harvest and woody biomass can be delivered at a variety of moisture contents. And so in terms of moisture content, a critical divide seems to exist at the interface between processing and pretreatment – for effective handling, storage and

comminution, biomass is often dried, while for optimum bioconversion yields wetter is thought to be better. Successfully bridging this divide by modifying biomass prior to pretreatment could be a means of increasing ethanol yields without modification of existing processes.

1.3.1 Biomass harvest and storage

Production of fuels and chemicals in a biorefinery must occur on a continuous basis in order to be economically viable. Unlike petroleum refineries where there is a constant supply of crude oil, most lignocellulosic biomass supplied to a biorefinery is harvested on a non-continuous basis. As a result, the biomass must be stored either on or off-site in a way that preserves as much of its value as possible. Figure 1-2 shows the steps involved in the harvest of herbaceous and wood biomass. Some feedstocks, like sugarcane, are processed continuously from stored cane so that the bagasse remaining after removing the cane juice is continually produced. However, storage of high moisture bagasse results in significant microbial growth detrimental to workers and yields [92]. The temperature of stored bagasse can rise, allowing the propagation of cellulytic microorganisms and resulting loss in material. The affliction bagassosis, caused by spores of the bacteria *Thermoactinomyces sacchari*, affects the lungs and impairs breathing. Both heating and bagassosis can be decreased by drying the biomass to under 25% moisture [93]. Other energy crops, like switchgrass, are harvested once or twice a year and dried on the stem or in windrows to less than 20% moisture content, then baled [94]. Woody biomass, depending on the climate, can be harvested more frequently. Storage of chips is often required, and as with herbaceous biomass, a high initial moisture content prior to storage is proportional to increased losses and increased greenhouse gas emissions [95].

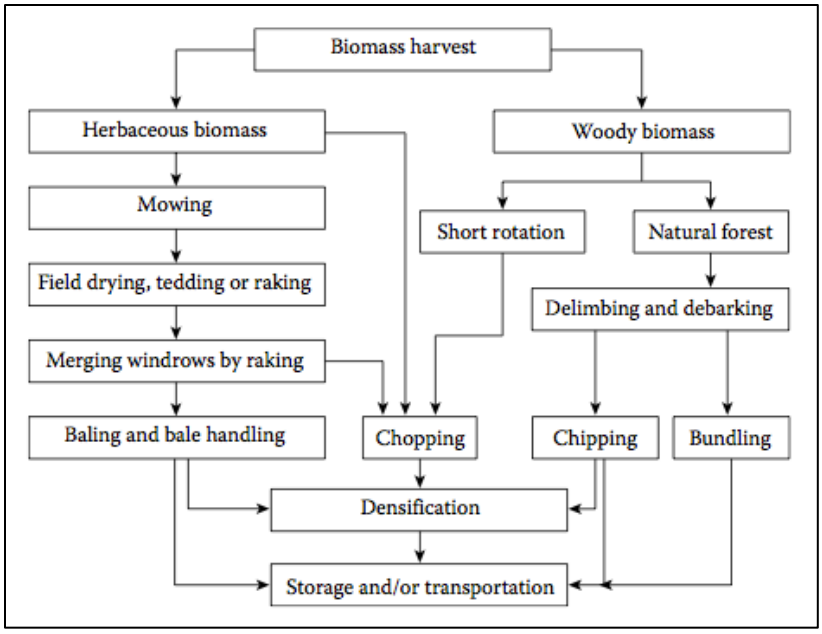

Figure 1-2. Harvest options for herbaceous and wood biomass. From [96]

After harvest, herbaceous biomass can be chopped and stored at either high or low moisture content. For dry storage in bales, moisture must be less than 20%. Biomass at greater than 40% moisture can be stored in sealed bags or silos, using a method known as ensilage. Anaerobic conditions in the container prevent microbial growth and decay [96].

Figure 1-3 shows two harvest pathways, a wet pathway and a dry one with the difference being whether biomass is dried on the field or transported immediately after harvest and stored in a wet state. Both pathways incur losses, but the storage losses are likely greater for the wet pathway as microbial growth is greatly enhanced at increased moisture.

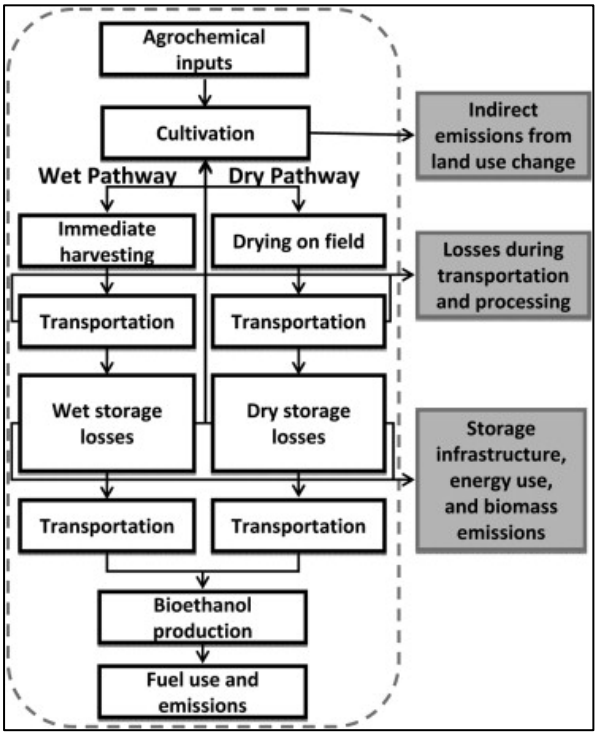

Figure 1-3. Flow diagram of the implications of wet and dry pathways on storage losses and biomass production. From [97].

One of the major limitations of using biomass for biofuels and biochemicals production is that the moisture content of biomass at the time of harvest or collection—whether agricultural or forest residues—is higher than desired and leads to degradation and decreased system efficiency. High moisture content can cause aerobic instability during storage, reduce the efficiency of transportation and pre-processing operations and can significantly affect the biomass storability [97].

1.3.2 Comminution

Comminution, or size reduction, of biomass is an important step in the biomass assembly process as it affects all downstream processes, including delivery, storage and conversion processes [98]. Size reduction of biomass along with transportation and storage costs make up between 13 and

28% of total feedstock costs [98]. Whether the biomass is wet or dry at the time of size reduction will affect the efficiency of the processes. Yancey et al. showed that increasing the moisture content of switchgrass from 10 to 25% decreased the grinding efficiency by 50% [99]. Woody biomass is generally chipped from whole logs at 50% or higher moisture content. The resulting chips are then stored until needed, during which time they must be carefully managed to minimize microbial growth. For long term storage, it is best to dry chips to below 20% moisture, but typically chips are not stored for long periods; rather logs are chipped closer to when they are needed [95].

1.3.3 Improving biomass heterogeneity

Some degree of variation in biomass characteristics is to be expected when large volumes are supplied to a biorefinery. In addition to fluctuating moisture content, there may be variability in particle size and general chemical characteristics of the biomass. Particle size can be maintained at a uniform level with advanced chipping and screening processes, and plantation grown biomass can help to homogenize chemical properties. Finding a way to homogenize the moisture content could go a long way toward effectively pretreating the majority of the biomass and minimizing over and under cooked particles. The simplest way to homogenize moisture content and improve product uniformity is to saturate all of the biomass with water [100]. The moisture can be increased by either steam or liquid water [101, 102]. Saturation of biomass is beneficial not only to homogenize the feedstock material, but also to improve catalyst and steam penetration by removing air from pore spaces of dried biomass. Steaming is commonly used prior to chemical addition [101, 103], but conditions need to be carefully chosen. At temperatures above 130 °C carbohydrate hydrolysis and lignin condensation can occur, so a practical limit for steaming is set at 120 °C [104].

Catalyst penetration

The chemical catalysts used for steam explosion pretreatment can be either aqueous or gaseous, but in either case they must be sufficiently absorbed into the biomass so as to allow the entire particle to be broken down during the relatively short time it is in the steam pretreatment reactor. Chemicals move into biomass in two possible ways: penetration under a pressure gradient or diffusion under a concentration gradient [104]. For pretreatment of many biomass types, sulfur dioxide (SO_2) impregnation is necessary prior to steam pretreatment to improve hydrolysability of solids and aid hemicellulose removal [46, 49]. In the case of gaseous SO_2, diffusion allows the gas to move to the center of the biomass particle during impregnation. Penetration occurs once the biomass is in the steam gun and steam moves to the center of the biomass particles across a pressure gradient. As a gas, SO_2 diffuses more rapidly in air than in water, but is highly soluble in water; SO_2 uptake and effectiveness could be therefore be improved by saturating biomass void volumes with water.

In dried or partially dried biomass, the lumen and pore structure in the biomass are no longer saturated with water and may have fully or partially collapsed. Below the fibre saturation point of 30% of oven dry weight, the structure of wood begins to collapse as it dries further [19]. Low chemical permeability and uptake often occur in dry biomass and could be explained not only by poor diffusion but by hornification, the fusing of cellulose fibrils upon removal of water from less ordered and more swollen areas [105]. The result is a decrease in free surface area and interstitial areas through which diffusion can occur. While hornification is said to be irreversible [105], it is hoped that re-wetting dried fibers can restore at least some of the permeability. This has been shown to be true in softwood and hardwood; increasing the moisture content of

Douglas-fir from 12% to 30% was found to improve the hydrolysability of the resulting steam pretreated substrate and reduce the effective severity of the pretreatment [106]. Similarly, fresh chipped poplar yielded a higher amount of glucose after steam pretreatment than dried chips [107]. The moisture content of biomass at the time of pretreatment is clearly an important determinant of overall process yields.

1.4 Analytical methods

In addition to homogenizing the physical properties of bioconversion feedstocks, another means of increasing yields is to improve overall process control by increasing the level of data collection for each process. Particularly in the case of a biorefinery producing bioethanol, monitoring each process can provide early detection of process upsets, contamination, and any deviation from normal and thereby increase yields and efficiency by maintaining a steady state environment.

Current chromatographic techniques to measure reactions like glucose liberation from cellulose and ethanol production from glucose are not capable of providing real-time, continuous measurements. Spectroscopic techniques such as Raman spectroscopy could be used to develop sensors to measure the concentration of reactants and products from each process, and potentially enable increased control over the unit processes. For example, such sensors could monitor reaction rates and determine when production of a desired compound has peaked and direct it to the next process.

1.4.1 Continuous measurement

Continuous monitoring of unit operations including pretreatment, saccharification, fermentation, and product purification is key to maintaining consistent product yields. To date, there are few methods for continuous monitoring of multiple compounds. Chromatographic methods like high performance liquid chromatography (HPLC) and gas chromatography (GC) can measure a wide variety of compounds in a single sample, but sample preparation, sample run time, and instrumentation not suitable for a process environment and preclude their use for continuous sampling. Continuous sampling methods have been developed utilizing sequential injection analysis (SIA), a refinement of flow injection analysis (FIA) wherein a sample of the reaction mixture is periodically injected into a flow path and a measured on a detector [108]. The injected sample is typically mixed with a solution that reacts with only the compound of interest to form a derivative that can be detected by the attached detector. For instance, mixing fermentation broth in a loop containing immobilized glucose oxidase and using a chemiluminescence detector to detect the hydrogen peroxide produced [109].

An important distinction between FIA/SIA and HPLC/GC is that for FIA/SIA components are not separated from each other before passing over the detector. This method is therefore less effective for mixtures of very similar compounds, such as carbohydrates, than it is for optically distinct molecules like ethanol or molecules that can be derivatized or reacted to form detectable compounds.

Ethanol is of particular interest as an analyte as it is often the final product and its production dictates the overall process. Ethanol can be measured chromatographically by GC and by HPLC with refractive index detection. Continuous measurement of ethanol in the fermentation

headspace is possible using an “electronic nose” electrochemical sensor, eliminating the need to separate ethanol from the rest of the reaction components and [110]. Measurement of headspace CO_2 can also approximate ethanol produced by employing the stoichiometric relationship between ethanol and CO_2 [111].

All of the above methods are hindered by the lack of ability to detect a variety of compounds simultaneously. In addition, they can be subject to interference and fouling by compounds present in lignocellulosic hydrolysates. Sample preparation can be time consuming and costly, leading many researchers to look for alternative analysis methods.

1.4.2 Spectroscopic methods

To measure mixtures of compounds (carbohydrates, ethanol, and other reactants and products) without the sample preparation and analysis time of chromatographic methods, spectroscopic methods provide a means to quickly and accurately identify and measure a range of compounds.

Raman spectroscopy

Raman spectroscopy has been utilized since its discovery in the 1920’s for measurement of a variety of compounds and reactions. However, it has not yet been extensively utilized to measure the progress of complex fermentations on a continuous, real-time basis. Raman is a complementary process to infrared (IR) spectroscopy; near-IR, mid-IR and Fourier Transform-IR (FT-IR) have been used to measure bioconversion processes [112–115]. Raman has also been utilized to monitor fermentation of lignocellulosic hydrolysates [116, 117] but not on a real-time, continuous basis. Raman and IR are considered complementary because IR measures asymmetric vibrations of polar groups while Raman detects symmetric stretching in non-polar groups [118].

Raman can be advantageous in aqueous environments, as water, a polar molecule, does not exhibit symmetric stretching.

One complication with using Raman spectroscopy to measure lignocellulosic materials is that the lignin-derived compounds present in many liquid and solid samples fluoresces at the wavelength used by the Raman laser, creating an elevated background signal. Known as laser-induced florescence (LIF), it can swamp out weaker features of the Raman spectrum [119]. Elevated background signals can also be caused by cell biomass and components of cell culture media in fermentation reactions [120]. Fortunately, data preprocessing methods can remove some or all of the background mathematically through the use of algorithms that correct the baseline [121]. Chemical extraction of biomass prior to fermentation can remove fluorescent material [117], but may not be practical for every application.

Utilizing Raman spectroscopy to follow the progress of fermentation of lignocellulosic hydrolysates will allow the concentration of both ethanol and numerous other compounds (such as carbohydrates, acetic acid, glycerol) to be measured and followed in real time so that process upsets can be spotted early and yields can be maximized.

1.5 Objectives

The overall objective of this body of research was to improve the yield of glucose from lignocellulosics, thereby increasing potential ethanol yields. This was approached in two ways. The first was to preprocess the biomass before it enters the bioconversion process by increasing the moisture content. The second was to continuously measure bioconversion processes, specifically fermentation, in real time through use of Raman spectroscopy.

Chapters 2 and 3 address the effect that the moisture content of raw lignocellulosic biomass has on the bioconversion of switchgrass, sugarcane bagasse and hybrid poplar to glucose and ethanol.

Chapter 4 details the work of a study demonstrating that 785 nm Raman spectroscopy can be used to measure the progress of a fermentation of the lignocellulosic hydrolysate obtained in the study in Chapter 2.

Chapter 5 contains a review of hydrothermal pretreatment of biomass.

Chapter 6 summarizes the work presented in this thesis and addresses future work needed to answer the questions raised by this research.

Chapter 2: The effect of biomass moisture content on bioethanol yields from steam pretreated switchgrass and sugarcane bagasse[1]

Abstract

This study aimed to determine the effect of moisture content of three different feedstocks on overall ethanol yield. Switchgrass and sugarcane bagasse from two sources were either soaked in water (~80% moisture) or left dry (~12% moisture), and half each of these were impregnated with 3% w/w SO_2 and all were steam pretreated. The twelve resulting substrates were compared based on overall sugar recovery after pretreatment, cellulose conversion following enzymatic hydrolysis, and ethanol yield following simultaneous saccharification and fermentation. The overall ethanol yield after simultaneous saccharification and fermentation of hexoses was 18-28% higher in samples that were soaked prior to SO_2 addition than in SO_2-catalyzed samples that were not soaked. In samples that were uncatalyzed, soaking made little difference, indicating that the positive effect of increased moisture content may be related to increased permeability of the biomass to SO_2.

[1] Published: Ewanick SM, Bura R: The effect of biomass moisture content on bioethanol yields from steam pretreated switchgrass and sugarcane bagasse. *Bioresource technology* 2011, 102:2651–2658.

2.1 Introduction

Following the success of first generation bioethanol made from corn starch and sugar cane, lignocellulosic ethanol can provide a transition to more sustainable fuel production by using non-food feedstocks. Bioconversion of biomass to ethanol is a rapidly growing field of research that encompasses many different methods for the fractionation, saccharification and fermentation of a number of different feedstocks. While a great deal of work has been done in the last 30 years on the effects of biomass composition, pretreatment and fractionation technology, and improvements in saccharification and fermentation, there has been less focus on the condition of raw materials entering the process; characteristics, for example, like particle size, bark content, and moisture content. Whereas moisture content can be relatively easily controlled and modified, other physical characteristics are often dictated by the equipment available for particle size reduction and debarking. Control and alteration of moisture content could be a means of increasing ethanol yields without modification of existing infrastructure.

Raw biomass varies widely depending on both the source and the time of year, with woody biomass more likely to be harvested year round and agricultural residues and grasses harvested on a seasonal basis [122]. This leads to issues with storage of seasonal biomass to minimize energy inputs for drying or freezing while preventing microbial contamination. Understanding exactly what effect moisture content has on the bioconversion properties of biomass could lead to improved storage and pre-pretreatment techniques to provide consistent results and maximize the ethanol yield from any given biomass. For example, increasing the moisture content of older, dried biomass at the facility could result in yields similar to those gained from fresh biomass.

Steam pretreatment was chosen for this study due to its ability to fractionate a wide variety of

biomass types, from softwood to hardwood to agricultural residues [46, 49, 123]. Biomass can be added to the reactor in either a wet or dry state while the reaction conditions remain the same with minimal extra time required to heat. For pretreatment of many biomass types such as softwoods and hardwoods, sulfur dioxide impregnation is necessary prior to steam pretreatment to improve hydrolyzability of solids and hemicellulose removal [46, 49]. As a gas, SO_2 diffuses more rapidly in water than in air. Accordingly, SO_2 uptake and effectiveness could be improved by saturating biomass void volumes with water. Reduced chemical permeability in dried biomass could also be explained by hornification, the fusing of cellulose fibrils upon removal of water from less ordered and more swollen areas [105]. The result is a decrease in free surface area, and interstitial areas through which diffusion can occur. While hornification is said to be irreversible [105], it is hoped that re-wetting dried fibers can restore at least some of the permeability. This has been shown to be true in softwood and hardwood; increasing the moisture content of Douglas-fir from 12% to 30% was found to improve the hydrolyzability of the resulting steam pretreated substrate and reduce the effective severity of the pretreatment [106]. Similarly, fresh chipped poplar yielded a higher amount of glucose after steam pretreatment than dried chips [107]. Neither of these studies measured the ethanol yield so it is unclear how moisture content prior to pretreatment affects the fermentation process. The effect of moisture content on the permeability and overall ethanol yield of agricultural residues and grasses is little studied.

Two types of biomass were chosen for this study. Switchgrass was selected for its ability to grow well on marginal land or double-cropped with agricultural crops [124]. It is typically harvested either once or twice per year and dried in the field, resulting in an inconsistent product for bioconversion [125]. Sugar cane bagasse is a byproduct of the sugar or sugar ethanol industry and is the material remaining after the cane has been pressed, with a yield of approximately 280

kg of bagasse for every ton of cane [126, 127]. In order to see whether different processing affects the resulting bagasse, samples were used from Brazilian and Hawaiian operations. Both switchgrass and sugarcane bagasse were steam pretreated at two different moisture levels, each with and without SO_2 impregnation. The resulting sugar recovery, enzymatic hydrolysis conversion and ethanol yield after simultaneous saccharification and fermentation were determined. The objective of this work was to determine whether increasing the moisture content of biomass prior to pretreatment would have a positive effect on the overall ethanol yield following simultaneous saccharification and fermentation.

2.2 Methods and materials

2.2.1 Pretreatment and processing conditions

Three types of biomass were used for this study. Air dried switchgrass was kindly provided by Weyerhaeuser. Air dried and washed sugar cane bagasse from Hawaii and Brazil was provided by Novozymes Inc. Both switchgrass and bagasse arrived cut to 1-2 inches in length, 1/8-1/4" in diameter.

The bioconversion process sequence is shown in Figure 2-1. Prior to pretreatment, half of each type of biomass was submerged in water for 48 hours. Each was then vacuum filtered to remove as much excess water as possible and the moisture content calculated and presented in Table 2-1. Gaseous sulfur dioxide (3% w/w) was added by weight to half of the soaked biomass and half of the unsoaked biomass based on the dry weight of the material. Specifically, for 200 g of dry biomass, 6 g of SO_2 was added by weight from a cylinder of gas to a plastic bag containing the biomass. 200 g dry weight of each type of biomass were impregnated, and both the catalyzed and uncatalyzed material were divided into 50 g portions which were pretreated sequentially using a

1.5 L batch steam gun (HM^3 Energy Inc, Gresham OR) at the time and temperature shown in Table 2-1. After the specified reaction time had elapsed for each portion of biomass, a pneumatic valve was opened between the pressurized reaction vessel and the collection vessel, blowing the pretreated slurry into the collection vessel. After all 4 shots had been discharged, the slurry of material was collected by opening a valve at the bottom of the collection vessel and allowing the material to drain into a bucket.

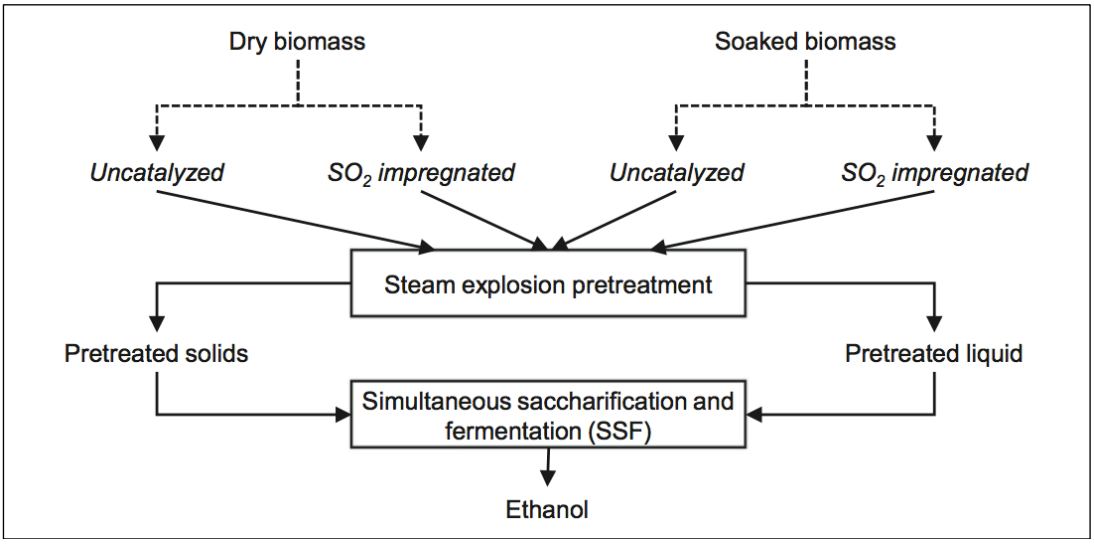

Figure 2-1. Process flow diagram for bioconversion of raw switchgrass and sugarcane bagasse into ethanol following soaking and SO2 impregnation.

The liquid and solid fractions were separated from the slurry by vacuum filtration, analyzed as described below, and used to construct a complete mass balance of carbohydrates and lignin. Solids were water-washed (with water equal to ten times the mass of solids) prior to analysis and saccharification.

Table 2-1. Biomass moisture content prior to pretreatment and subsequent pretreatment conditions for switchgrass and sugarcane bagasse.

	Moisture content (%)		Pretreatment conditions	
	Initial	Soaked	Temperature (°C)	Time (min)
SG	9	80	195	7.5
SCB-B	13	79	205	10
SCB-H	9	79	205	10

2.2.2 Instrumental analysis

HPLC

Carbohydrates were measured by pulsed amperometric electrochemical detection on a Dionex ICS 3000 HPLC. The method used a flow rate of 1 ml/min and mobile phase of deionized water for the first 30 minutes followed by 10 min of 0.2 M NaOH, followed by 10 minutes of deionized water. Samples were diluted as appropriate, spiked with fucose as an internal standard and filtered through 0.22 μm syringe filters. 10 μL of sample were injected onto the column, a Dionex Carbopac PA1 fitted with a guard column. After separation of the injected sample on the column, 0.2 M NaOH was added to a T-junction at 0.5 ml/min using a post-column AXP pump and mixed with the sample prior to electrochemical detection. Samples were measured against standards consisting of arabinose, galactose, glucose, xylose, and mannose.

Ethanol, glycerol, acetic acid, and furfurals were measured using refractive index detection on a Shimadzu Prominence LC. Samples were diluted as appropriate, filtered through 0.22 μm syringe filters and 20 μL of sample were injected run on a Phenomenex Rezex RHM H^+ column at 63 °C with an isocratic mobile phase elution of 0.05 mM H_2SO_4. Standards were prepared and used to quantify the unknown samples.

2.2.3 Compositional analysis

Ash

Ash content of raw biomass samples was measured gravimetrically by heating 20-mesh-milled dry biomass to 550 °C for 20 hours [128].

Insoluble carbohydrates and lignin

Solids were analyzed gravimetrically for lignin content, photometrically for soluble lignin, and by HPLC for carbohydrate content using the TAPPI method T-222 om-98 [129]. Briefly, 0.2 g of 40-mesh ground oven dried sample was mixed with 3 ml of 72% H_2SO_4 for 120 minutes, diluted with water to 120 ml total volume, and autoclaved at 121 °C for 60 minutes. The samples were then filtered through tared glass fritted crucibles which were oven dried and weighed to determine acid insoluble lignin. Since the acid insoluble material included ash, the ash content was subtracted from the total acid insoluble lignin amount. The filtrate was analyzed by HPLC for carbohydrate composition and by UV at 205 nm for acid-soluble lignin content.

Soluble carbohydrates

Monomeric and oligomeric soluble carbohydrates were determined using NREL LAP TP-510-42623 [130]. Briefly, samples were diluted by half and 72% H_2SO_4 added to reach a pH of 0.07. These samples were then autoclaved at 121 °C for 60 minutes to determine the total sugar concentration. Monomeric sugars were determined by analyzing the original samples by HPLC without acid hydrolysis. Oligomeric sugar was calculated by subtracting monomeric sugar content from total sugar content.

2.3 Saccharification

Enzymatic hydrolysis of washed solids was done at 5% w/v solids in a total volume of 50 ml in 125 ml Erlenmeyer flasks. The solution was buffered at pH 4.8 with 0.05 M sodium acetate buffer and the hydrolysis was completed at 50 °C and 150 rpm shaking on an orbital shaking incubator (New Brunswick). Cellulase (Spezyme-CP, 26 FPU/ml, Sigma) was added at 10 FPU/g cellulose and supplemental beta-glucosidase (Novozym 188, 492 CBU/ml, Sigma) was added at 20 CBU/g cellulose. 1 ml samples were periodically removed and analyzed for glucose and xylose.

2.4 Simultaneous saccharification and fermentation (SSF)

Saccharomyces cerevisiae ATCC 96581 isolated from spent sulfite liquor [72] (obtained from ATCC) was streaked onto YPD agar plates and allowed to grow for 48 hours. Prior to fermentation, preculture cells were grown by adding one colony from the plate to liquid media containing 10 g/L each of glucose, yeast extract and peptone. After 24 hours of growth at 30 °C and 150 rpm shaking, the cells were centrifuged and the spent supernatant removed and replaced with fresh media. The cells were then grown for another 24 hours under the same conditions, the cells were again spun down, washed twice in water, and then resuspended in a small volume of 0.9% sodium chloride. Cell concentration was determined by measuring the optical density of the suspension at 600 nm and comparing to a calibration curve prepared using oven dried cells at different optical densities.

SSF was carried out at 5% w/v washed, never dried solids, 5 g/L yeast, and enzyme loading of 10 FPU/g cellulose and 20 CBU/g cellulose. The total reaction volume was 50 ml in 125 ml Erlenmeyer flasks. Ammonium phosphate (2 g/L), sodium phosphate (0.2 g/L) and sodium

nitrate (2 g/L) were added to each flask. Prior to mixing with the solids, the pretreated liquid stream was adjusted to pH 5.5 with 10% NaOH. The pH-adjusted liquid stream was added to each flask along with yeast, enzymes, and nutrients such that the final volume including the moisture in the pretreated solids was 50 ml. Flasks were incubated at 37 °C with 150 rpm orbital shaking. 1 ml samples were removed periodically for ethanol and glucose analysis.

2.5 Results and discussion

In this study, we altered the moisture content of two types of biomass by soaking the material in water to determine the implications for subsequent enzymatic hydrolysability and overall ethanol yield. In particular, this research aimed to assess whether increasing the moisture content of the biomass prior to impregnation and subsequent pretreatment could stimulate an increase in overall ethanol yield. It is well known that the hydrolyzability of some types of biomass is improved by addition of SO_2 [49]; what is lesser known is whether similar improvements can be achieved by altering the moisture content. Switchgrass and two types of sugarcane bagasse were used to see if any observed effects were consistent across different types of biomass.

2.5.1 Compositional analysis

Raw biomass

Table 2-2. Composition of raw switchgrass (SG) and sugarcane bagasse (SCB) presented as a percentage of total biomass analyzed prior to pretreatment.

	Arabinan	Galactan	Glucan	Xylan	Mannan	Ash	Lignin	
							Acid insoluble	Acid soluble
SG	2.8	0.9	35.2	21.7	0.2	3.7	24.1	3.3
SCB-B	1.8	0.5	41.3	21.8	0.3	4.1	20.5	2.9
SCB-H	1.4	0.3	40.5	21.9	0.3	1.4	23.6	2.9

Switchgrass (SG) and two subtypes of sugar cane bagasse (SCB-B and SCB-H) were chosen for their similar composition (Table 2-2) and for their potential use as sustainable bioethanol feedstocks. The relatively similar composition was important, as it enabled any differences in characteristics after pretreatment to be seen as a result of the pretreatment or characteristics of the biomass rather than chemical composition. Switchgrass and bagasse differ in terms of their level of pre-processing; switchgrass is either dried in the field or harvested and then dried [122]. Sugar cane is harvested, mechanically pressed to extract sucrose, and the remaining fiber, the bagasse, can be dried for transport or used immediately [122].

The total polysaccharide content of all of the biomass proved to be very high (61-66%) with only 23-27% lignin, making both the switchgrass and sugar cane bagasse attractive material for saccharification and fermentation processes. The composition of the biomass was similar to compositions observed by other investigators [51, 131]. Glucan was shown to be the most abundant component in the feedstocks as determined by secondary acid hydrolysis of constituent polysaccharides with the remainder of the biomass composed of 35-41% lignin, 22% xylan, 1.5-3% arabinan and minor amounts of galactan and mannan. As glucose and xylose made up the majority of carbohydrates in the raw material, only their behavior was reported in subsequent analysis.

2.5.2 Solids and liquid composition and sugar recovery after pretreatment

Switchgrass and sugarcane bagasse, while similar in chemical composition, differed in their response to steam pretreatment. Initial experiments (data not shown) showed that more severe conditions were required to pretreat the bagasse samples to an acceptable level of enzymatic digestibility (10 min at 205 °C for sugar cane bagasse compared to 195 °C for 7.5 minutes for

switchgrass). As a result, there were significant differences between the two feedstocks in the amount of sugar remaining in solids and liquids after pretreatment (sugar recovery), enzymatic cellulose conversion of the solids (digestibility) and subsequent overall ethanol yield after simultaneous saccharification and fermentation (SSF).

Table 2-3. Composition of pretreated switchgrass (SG) and sugarcane bagasse (SCB) determined by gravimetric analysis of solids and acid hydrolysis and analysis of liquids.

			Water insoluble material g/100g pretreated solids			Water soluble material g/100 g raw biomass					
			Glucan	Xylan	Lignin	Glucose		Xylose			
					Total	Total	% oligomeric[1]	Total	% oligomeric	FF[2]	HMF[3]
SG	uncat	dry	47.2	11.1	33.0	6.2	89	23.6	93	0.25	0.08
		soaked	47.1	13.4	32.6	5.1	90	25.7	93	0.26	0.06
	SO_2	dry	46.5	3.9	40.4	5.0	75	17.7	73	0.76	0.21
		soaked	49.1	2.5	37.9	7.8	62	36.2	50	1.42	0.21
SCB-B	uncat	dry	51.3	3.6	33.9	2.0	82	13.2	68	1.07	0.11
		soaked	51.9	4.5	32.4	1.9	88	20.7	71	0.77	0.07
	SO_2	dry	48.7	2.1	37.9	2.6	76	12.7	65	0.20	1.28
		soaked	48.9	0.3	37.2	7.1	26	16.0	21	0.72	2.52
SCB-H	uncat	dry	50.1	4.0	35.7	1.7	85	19.8	73	0.08	0.69
		soaked	51.5	4.3	33.7	1.7	84	22.0	64	0.09	0.83
	SO_2	dry	45.8	1.3	43.3	2.6	70	12.9	60	0.25	1.44
		soaked	46.9	0.3	44.4	6.2	29	14.1	23	0.69	2.09

[1] "% oligomeric" describes the percentage (w/v) of soluble sugar not present in monomeric form
[2]furfural
[3]5-hydroxymethyl furfural

Following pretreatment and liquid-solid separation of all 12 samples, the compositions of the solid, water-insoluble fraction and the liquid, water-soluble fraction, were analyzed. The glucan content of the resulting solids for all samples was between 45.8 and 51.9 g glucan/ 100g pretreated solids (Table 2-3). Along with lignin, this comprised at least 80% of the pretreated solids with the majority of the hemicellulosic sugars solubilized into the liquid fraction. Xylan content in all of the samples was low – only uncatalyzed switchgrass contained more than 5%

xylan (11-13%). Both presoaking and the addition of SO_2 had a significant impact on the composition of the resulting biomass (Table 2-3). Simply adding SO_2 to dry biomass had the expected result – a large decrease in xylan, a small decrease in glucan and resulting increase in lignin. However, by soaking prior to adding SO_2, the effect of SO_2 was enhanced - xylan was further reduced in the solids with minimal change to glucan (Figure 2-2, Table 2-3). In the liquid, soluble glucose was increased by 56-170% in samples treated with SO_2 following soaking compared to dry samples (Figure 2-2, Table 2-3).

The pretreatment conditions were chosen to be severe enough to produce a solid substrate that could be relatively quickly and completely hydrolyzed but not so severe that the sugars in the starting material were degraded. As glucose recovery was over 80% in the two bagasse samples, and over 96% in the switchgrass samples (Table 2-4), it seemed to indicate that the pretreatment conditions were adequate and not overly severe.

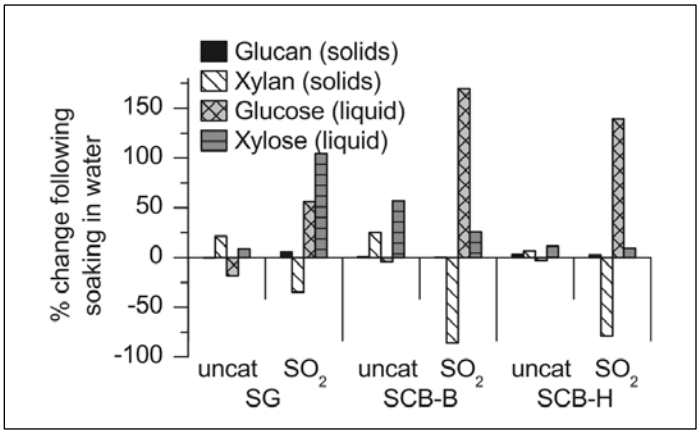

Figure 2-2. Percent change in glucan, xylan, glucose and xylose in SO_2-catalyzed or uncatalyzed pretreated switchgrass (SG) and sugarcane bagasse (SCB) as a result of increased moisture content.

Table 2-4. Overall carbohydrate recovery as a percentage of each component present in the original material following pretreatment of switchgrass (SG) and sugarcane bagasse (SCB).

			Glucose	Xylose	Glucose and xylose
SG	uncat	dry	99	82	92
		soaked	96	93	95
	SO_2	dry	100	51	93
		soaked	100	82	100
SCB-B	uncat	dry	100	40	80
		soaked	83	55	73
	SO_2	dry	96	33	74
		soaked	84	33	66
SCB-H	uncat	dry	97	54	82
		soaked	95	58	82
	SO_2	dry	80	30	62
		soaked	88	29	67

Furfural and 5-hydroxymethyl furfural (HMF), degradation products of pentoses and hexoses respectively, were present in low concentrations (Table 2-3). Furfural was below 2.6 and HMF was less than 0.7 g/100g original biomass in all twelve samples. These relatively low levels of furan formation are a result of minimal sugar degradation during pretreatment. Minimizing their formation serves to improve sugar yields and increase potential ethanol yield by reducing microbial inhibition during fermentation. Carrasco et al. pretreated sugar cane bagasse using steam pretreatment under similar conditions on high moisture (75-77%) biomass with and without SO_2, with the major difference being almost twice as much SO_2 added to the biomass [51]. Despite this additional SO_2, pretreated liquid hydrolysates in this study had less than 0.7 g/100 g original biomass of furfural and nearly no HMF present. Comparable samples in our study contained at least 3 times as much furfural and 0.7 g/100g of HMF, a result of either a lack of further degradation to formic and levulinc acids or different pretreatment equipment.

2.6 Hydrolysis

Following pretreatment, all 12 samples were enzymatically hydrolyzed at 5% consistency and 10 FPU/g cellulose cellulase enzyme loading (Figure 2-3). The extent of cellulose conversion highlighted the differences in digestibility between soaked and unsoaked, and catalyzed and uncatalyzed. Of all of the samples, the maximum cellulose conversion after 10 hours was 92-94%, for all three soaked and SO_2 catalyzed substrates. For each of the three feedstocks, soaking improved the extent of conversion after 10 hours by 10-19% for SO_2-catalyzed samples, whereas soaking reduced digestibility in uncatalyzed samples by 1-9% (Figure 2-4). In switchgrass, the addition of SO_2 to dry biomass increased the hydrolysis conversion by 43%, while soaking prior to SO_2 addition increased the yield by 82% over the soaked, uncatalyzed sample. Similarly, the two bagasse samples showed a 2% increase in conversion for Brazilian and 28% increase for Hawaiian bagasse following the addition of SO_2 to dry biomass. A far more substantial increase in conversion was generated by adding SO_2 to soaked biomass - the glucose yield increased by 22-42% for Brazilian and Hawaiian, respectively.

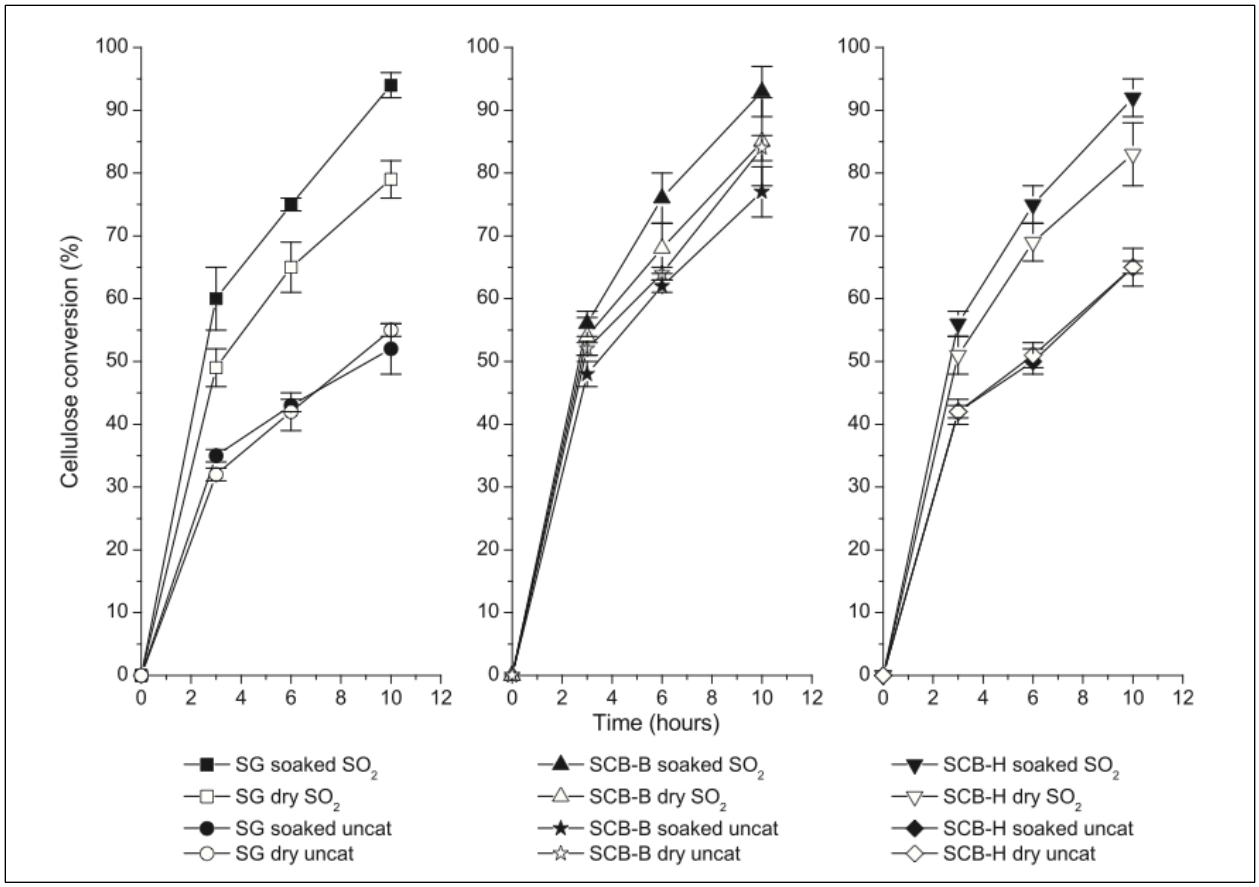

Figure 2-3. Cellulose conversion of pretreated switchgrass (SG) and sugarcane bagasse (SCB) to glucose during enzymatic hydrolysis at 5% solids consistency and 10 FPU/g cellulose cellulase loading.

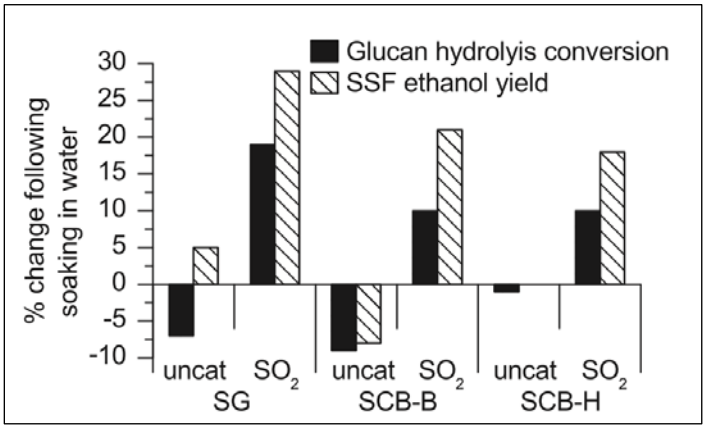

Figure 2-4. Percent change in hydrolytic glucan conversion and SSF ethanol yield in SO_2-catalyzed or uncatalyzed pretreated switchgrass (SG) and sugarcane bagasse (SCB) as a result of increased moisture content.

The increase in glucan conversion in soaked, SO_2-catalyzed samples appears to correlate to the increased reduction in xylan content in these samples (Figure 2-5). Conversely, as xylan content in the pretreated solids increases, the extent of glucan conversion decreases. The relationship between these two variables has an R^2 value of 0.781, indicating a strong correlation. While xylan removal has been previously shown to improve the cellulose digestibility (Bura et al., 2009), many other factors influence hydrolyzability, including lignin content, particle size, available surface area and cellulose crystallinity. With so many variables it is difficult to determine the extent of the role that xylan plays [132, 133], but it is possible that increased moisture content allows better penetration of SO_2 into the cell wall. Xylan present in cell wall hemicelluose is susceptible to acid hydrolysis and is solubilized by the SO_2 and other acids formed during steam pretreatment [49]. Removal of xylan from the cellulose matrix increases cellulose accessibility and subsequently improves enzymatic saccharification.

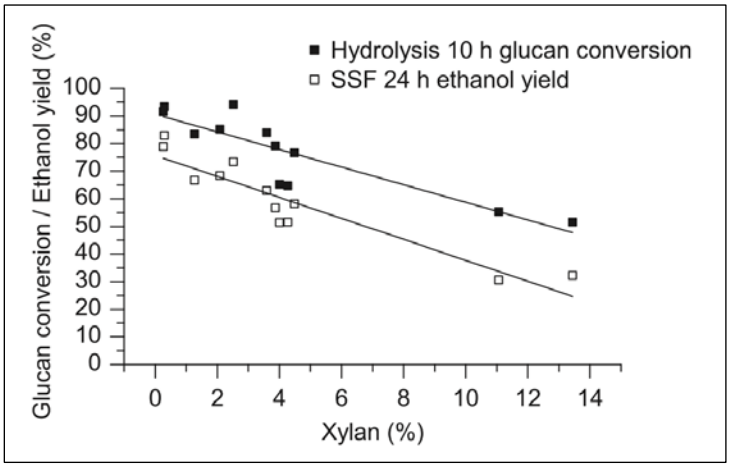

Figure 2-5. Effect of the xylan content of pretreated solids on the enzymatic cellulose conversion after 10 hours of hydrolysis and 24 hours of simultaneous saccharification and fermentation (SSF).

2.7 SSF

Simultaneous saccharification and fermentation was carried out using the same enzyme loading and solids consistency as for enzymatic hydrolysis with the addition of the pH-adjusted pretreated liquid stream and 5 g/L of *S. cerevisiae*. Only hexoses were utilized by this organism, and since galactose and mannose made up 4% or less of the six-carbon sugars present in the reaction, only glucose was measured. The production of ethanol and consumption of glucose were analyzed over time and compared after 24 hours of saccharification and fermentation.

As shown in Figure 2-6, soaking prior to adding SO_2 produced the highest yield of ethanol, while adding SO_2 to dry biomass generated higher yields than uncatalyzed samples. Switchgrass showed the greatest increase in ethanol production, with over twice as much ethanol produced after soaking and SO_2 than soaking without SO_2. This result is likely a result of the reduced enzymatic digestibility of the unsoaked samples (Figure 2-3) rather than a difference in fermentability of the liquid stream; analysis of the liquid stream (Table 2-3) showed that levels of 5-hydroxymethyl furfural and furfural were below levels shown to be inhibitory to *S. cerevisiae* [134]. In all samples, glucose was consumed after 6 hours (Figure 2-6) while the small amount of galactose and mannose present took up to 24 hours to be consumed (data not shown).

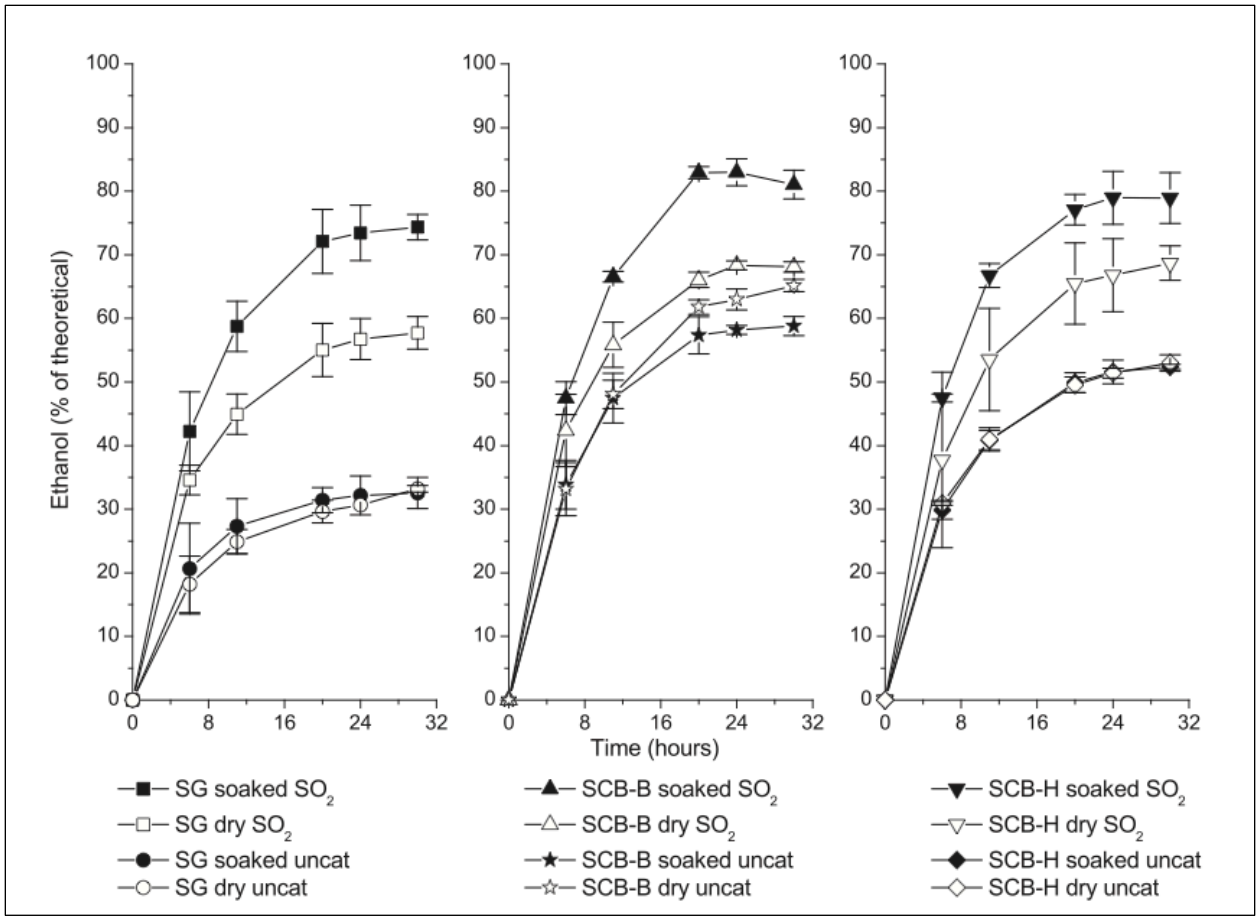

Figure 2-6. Ethanol yield as a percent of maximum theoretical ethanol yield following simultaneous saccharification and fermentation (SSF) at 5% solids consistency, 10 FPU/g cellulose cellulase loading, and 5 g/L *Saccharomyces cerevisiae* of pretreated switchgrass (SG) and sugarcane bagasse (SCB)

Linear regression of the relationship between xylan content in the pretreated solids and the ethanol yield following SSF had a slope very close to the plot of hydrolysis glucan conversion, with an R^2 value of 0.869 (Figure 2-5). This indicated that the difference in ethanol yield between all of the samples is due to the same factors that affect hydrolytic conversion, likely the extent of xylan removal.

Overall ethanol yields were calculated based on the sugar recovery following pretreatment and the amount of ethanol produced after 24 hours of SSF of the pretreated material. Not

surprisingly, based on the above results, overall ethanol yields for all three feedstocks were higher for SO_2-catalyzed than uncatalyzed. For SO_2-catalyzed samples, overall yields were as much as 28% higher for soaked biomass than for dry. In uncatalyzed samples, soaking had a negligible or negative effect on overall ethanol yield (Table 2-5). The highest ethanol yield, from Brazilian sugarcane bagasse was 52 gallons per ton of raw biomass, followed by 51 gal/ton for Hawaiian bagasse and 45 gal/ton for switchgrass. Since only hexoses were utilized, these yields are lower than if both pentoses and hexoses were used. The maximum yields for each biomass, assuming 100% recovery of sugars after pretreatment and full conversion of only hexoses to ethanol is 63, 72, and 71 gal ethanol/ton for switchgrass, Brazilian bagasse, and Hawaiian bagasse, respectively. The highest ethanol yield for each feedstock is therefore 71% of the maximum possible.

Table 2-5. Theoretical ethanol yields from raw biomass following pretreatment of switchgrass (SG) and sugarcane bagasse (SCB) following 24 hours of simultaneous saccharification and fermentation (SSF).

			Theoretical ethanol yield	
			Gal/ton	L/tonne
SG	uncat	dry	18.6	77.6
		soaked	19.5	81.4
	SO_2	dry	34.4	143.5
		soaked	44.5	185.7
SCB-B	uncat	dry	44.8	187.0
		soaked	36.9	154.1
	SO_2	dry	48.6	202.8
		soaked	51.6	215.3
SCB-H	uncat	dry	35.9	149.6
		soaked	35.7	149.1
	SO_2	dry	39.2	163.5
		soaked	50.2	209.4

The surprising increase in overall ethanol yield brought on by increasing the moisture content of SO_2-catalyzed biomass is hypothesized to be due to an increase in permeability, allowing improved penetration of SO_2. Soaking alone did not produce an increase in ethanol yield, so it seems that the improved yields are due solely to increased efficacy of the added SO_2. The lower ethanol yield of dried biomass compared to soaked is thought be due at least partially to hornification of the biomass during drying preventing thorough uptake of SO_2. The cause of hornification is not well understood, but the effects are reduced pore size and surface area [135], both of which could be responsible for reduced efficacy of pretreatment and subsequent reduced enzymatic hydrolyzability. The most established definition of hornification is that cellulose fibrils are brought closer together upon drying and crosslinked by formation of hydrogen bonds[136]. Suchy et al propose an alternative mechanism to hornification to explain ultrastructural rearrangements seen after drying – an irreversible stiffening of the hemicellulose-lignin matrix in regions that typically swell when exposed to water [137]. Hornification has been well studied on pulp fibers, but is less understood on whole biomass. It is thought to be less likely that untreated biomass (such as the raw switchgrass and bagasse used in this study) would experience cellulose microfibril aggregation due the substantial presence of lignin and hemicellulose, and would experience only low level hornification and stiffening of the hemicellulose-lignin matrix [137]. This would still allow penetration of water into the cell walls and improve the transfer of SO_2 throughout the biomass, thus increasing hemicellulose solubilization and improving enzymatic hydrolysis. Full reversal of hornification requires harsh treatments such as beating, addition of bulking agents, or derivitization [135]. As such, increasing moisture content is likely not reversing hornification, only improving the passage of SO_2 through the biomass.

It is difficult to determine whether the improvement in ethanol yield shown by increased moisture content and SO_2 addition is a result of the moisture content increasing pore spaces and surface area and for SO_2, or simply filling pore spaces with water and allowing better diffusion of SO_2. Increasing the moisture content to 150% of corn fiber showed an improvement in hydrolyzablity following AFEX pretreatment [138]. In this case, the improvement was thought to be related to the formation of ammonium hydroxide within the biomass. However, the fact that the chemicals could still penetrate despite dilution by void water content was notable. Sassner et al found that increasing the moisture content of steam pretreated *Salix* to 59% resulted in lower loss of sugar during pretreatment and liquid streams that were more easily fermentable [139].

The implications of this work cannot be understated. By simply increasing the moisture content of biomass prior to SO_2-catalysis and steam pretreatment, the yield of ethanol can be increased by over 25%. This represents a promising means of increasing commercial ethanol yields through simply monitoring and altering moisture of biomass as it enters the process. Improved solids digestibility also represents a potential cost savings in that reduced enzyme loadings are required for the same ethanol yield. These results also go a long way towards explaining discrepancies in the literature in overall ethanol yields from similar feedstocks in different labs. Unless the moisture content of the starting biomass is the same, it is difficult to compare the results of experiments utilizing the same biomass.

Future work will investigate whether soaked biomass is equivalent to fresh cut biomass in terms of the final achievable ethanol yield. In particular, examination of fresh, dried and soaked biomass at the microscopic level might reveal changes in structure upon drying and explain the differential efficacy of SO_2 amongst otherwise similar samples. Secondly, both feedstocks used

in this study required SO_2 for effective pretreatment. A feedstock that could be successfully treated without catalysis, such as corn stover [49], would demonstrate whether soaking the biomass prior to uncatalyzed pretreatment increases overall ethanol yield or if increased moisture only helps when in conjunction with SO_2. Finally, techno-economic analysis of the feasibility of increasing the moisture content of materials like switchgrass that are typically shipped to the mill after drying in the field would determine if the increase in ethanol yield surpasses the additional cost of hydration.

2.8 Conclusions

The moisture content of biomass at the time of SO_2 impregnation and subsequent steam pretreatment has a major impact on the final ethanol yield, with water soaked, SO_2-catalyzed biomass providing an 18-28% increase in the amount of ethanol produced after SSF. These higher ethanol yields are thought to be due to improved efficacy of SO_2 catalysis. This results in increased xylan removal, increasing cellulose accessibility and eventual hydrolyzability of the pretreated biomass. These findings have the potential to improve reproducibility of laboratory scale research and reduce costs at the industrial scale by reducing enzyme loadings and improving ethanol yields.

2.9 Acknowledgements

We'd like to thank Andrew Green, Rich Palmer and Hiroshi Morihara at HM3 Inc. for allowing us to use their steam gun and Novozymes and Weyerhaeuser Inc. for providing the raw biomass. Thanks also to Lisa Lai, Azra Vajzovic and Frankie McCaig for all of their help in the lab along with Rick Gustafson and Bill McKean for their invaluable insights.

Chapter 3: The effect of moisture content on steam explosion pretreatment and enzymatic hydrolysis of hybrid poplar

Abstract

Hybrid poplar is an ideal feedstock for bioconversion to ethanol as it can be grown sustainably in many regions with minimal energy inputs. By converting poplar to bioethanol, the growing ethanol industry can more readily produce the large volumes of ethanol required by federal law without utilizing agricultural crops. Hardwoods like poplar can be converted to ethanol by acid-catalyzed steam pretreatment followed by enzymatic hydrolysis and fermentation. It has been shown that the moisture content of the biomass has a significant effect on the digestibility and subsequent glucose yield of the pretreated solids. However, biomass supplied to a future biorefinery will vary in moisture content, potentially resulting in inconsistent yields. This study examines the effect of introducing moisture to dried chips on the digestibility of the resulting solids.

Poplar chips at different moisture contents were pretreated with SO_2-catalyzed steam explosion. Prior to SO_2 impregnation at high and low concentrations the chips were subjected to soaking and steaming in order to increase the moisture content. Chips that were impregnated with less than 2% SO_2 at less than 30% moisture content were 30% less digestible than those with 57% moisture content. However, when 2.5% or higher SO_2 was added, the moisture content did not affect the subsequent digestibility of the pretreated solids. Chips with lower moisture content did, however, have a greater amount of uncooked material (rejects), which was shown to hydrolyze poorly. This work shows that the hydrolysability of low moisture content hybrid poplar can best be improved by increasing the chip moisture content to above 50% and utilizing 1.5% SO_2.

3.1 Introduction

Bioethanol has the potential to offset the use of fossil fuels and alleviate the many associated environmental, economic and social concerns. First generation bioethanol produced from starch and sucrose currently makes up nearly all of the bioethanol produced worldwide. However, as ethanol demand continues to increase, concerns over using resource-intensive food crops for fuel have led to the development of second generation bioethanol produced from cellulosic feedstocks [4, 140]. In 2011, cellulosic ethanol production in the United States was only 7 million gallons. Production must increase dramatically in order to reach the federally mandated 16 billion gallons per year of ethanol by 2020 [1]. To meet this demand, all aspects of ethanol production must improve, but the most dramatic change will be the increase in cellulosic biomass required to meet the raw feedstock requirements. To avoid land and end use conflicts with existing crops, it is essential to make efficient use of marginal land which is not currently being utilized. Many crops can be grown on marginal land to a high density, but the ideal crop depends on the land available in the geographical region in which it will be used [141].

Lignocellulosic feedstocks include woody and herbaceous material like softwoods, hardwoods, agricultural residues and energy crops. Poplar grows well in temperate climates as a short rotation woody crop; in the Pacific Northwest, 1.2 million acres of land are suitable for hybrid poplar plantings [142]. It can be grown plantation-style to high density with very high yields per acre and a 6- to 10-year rotation [143]. In fact, the yield of biomass from commercial Washington plantations managed on six-year rotations is 10-12 tonne/ha (4.4-5.5 ton/acre) [142, 144]. Hybrid poplar can also be utilized as a pulp and paper feedstock [145], for bioremediation of contaminated soil [146] and for lumber and engineered wood products [147], making it a

versatile and valuable resource.

Poplar and other woody feedstocks are likely to be harvested and brought to the biorefinery from different locations at different times of the year, resulting in a non-uniform and changeable feedstock supply. The moisture content in particular will vary significantly. While most herbaceous feedstocks are dried before storage to prevent storage losses, woody feedstocks like poplar can be chipped and stored at either high or low moisture content [97]. The moisture content has an impact on every upstream process from the biorefinery, including harvest, shipping, comminution and storage. It also impacts downstream processes; enzymatic hydrolysis has been shown to be less effective due to collapse of cell wall capillaries and resulting decrease in pore size due to drying [148, 149]. In herbaceous residues, ethanol production has been shown to be lower in biomass that was dried prior to pretreatment [50].

The moisture content of biomass is known to be very important, both with regards to upstream processing and downstream bioconversion. Although dry chips are less expensive to ship and less susceptible to microbial contamination, they are thought to be more difficult to break down and be converted to sugar, ethanol and other products. This study will explore whether altering the moisture content of dried chips can bring them back to their never-dried state in terms of SO_2 uptake, composition and enzymatic digestibility.

3.2 Methods and materials

Figure 3-1, below, illustrates the processes used in this work.

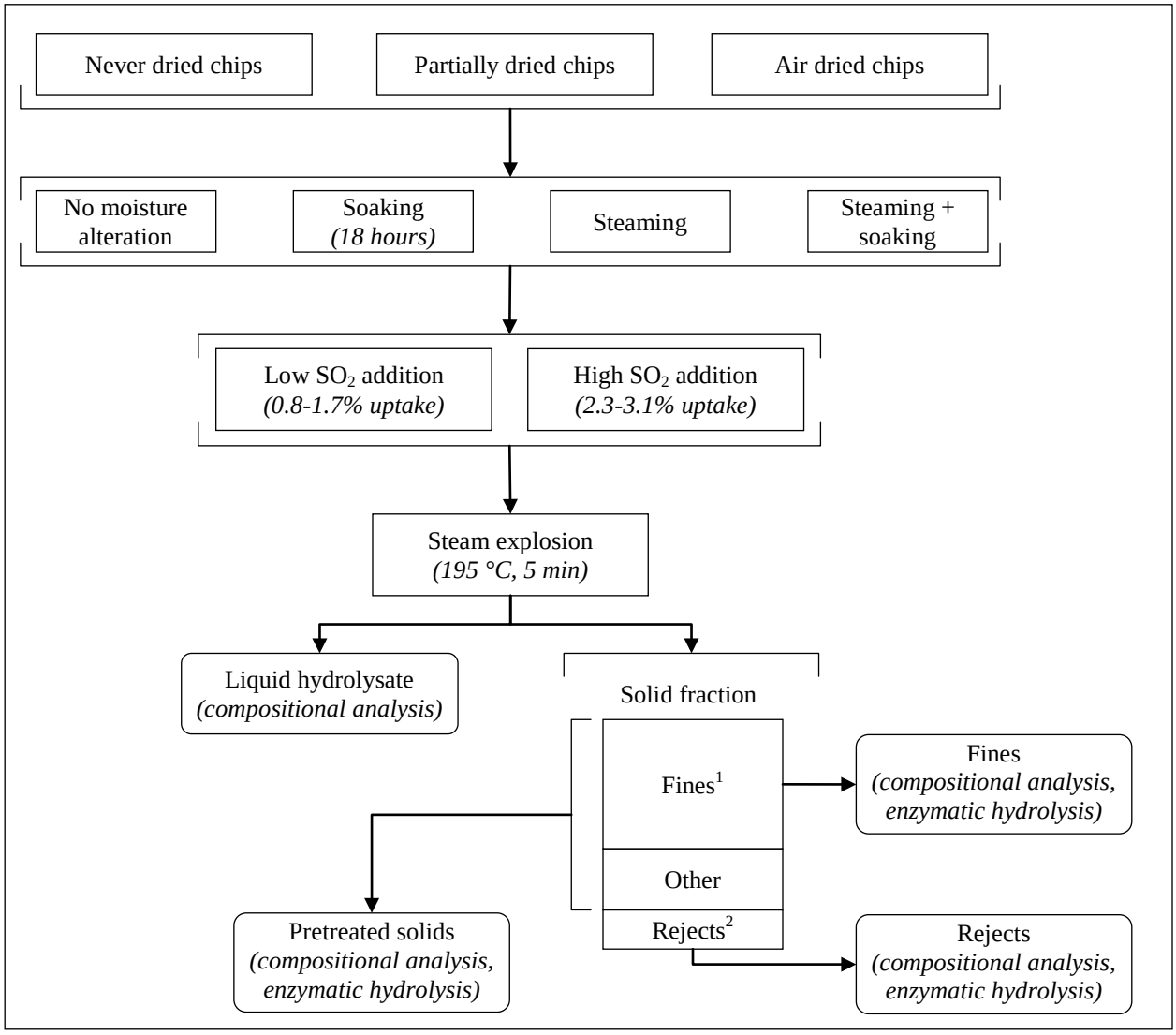

Figure 3-1 Process flow diagram detailing drying, moisture alteration, SO_2 impregnation pretreatment and hydrolysis of hybrid poplar. [1]Fines were separated only from the air-dried, high-SO_2 solids and made up approximately 50% of the dry weight of the pretreated solids. [2]Not all samples contained rejects.

3.2.1 Biomass preparation

Hybrid poplar was obtained from Forest Concepts (Auburn WA) as whole peeled logs 15-20 cm in diameter. They were chipped the day following harvest at the Acrowood test facility (Everett WA) in a slant disc chipper and screened to approximately 5mm thickness and 1-3 cm length and width. Chips were bagged and stored at -20 °C.

Prior to pretreatment, chips were thawed at 25 °C and thoroughly mixed to ensure even distribution of moisture. Never dried (ND) chips at 55% moisture were used immediately. Partially dried (PD) chips were spread in a thin layer and allowed to dry for 2 days at 25 °C, after which they were combined in a plastic bag for 16 hours to allow the moisture content to equilibrate. Chip moisture was found to be 24-31% (Table 3-2). Air dried (AD) chips were treated in the same manner, but allowed to dry for 4 days to a final moisture content of 8%.

Soaking of chips was carried out by submerging a cheesecloth bundle containing 800 g OD of chips in a bucket of water at 4 °C for 18 hours. Chip moisture content after soaking was measured, as was the composition of the soaking liquid. Steaming of chips was carried out in a Thermo Scientific SterileMax Tabletop Sterilizer set to 105 °C for 10 minutes as follows. Cheesecloth bundles of 800 g OD chips were placed in the preheated autoclave. The cycle heated for approximately 15 minutes, held at 105 °C for 10 minute and vented for 5 minutes, at which time the chips were removed and either immediately submerged in a bucket of water for 10 minutes or placed in a zip-top bag. Following all soaking procedures, chips were spun in a Bock spin-dryer for 30 seconds to remove excess moisture. Moisture content of all samples was measured before SO_2 impregnation.

3.2.2 Pretreatment and processing conditions

Low SO_2 samples were impregnated with SO_2 as follows. 800 g OD chips were placed in plastic zip-top bags and 24 g of gaseous SO_2 added from a 454 g lecture bottle (Sigma) as determined by weighing the bag before and after SO_2 addition. The bags were sealed and left for 18 hours in the fume hood either uncontained (Low-SO_2 samples) or sealed in a 20 L polypropylene bucket. After 18 hours the bags were opened for 5 minutes to allow unabsorbed SO_2 to escape and then weighed to determine actual SO_2 absorption. The contents of each bag were split into two 400 g OD portions for subsequent steam explosion pretreatment.

Each 400 g OD portion was steam pretreated for 5 minutes at 195 °C (188 psi) in a 2.7 L steam gun (Aurora Technical, Savona BC) with explosive decompression into a water-jacketed catch tank. Both 400 g shots were collected together and the steam gun washed with water, which was collected for a complete mass balance.

The resulting slurry was separated by vacuum filtration in a Buchner funnel to yield solid and liquid fractions. The liquid hydrolysate fraction, along with the wash fraction, was analyzed for monomeric and oligomeric carbohydrates, acetic acid, HMF and furfural. The solid fraction was first washed with sufficient water to equal approximately 15 times the OD weight of solids being washed. During washing, any large rejects which floated to the surface were removed and weighed. Large rejects were defined as the material that floated; visibly, they appeared to be chip centers and were as large as 5 x 10 x 5 mm in size. The rejects were disintegrated in a 2 L disintegrator for 5000 rpm at 1.2% consistency prior to compositional analysis and enzymatic hydrolysis. Rejects were not removed from or measured in the one low-SO_2 substrate that contained them because they became too waterlogged after storage to be separated.

The washed, reject-free solids were analyzed for carbohydrates, lignin and acetate groups and then enzymatically hydrolyzed as described below.

Separation of fines

400 g OD of unwashed, steam pretreated air-dried chips (high SO_2) were placed in open-weave cheesecloth (10 mesh) and submerged repeatedly in water so that fine particles could wash through the cheesecloth. The fines were collected by vacuum filtration, weighed and determined to make up approximately 50% of the total weight of the solids.

Severity factor calculation

The severity factor for all the pretreated sample was the same, 3.50, and was calculated using the formula $\mathbf{R_o = te^{(T-100)/14.75}}$ [54]. The combined severity factor (CS) was calculated using the formula $\mathbf{CS=logR_o - pH}$ [55] using the pH of the liquid hydrolysate after pretreatment.

3.2.3 Instrumental analysis

HPLC

Monomeric carbohydrates were measured by pulsed amperometric electrochemical detection on a Dionex ICS 3000 HPLC. The method used a flow rate of 1 ml/min and mobile phase of deionized water for the first 30 minutes followed by 10 min of 0.2 M NaOH, followed by 10 minutes of deionized water. Samples were diluted as appropriate, spiked with fucose as an internal standard and filtered through 0.22 μm syringe filters. 5-10 μL of sample were injected onto the column, a Dionex Carbopac PA1 fitted with a guard column. After separation of the injected sample on the column, 0.2 M NaOH was added to a tee-junction at 0.5 ml/min using a

post-column AXP pump and mixed with the sample prior to electrochemical detection. Samples were measured against standards consisting of arabinose, galactose, glucose, xylose, and mannose.

Acetic acid, HMF and furfural were measured using refractive index detection on a Shimadzu Prominence LC. Samples were diluted as appropriate, filtered through 0.22 μm syringe filters and 20 μL of sample were injected run on a Phenomenex Rezex RHM H^+ column at 63 °C with an isocratic mobile phase elution of 0.05 mM H_2SO_4 at 0.6 ml/min. Standards were prepared and used to quantify the unknown samples.

3.2.4 Compositional analysis

Insoluble carbohydrates and lignin

Solids were analyzed gravimetrically for lignin content, photometrically for soluble lignin, and by HPLC for carbohydrate content using the TAPPI method T-222 om-98 [129]. Briefly, 0.2 g of 40-mesh ground oven dried sample was mixed with 3 ml of 72% H_2SO_4 for 120 minutes, diluted with water to 120 ml total volume, and autoclaved at 121 °C for 60 minutes. The samples were then filtered through tared fritted glass crucibles which were oven dried and weighed to determine acid insoluble lignin. Since the acid insoluble material included ash, the ash content was subtracted from the total acid insoluble lignin amount. The filtrate was analyzed by HPLC for carbohydrate composition and by UV at 205 nm with an extinction coefficient of 110 L/g-cm for acid-soluble lignin content.

Soluble carbohydrates

Monomeric and oligomeric soluble carbohydrates were determined using NREL LAP TP-510-42623 [130]. Briefly, samples were diluted by half and 72% H_2SO_4 added to reach a pH of 0.07. These samples were then autoclaved at 121 °C for 60 minutes to determine the total sugar concentration. Monomeric sugars were determined by analyzing the original samples by HPLC without acid hydrolysis. Oligomeric sugar was calculated by subtracting monomeric sugar content from total sugar content.

3.2.5 Saccharification

Enzymatic hydrolysis of washed solids was done at 5% w/v solids in a total volume of 50 ml in 125 ml Erlenmeyer flasks. The solution was buffered at pH 4.8 with 0.05 M sodium acetate buffer and the hydrolysis was completed at 50 °C and 150 rpm shaking on an orbital shaking incubator (New Brunswick). Cellulase (Spezyme-CP, 26 FPU/ml, Sigma) was added at 10 FPU/g cellulose and supplemental beta-glucosidase (Novozym 188, 492 CBU/ml, Sigma) was added at 20 CBU/g cellulose. 1 ml samples were periodically removed and analyzed for glucose and xylose.

3.3 Results and Discussion

3.3.1 Moisture adjustment

To investigate the effect of moisture on hybrid poplar bioconversion, firstly never-dried (ND, 55% MC) poplar chips were dried at 25 °C to two different moisture levels: partially dried (PD, 24-31% MC) and air dried (AD, 8% MC). These chips were then subjected to moisture adjustment in two different ways: steaming and soaking (Figure 3-1). Soaking in water has been

used with success previously to increase hydrolysability of sugarcane bagasse and switchgrass [50] and also found to increase water retention in steam exploded eucalyptus chips [102]. In order to ensure full saturation of the chips, they were soaked overnight under refrigeration. HPLC analysis showed there was no sugar present in the soaking liquid (data not shown).

Steaming is well established as a way of removing entrapped air from chips by replacing it with water vapor [150]. It was hoped that this would aid in both the diffusion of SO_2 and penetration of steam into the center of the chips. Cooling of steamed chips to ambient temperature causes any water vapor in the cell pores to condense and create a slight vacuum, so immediately submerging steamed chips in cold water was thought to cause water to be drawn into the pore spaces. To investigate this, a portion of chips was steamed and cooled to ambient temperature prior to impregnation while a second portion was steamed and immediately submerged in cold water.

Steaming alone increased the weight of the chips by only 0-3% (Table 3-1), indicating that the amount of condensed steam was minimal. Chips that were soaked and steamed + soaked had moisture contents 1-10% higher than the never-dried chips. This could be due to experimental error, but could also be due to displacement by water of air bubbles in the never-dried wood. Chen [151] found that an increase of 7% moisture content could be observed by re-wetting air-dried yellow poplar.

Table 3-1. Moisture content of chips before and after moisture adjustment and amount of SO_2 absorbed by hybrid poplar chips which were never dried (ND), partially dried (PD) or air dried (AD) prior to moisture adjustment, SO_2 impregnation and steam pretreatment.

	Chip moisture content		Absorbed SO_2
	Initial	After moisture adjustment	
	%	%	%
Low-SO_2 ND	55	55	1.6
Low-SO_2 PD	31	31	0.8
Low-SO_2 ND soaked	55	57	1.6
Low-SO_2 PD soaked	31	54	1.7
ND	53	53	2.7
PD	24	24	2.5
AD	8	8	3.1
ND soaked	53	57	2.9
PD soaked	24	57	2.6
AD soaked	8	58	2.7
ND steamed	53	52	2.8
PD steamed	24	25	2.3
AD steamed	8	11	3.1
AD steam+soak	8	55	2.3
PD steam+soak	24	63	2.7

3.3.2 SO_2 absorption

All samples were impregnated in plastic zip-top bags with 3% wt/wt SO_2. By allowing excess SO_2 to diffuse out of the bags, the four low-SO_2 samples retained only a third to a half of the added SO_2 (Table 3-2). The remaining 11 samples retained nearly all of the added SO_2 because the zip-top bags were sealed inside a bucket after the SO_2 was added, preventing the gas from diffusing out of the bags and being lost to the atmosphere. The low-SO_2 sample bags were not sealed inside a bucket and over the course of 18 hours, a significant amount of SO_2 was lost.

This created two subsets of samples with both low and high SO_2 amounts. All samples were steam pretreated with the same conditions; 195 °C for 5 minutes. The pH of the liquid

hydrolysate fraction after vacuum filtration of the pretreated slurry was measured. Not surprisingly, it was found that there was a strong correlation between the amount of SO_2 absorbed by the chips and the resulting pH of the liquid hydrolysate (Table 3-2). When the pH was used to calculate the combined severity factor (CS) for each sample, a linear fit to the plot of absorbed SO_2 vs pH resulted in an R^2 value of 0.84. This confirms that the amount of SO_2 absorbed by the chips directly determines the pH of the pretreated liquid hydrolysate.

Table 3-2. Hydrolysate consistency and pH, pretreatment severity and solids reject content of steam pretreated hybrid poplar chips which were never dried (ND), partially dried (PD) or air dried (AD) prior to moisture adjustment, SO_2 impregnation and steam pretreatment.

	Hydrolysate		Severity		Rejects
	Consistency %	pH	log R_o	logR_o-pH	%
Low-SO_2 ND	21	1.66	3.50	1.84	0
Low-SO_2 PD	26	2.00	3.50	1.50	present*
Low-SO_2 ND soaked	25	1.61	3.50	1.89	0
Low-SO_2 PD soaked	20	1.76	3.50	1.74	0
ND	24	1.50	3.50	2.00	0
PD	33	1.39	3.50	2.11	7
AD	34	1.44	3.50	2.06	19
ND soaked	21	1.45	3.50	2.05	0
PD soaked	21	1.46	3.50	2.04	0
AD soaked	21	1.50	3.50	2.00	0
ND steamed	24	1.41	3.50	2.09	0
PD steamed	35	1.50	3.50	2.00	5
AD steamed	38	1.39	3.50	2.11	11
AD steam+soak	22	1.58	3.50	1.92	0
PD steam+soak	21	1.40	3.50	2.10	0

***rejects were determined to be present in the solids, but were not quantified**

The consistency of the slurry recovered from the steam gun varied considerably (Table 3-2), but was directly correlated to the moisture content of the biomass. The higher the moisture content of

the biomass entering the steam gun, the lower consistency of the slurry, with a linear fit of the data providing an $R^2 = 0.869$. It has been reported that higher moisture content biomass results in increased steam consumption and therefore increased costs [152]. However, examining the amount of added water coming from condensed steam compared to the initial moisture content reveals that there is no correlation between the moisture content of material entering the steam gun and the resulting steam condensate produced ($R^2 = 0.009$).

3.3.3 Compositional analysis

Table 3-3. Composition (in g/100 g) of raw hybrid poplar and the solids resulting from of steam pretreated chips that were never dried (ND), partially dried (PD) or air dried (AD) prior to moisture adjustment, SO_2 impregnation and steam pretreatment. Standard deviations were determined from triplicate measurements to be less than 3%.

	Glucose	Xylose	Lignin		Acetate
			insoluble	soluble	
Raw poplar	51	17	22	3.0	4.02
Low-SO_2 ND	70	1.0	33	2.9	0.14
Low-SO_2 PD	69	3.5	32	3.4	0.41
Low-SO_2 ND soaked	67	2.8	32	3.4	0.12
Low-SO_2 PD soaked	71	2.7	32	2.9	0.12
ND	72	0.5	32	2.1	0.00
PD[1]	66	0.7	32	2.5	0.08
AD[1]	67	1.1	32	2.3	0.22
ND soaked	72	0.4	33	2.2	0.00
PD soaked	67	0.3	30	2.1	0.06
AD soaked	68	0.3	30	2.1	0.05
ND steamed	71	0.6	31	2.4	0.00
PD steamed[1]	64	0.7	31	2.7	0.08
AD steamed[1]	64	1.0	32	2.6	0.21
AD steam+soak	70	0.5	29	2.2	0.07
PD steam+soak	69	0.4	28	2.6	0.03

[1]Rejects were removed prior to compositional analysis and hydrolysis

The pH of the hydrolysate and the range of severities used had little effect on the composition of the pretreated solids (Table 3-3), all of which contained 66-72% glucose and 28-33% acid insoluble lignin. The amount of xylose in the solids was more variable, but very low in all samples. Acetate groups were slightly higher in samples containing more xylan, but generally low in all samples.

The amount of HMF, furfural and acetic acid in the liquid stream is shown in Table 3-4. HMF concentrations ranged from 0.12-0.33 g/100 g original biomass. Furfural ranged from 0.64-1.7 and acetic acid from 2.6-4.4 g/100 g original biomass. The actual concentrations of each inhibitor were much more variable, with more concentrated liquid streams generated by the low moisture biomass. In particular, acetic acid concentrations in the liquid hydrolysate ranged from 8.9-22.5 g/L. It has been shown that acetic acid is inhibitory to *Saccharomyces cerevisiae* at concentrations above 10 g/L [153], so most of the hydrolysates produced would need to be detoxified, diluted or fermented with a more tolerant organism. The hydrolysate inhibitor concentrations are high, but so are the sugar concentrations, which range from 11-34 g/L glucose, and 31-57 g/L xylose (data not shown). One reason for this is that the steam pretreatment reactor used for this research employs a trap that removes steam condensate from steam before it enters the reactor. This results in a higher consistency slurry than what has been observed coming from other steam guns, and a correspondingly more concentrated hydrolysate.

Table 3-4. Liquid fraction concentrations of HMF, furfural and acetic acid in g per 100 g of raw biomass following pretreatment of hybrid poplar treated with different moisture regimes.

	HMF		Furfural		Acetic acid	
	g/100g	g/L	g/100g	g/L	g/100g	g/L
Low-SO_2 ND	0.30	0.93	1.13	3.38	4.09	12.65
Low-SO_2 PD	0.12	0.44	0.64	2.28	2.64	9.58
Low-SO_2 ND soaked	0.20	0.65	0.70	2.08	2.83	9.25
Low-SO_2 PD soaked	0.16	0.43	0.91	2.35	3.37	8.86
ND	0.29	1.01	1.62	5.55	3.86	13.60
PD	0.27	1.32	1.23	5.55	4.03	20.03
AD	0.18	0.95	0.84	4.02	3.29	17.03
ND soaked	0.31	0.97	1.65	5.07	4.06	12.89
PD soaked	0.24	0.71	1.34	3.71	4.28	12.87
AD soaked	0.21	0.66	1.30	3.80	4.40	13.57
ND steamed	0.33	1.22	1.70	6.24	4.04	15.19
PD steamed	0.26	1.40	1.36	6.62	3.82	20.42
AD steamed	0.21	1.34	1.05	5.61	3.63	22.52
AD steam+soak	0.20	0.62	1.03	3.06	3.70	11.64
PD steam+soak	0.26	0.77	1.35	3.76	4.39	13.32

3.3.4 Hydrolysis (low SO_2)

Hydrolysis of the low-SO_2 substrates, shown in Figure 3-2, revealed that there were significant differences between them. The partially dried (PD) material provided significantly lower cellulose conversion as compared to the other three samples, which averaged 73% conversion of cellulose conversion to glucose. These results showed that soaking the PD chips prior to pretreatment improved the hydrolysability of the resulting solids by 30%. It is unclear from these results whether soaking improves the hydrolysability because of the increased moisture content alone or because low moisture chips absorbed half of the SO_2 of the soaked chips. To further investigate the effect of SO_2, the hydrolysability of the chips that were "forced" to absorb more than 2.5% SO_2 was measured.

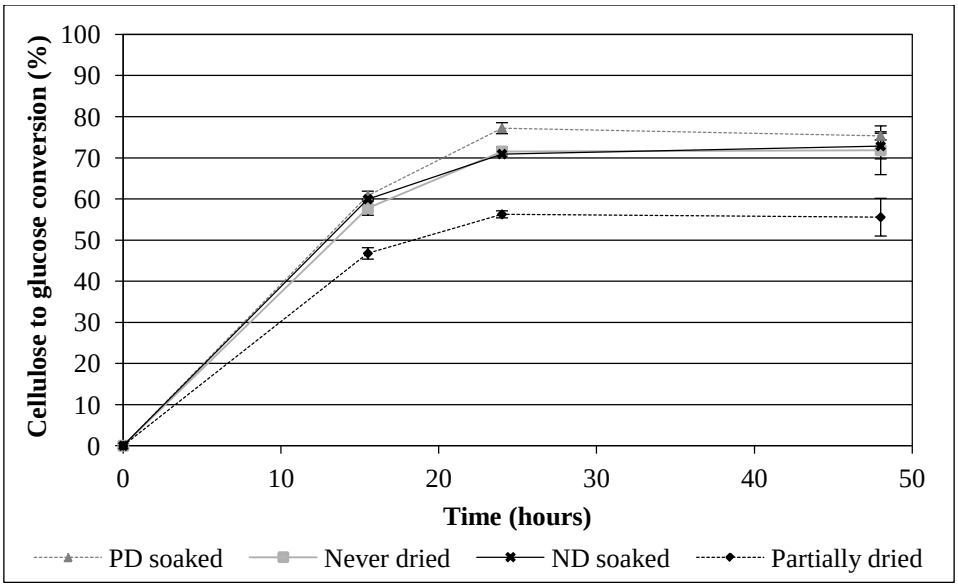

Figure 3-2. Cellulose to glucose conversion in for hybrid poplar chips which were never dried (ND) or partially dried (PD) prior to moisture adjustment, low SO_2 impregnation and steam pretreatment.

3.3.5 Hydrolysis (high SO_2)

Prior to compositional analysis and hydrolysis of the high SO_2 substrates, large uncooked particles (rejects) were observed in and removed from four samples (PD, AD, PD-steamed and AD-steamed, Figure 3-1, Table 3-2) in order to improve the reproducibility and uniformity of the reactions. No such particles were observed in any of the other samples.

Rejects can come from either the feedstock as uncooked knots, contaminants or large particles, or be a consequence of the pretreatment conditions and represent uncooked material. Since the same amount of reject material was not present in all pretreated samples, their presence in only some of the samples must be attributable to the pretreatment process and not the feedstock material. Specifically, rejects were present in partially dried, air dried, PD-steamed and AD-steamed pretreated solids. These were the four chips that were below 25% moisture content at the time of impregnation and pretreatment, and Figure 3-3 does show that there is a direct

correlation between the moisture content at the time of pretreatment and the amount of reject material recovered from the sample (R^2 = 0.841). Interestingly, AD and PD chips that were steamed and immediately soaked showed no sign of rejects; the same was true for chips that were soaked overnight. The reject content of the low-SO_2 chips was not quantified, but none were visible in ND, ND soaked and PD soaked. Some rejects were present in the PD substrate, but were not quantified.

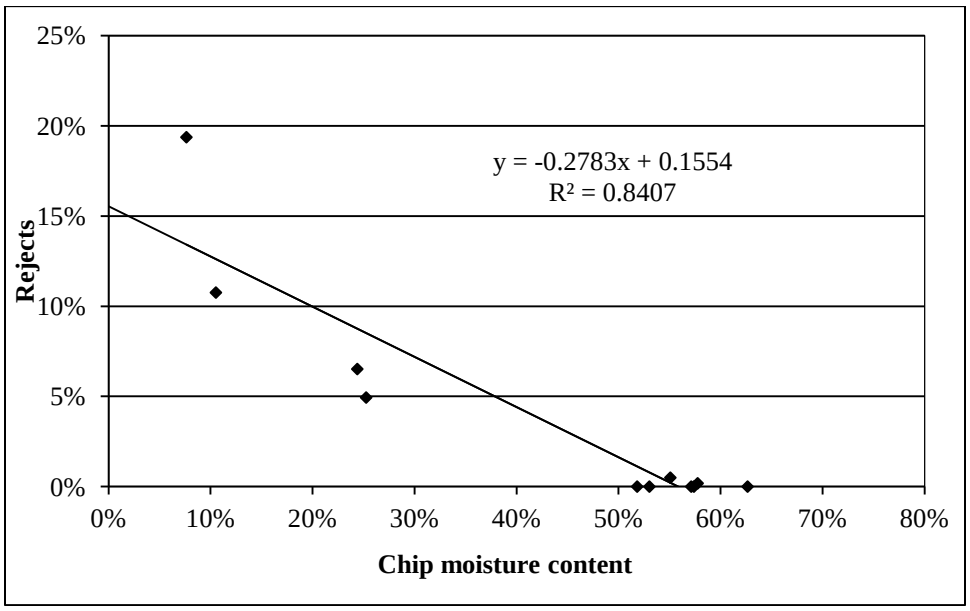

Figure 3-3. Relationship between chip moisture content prior to pretreatment and amount of rejects found in the pretreated solids.

The visible rejects in the high-SO_2 substrates are likely the undercooked centers of chips in which the catalyst did not penetrate to the center [100]. These samples absorbed the same amount of SO_2 as the other samples, so the only variable was the moisture content. The relatively low moisture within the chips may have prevented the SO_2 from penetrating to the center of the chip. The SO_2 then concentrated on the outer surface of the chips leading to overcooking of the

outside and undercooking of the inside.

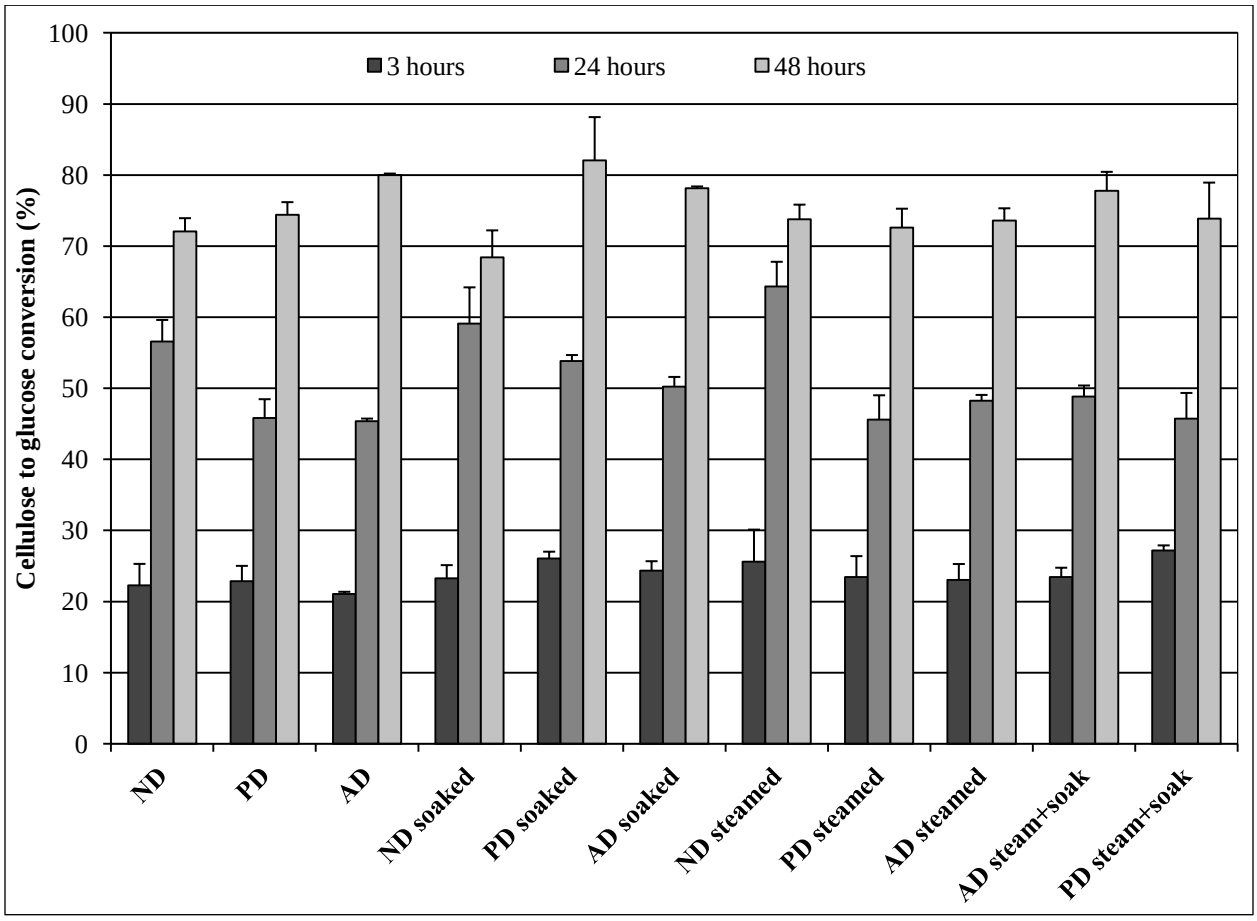

Figure 3-4. Cellulose to glucose conversion in 3, 24, and 48 hours for hybrid poplar chips which were never dried (ND), partially dried (PD) or air dried (AD) prior to moisture adjustment, SO_2 impregnation and steam pretreatment.

To determine whether there were any differences in digestibility between the different moisture regimes, the reject-free substrates were hydrolyzed. Hydrolysis conversions were not significantly different between any of the samples (Figure 3-4). The low moisture samples (AD, PD, AD-steamed, and PD-steamed) did now show significantly lower conversion than their higher-moisture counterparts. This is in contrast to the low-SO_2 samples, in which the lower moisture content chips produced a substrate that was 30% less digestible. This could be due to

the fact that rejects were present in the low-SO_2 hydrolysis or it could be that the increased amount of SO_2 is able to overcome the low moisture content limitation.

The low-SO_2 air dried chips generated the greatest amount of rejects, so in an effort to understand the effect of the rejects on enzymatic hydrolysis, rejects and fines were fractionated from the material, analyzed and hydrolyzed. Fines were defined in this case as particles small enough to fit through the pores of 10 mesh cheesecloth and made up approximately 50% of the total solids. As shown in Table 3-5, the composition of the fines was very similar to the reject-free solids in Table 3-3. The rejects contained three times more xylose and detectable amounts of arabinose, galactose and mannose, but were otherwise very similar to the fines.

Table 3-5. Composition in g/100 g raw biomass of reject fractions of air dried, steam pretreated hybrid poplar. Standard deviations were determined from triplicate measurements to be less than 3%.

	Arabinose	**Galactose**	**Glucose**	**Xylose**	**Mannose**	**Lignin**	
						insoluble	soluble
Fines	0.0	0.0	68.6	1.1	0.0	31.2	2.4
Rejects	0.2	0.2	68.9	3.0	0.3	27.7	3.0

3.3.6 Reject hydrolysis

The hydrolysability of fines and rejects is shown in Figure 3-5. Prior to hydrolysis, the rejects were disintegrated to allow the best possible hydrolysis. The fines fraction showed the highest conversion (87%), which was significantly higher than the conversion of the whole substrate (81%). This shows that even though the rejects clearly hydrolyze poorly (57% conversion), the remaining solids are well pretreated. Seemingly, the excess SO_2 absorbed by the chip exterior produced highly digestible solids. Fines in particular have been shown to hydrolyze more readily

than larger fibers, likely due to a combination of their increased surface area and pore volume [154].

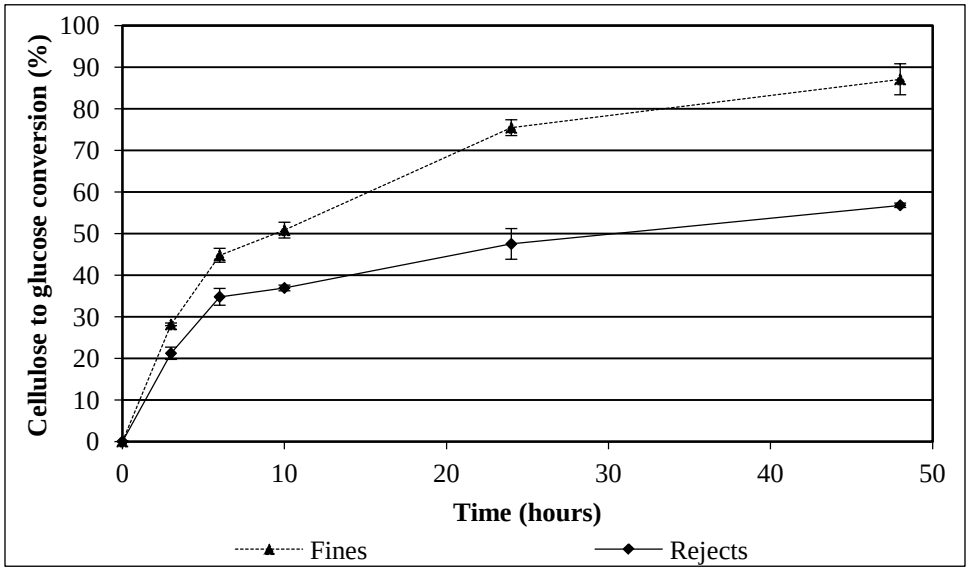

Figure 3-5. Enzymatic conversion of cellulose to glucose of the fines and reject fractions of air dried, steam pretreated hybrid poplar

3.4 Conclusions

The moisture content of hybrid poplar at the time of SO_2 impregnation is important in ensuring that the SO_2 is absorbed and can diffuse to the chip center. With less than 25% moisture and sealed impregnation conditions, SO_2 can be fully absorbed by the chip but seemingly not reach the center, leading to 5-19% rejects, presumably from the centers of larger chips. Rejects were removed from the four samples in which they were observed prior to compositional analysis and hydrolysis. The composition and hydrolysability of the reject-free steam pretreated solids were not affected significantly by the different moisture alteration regimes; hydrolysis yields of 70-80% conversion could be achieved in 48 hours.

The composition of rejects and fines separated from pretreated, air-dried chips were measured and it was found that that the compositions were not significantly different. The hydrolysis of each was significantly different, however, with fines reaching 87% conversion in 48 hours and the rejects only 57%; the difference in hydrolysability is therefore related to the structure of the pretreated solids rather than composition.

This work shows that the hydrolysability of low moisture content hybrid poplar can be improved in two ways: either by impregnating chips with 3% SO_2 or by increasing the chip moisture content to above 50% and adding only 1.5% SO_2. However, adding high SO_2 to low moisture chips may result in the formation of poorly digestible rejects which will be detrimental to overall process yields.

Chapter 4: Real-time understanding of lignocellulosic bioethanol fermentation by Raman spectroscopy[2]

Abstract

A substantial barrier to commercialization of lignocellulosic ethanol production is a lack of process specific sensors and associated control strategies that are essential for economic viability. Current sensors and analytical techniques require lengthy offline analysis or are easily fouled in situ. Raman spectroscopy has the potential to continuously monitor fermentation reactants and products, maximizing efficiency and allowing for improved process control.

In this paper we show that glucose and ethanol in a lignocellulosic fermentation can be accurately monitored by a 785 nm Raman immersion probe, even in the presence of an elevated background thought to be caused by lignin-derived compounds. Chemometric techniques were used to reduce the background before generating calibration models for glucose and ethanol concentration. The models show very good correlation between the real-time Raman spectra and the offline HPLC validation.

Our results show that the changing ethanol and glucose concentrations during lignocellulosic fermentation processes can be monitored in real-time, allowing for optimization and control of large scale bioconversion processes.

[2] Manuscript submitted for publication in October 2012: Ewanick SM, Thompson WJ, Marquardt BJ, Bura R: **Real-time understanding of lignocellulosic bioethanol fermentation by Raman spectroscopy**. *Biotechnology for biofuels* 2012.

4.1 Background

The bioethanol industry produced 22.3 billion gallons of starch and sucrose-based ethanol worldwide in 2011 [15] and is replacing many non-renewable products with products derived from biomass. The cost to produce many of these products, however, is still not competitive with petroleum-derived counterparts. Processes to produce fuels and chemicals from petroleum have a wealth of online analytical sensors that permit them to operate at or near capacity with optimal process yields. This hyper efficiency is a necessary condition for profitability in producing high volume, low value products such as fuels and some commodity chemicals. The need for process efficiency – and hence the need for online sensors – is especially acute in biomass fed biorefineries due to the complexity and expense of the feedstock. Development of robust sensors for lignocellulosic biorefineries is as critical as the research that has gone into developing the processes themselves, but has received little or no attention. Process improvements (pretreatments, microorganisms, enzymes, etc.) will likely reduce costs in the future, but in both the short and long term, improving the efficiency of existing operations will have the greatest effect on overall process economics.

In a typical ethanol production process, raw biomass is first pretreated, then saccharified, fermented and purified. The liquid fraction following acidic pretreatment and saccharification is high in soluble lignin, phenolics, sugar degradation products (e.g. furfural) and monomeric and oligomeric hemicellulosic sugars. Monomeric sugars are fermented using microorganisms that primarily produce ethanol, so both high and low concentrations of sugar and ethanol must be monitored over the course of fermentation in order to ensure that the fermentation is proceeding optimally. Such a diverse mix of compounds can pose a challenge to current analytical methods;

high performance liquid chromatography (HPLC) with refractive index detection is one of the only methods currently in use that can measure ethanol and carbohydrates simultaneously in the presence of the aforementioned compounds. Although capable of very high sensitivity, good separation and quantification of multiple component mixtures, sample preparation and analysis can be time consuming, costly and not suitable for a process environment. As such, HPLC is usually limited to offline analysis of samples, precluding its use for real-time, continuous analysis. Spectroscopic methods have the potential to rapidly and non-destructively analyze multiple components of a reaction mixture. Raman spectroscopy in particular is an established vibrational spectroscopy technique useful for determining both qualitative and quantitative molecular information from almost any type of sample (e.g. solid, liquid or gas) [155, 156]. A Raman spectrum is obtained by exciting a sample with a laser and measuring the inelastic scattering of photons from the vibrations within the molecules. Raman spectroscopy has been used successfully to measure ethanol alone during fermentations [112, 120, 157], but these techniques have as yet not been fully utilized to provide on-line, real time measurements of lignocellulose-derived materials.

Our objective in this research was to evaluate the possibility of real-time, continuous lignocellulosic fermentation monitoring using Raman spectroscopy. We monitored the progress of fermentation of both synthetic sugars and steam-pretreated switchgrass hydrolysate in a controlled bioreactor using a novel Raman immersion probe inserted in a fast loop parallel sampling system. Chemometric analysis of the reactants and products was done using Principal Component Analysis (PCA) of the Raman spectra and a Partial Least Squares (PLS) model was developed and validated using HPLC data.

4.2 Results

4.2.1 Synthetic glucose fermentation

To first evaluate the effectiveness of the Raman probe under ideal fermentation conditions, a synthetic glucose solution was fermented using *Saccharomyces cerevisiae.* Although fermentation of glucose to ethanol is typically carried out as a batch process, a stepwise fed-batch experiment was run to follow the product consumption and formation rates more clearly over short periods of time. The experiment began with a glucose concentration of 5 g/L and additional aliquots of glucose were added at regular intervals when ethanol production had ceased to increase (determined by monitoring the intensity of the ethanol Raman peak at 883cm^{-1} in real time) until a total of 25 g/L had been added. HPLC validation samples for determination of ethanol and glucose were withdrawn from the vessel at 10-15 minute intervals and Raman spectra were measured automatically every 30 seconds.

Figure 4-1 (inset) shows the full Raman spectrum of the reaction mixture. The region from 350-1800 cm^{-1} shows an increased background due to water and some evidence of cosmic ray interference. To mitigate these effects and evaluate the elements of the spectra changing over time it was necessary to process the data with in-house data pre-treatment algorithms. The water background removal algorithm is modified from the polyfit algorithm [121] by applying a moving window to the polynomial subtraction routine that reduces the effects of baseline shifts and fluctuating background. Data were also processed using a cosmic ray removal filter [158] which compares each spectrum to the ones preceding and proceeding it, identifying the transient cosmic spikes and removing them. The remainder of Figure 4-1 shows the region of interest after all pretreatment algorithms. These data were used for the development of multivariate models.

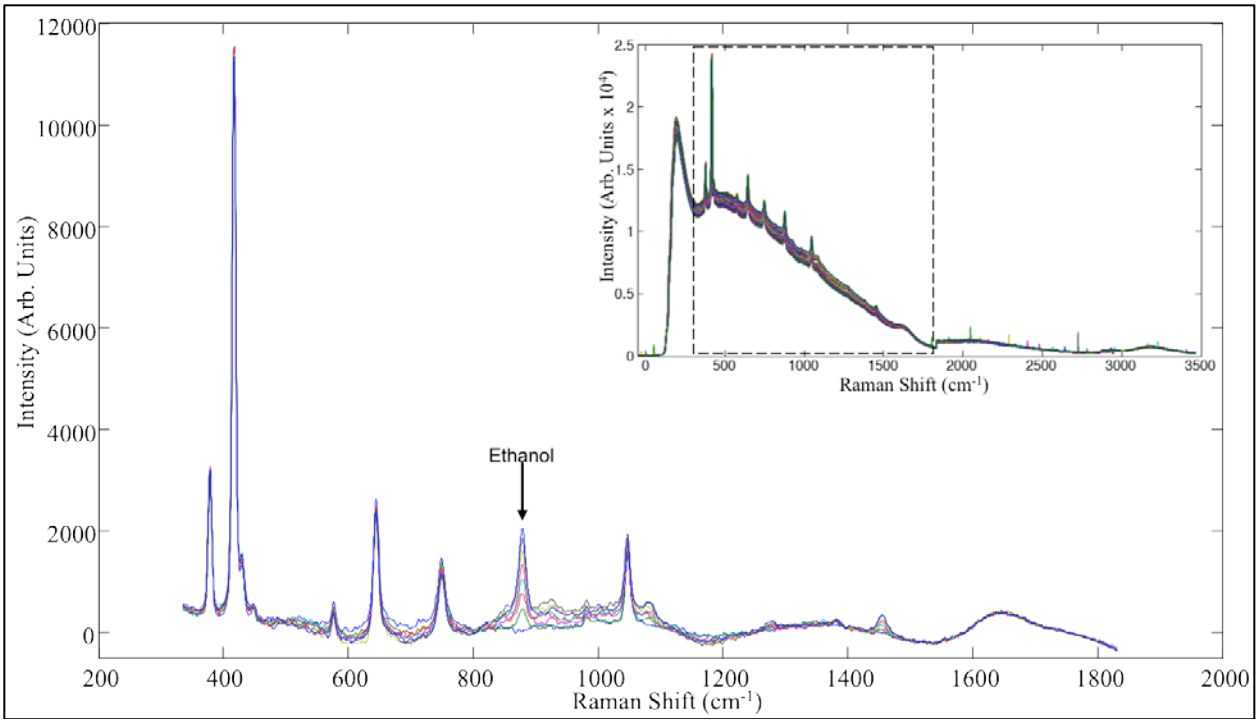

Figure 4-1. Raw spectra (inset) were pretreated with a polynomial fitting routine to reduce the elevated background and a cosmic ray removal algorithm to remove spurious peaks caused by the high energy particles from the sun. In the pretreated spectra, the ethanol peak can be easily seen at 883 cm^{-1}.

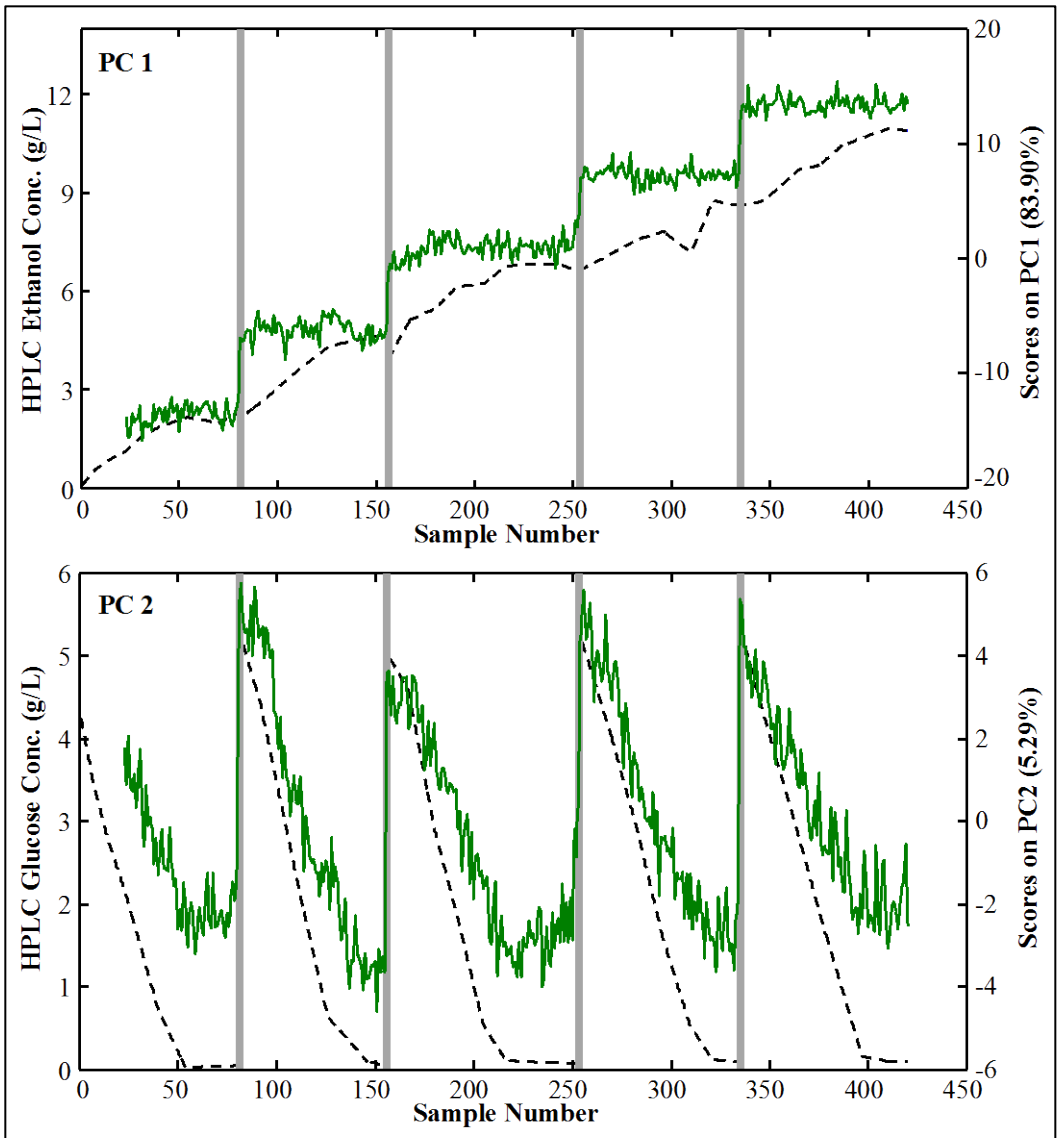

Figure 4-2. Correlation between scores data and reference ethanol and glucose concentrations measured by HPLC. The dashed lines show the concentration of the analytes by HPLC while the solid trace shows the principal component score of the Raman data. Ethanol correlates to principle component one (PC1, top) while glucose can be seen on principle component two (PC2, bottom). The vertical grey lines indicate added aliquots of glucose.

Principal Component Analysis (PCA) of the Raman data determined two components comprised 89% of the variance in the data and the scores of these components correlated well to the HPLC concentration data of ethanol on the first principal component (PC) and glucose on the second PC (Figure 4-2).

While simple monitoring of the fermentation rates provides some information, determining the actual concentration of the reactants is essential to compare the process to past and future processes. The Raman data were evaluated by Partial Least Squares (PLS) using the HPLC results as the calibration concentration data set (Figure 4-3). The data were pretreated with Orthogonal Signal Correction (OSC) and mean centering to mitigate any non-relevant variation. The PLS models were cross validated by removing random prediction subsets. Cross validation provides a means to evaluate the performance of the models by removing a subset of the data, generating a model from the remaining data and applying the subset as a test set. The Root Mean Square Error of Cross Validation (RMSECV) defines the model's ability to accurately predict the test set samples. The models correlated well with the HPLC data over the full range of concentrations (0.1-11 g/L – ethanol and 0.1-5.5 g/L – glucose), and a standard limit of detection as defined as 2x the RMSECV allows quantification of 1 g/L and above for either ethanol or glucose (Table 4-1). These results indicate that even with the varied rates of glucose uptake and ethanol production, the reaction components were detected and followed over the course of the reaction.

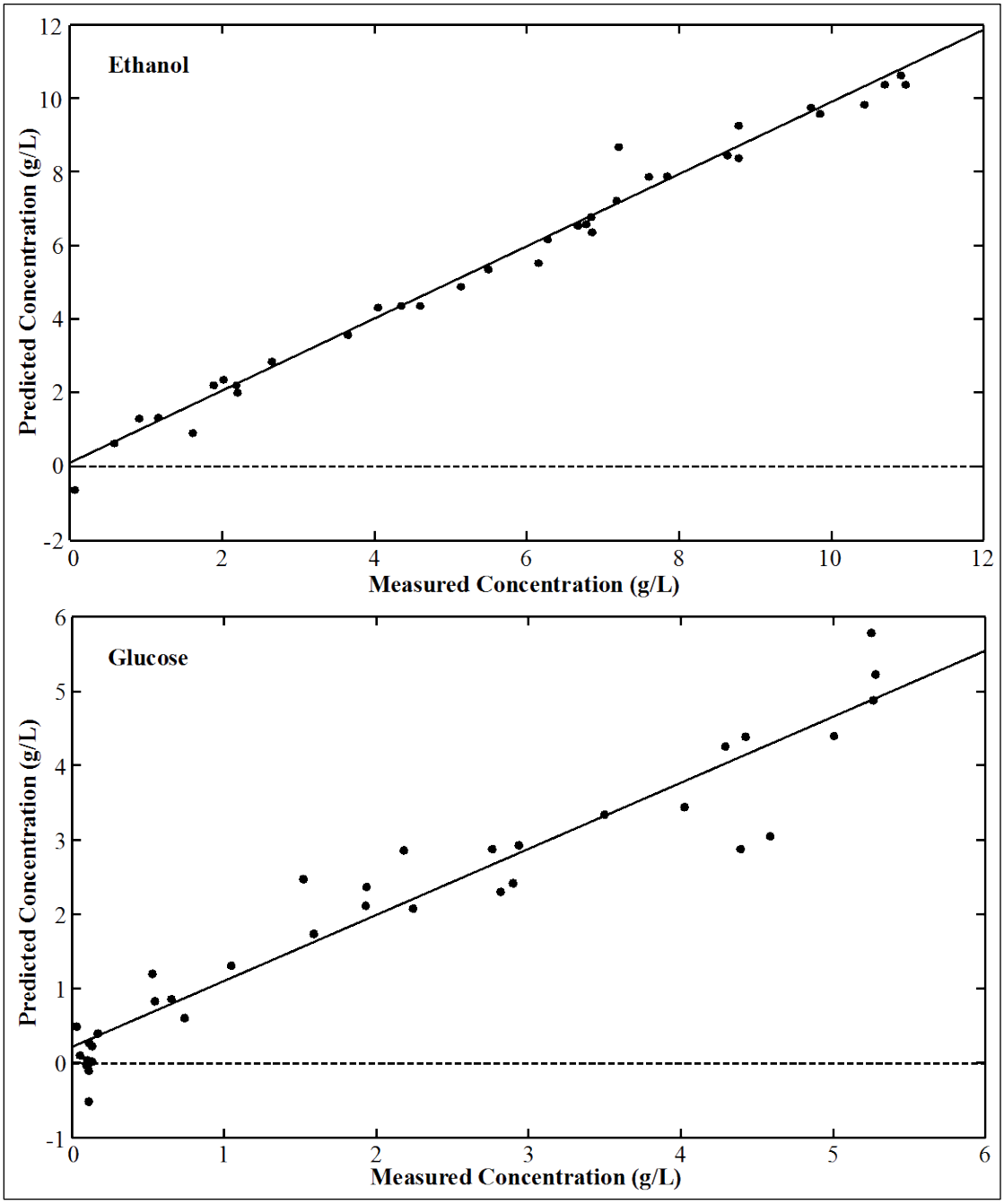

Figure 4-3. Partial Least Squares models from the synthetic glucose fermentation. Ethanol (top) and glucose (bottom) models were pretreated with orthogonal signal correction and cross validated using random subsets.

4.2.2 Lignocellulosic hydrolysate fermentation

The initial experiments demonstrated that Raman could identify glucose and ethanol within a synthetic glucose fermentation spectrum and monitor the process. The same sampling, pre-processing, modeling and data analysis techniques were then applied to the fermentation of steam exploded switchgrass hydrolysate; a dark brown solution produced by the reaction of switchgrass for a short time under high heat and pressure in the presence of SO_2 [50]. The hydrolysate is high in lignin and sugar degradation products, as well as monomeric and oligomeric carbohydrates from cellulose and hemicellulose. The hydrolysate was fermented in the same step-wise fashion as the synthetic glucose. The concentration of monomeric glucose in the hydrolysate was relatively low (1.5 g/L), so additional synthetic glucose (3.5 g/L) was added at the beginning and 5 g/L of glucose added at regular intervals. Fermentation of each added amount of glucose proceeded until the glucose was consumed and ethanol production had ceased to increase as determined by monitoring the intensity of the ethanol Raman peak at 883 cm^{-1} in real time and verified by offline HPLC analysis.

As expected, the switchgrass hydrolysate spectra exhibited a highly elevated background compared to the synthetic sugar fermentation, presumably due to the presence of fluorescent lignin-derived compounds (Figure 4-4). These spectra have increased noise compared to the synthetic spectra due to the heteroscedastic nature of the noise remaining after pretreatment. Future work will focus on the reduction of background signal to improve our signal to noise ratio and modeling ability.

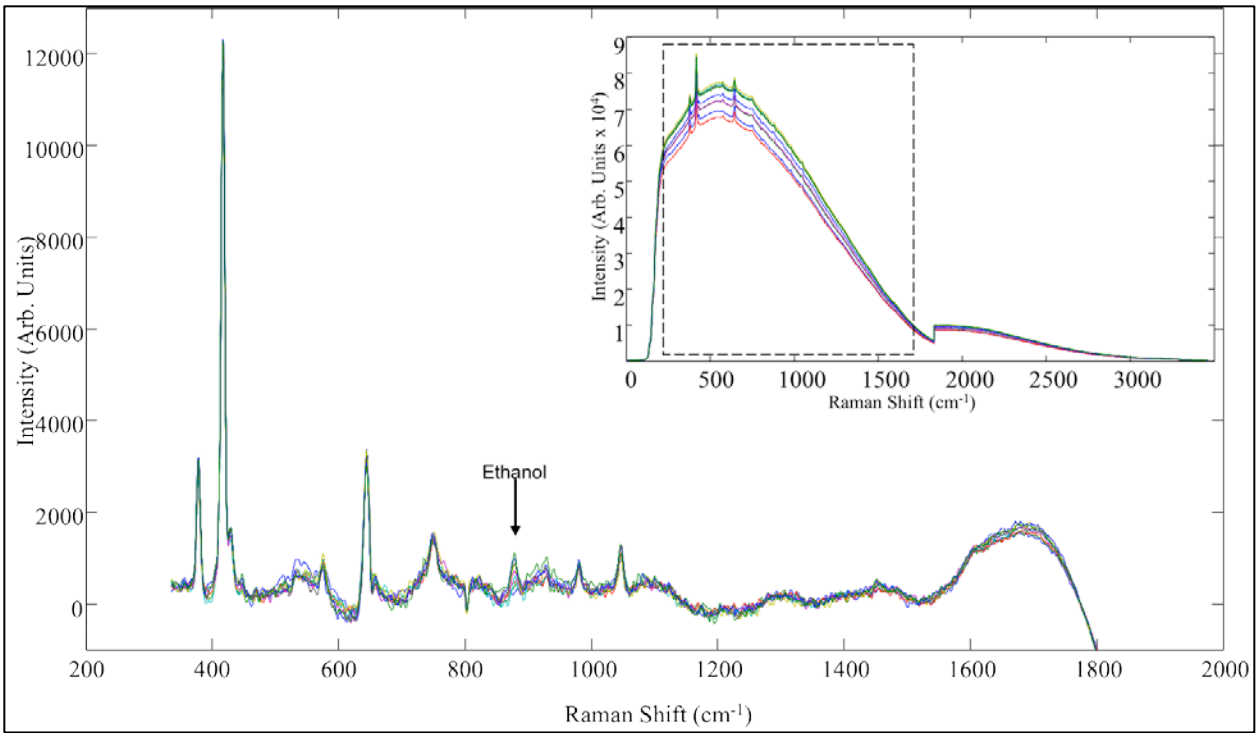

Figure 4-4. Raw spectra from the hydrolysate fermentation (inset) were treated similarly to the synthetic fermentation data to remove the elevated background. Noise is intensified in the pretreated spectra due to the greater intensity of the background signal and the heteroscedastic nature of the noise.

The hydrolysate fermentation spectra correlated well with the offline HPLC analysis for ethanol (Figure 4-5). The ethanol peak at 883 cm^{-1} is visually distinct from the baseline and models well even in the presence of an elevated background, with a RMSECV only 0.2 g/L lower than the synthetic glucose fermentation (Table 4-1). The concentration of glucose was more difficult to predict after the background pretreatment. The low concentration of glucose in our fermentation, combined with a high loading of yeast cells, yielded a glucose concentration that rapidly decreased culminating in calibration HPLC data below the Raman limit of detection (LOD). The synthetic fermentation glucose models had a limit of detection of about 1 g/L. Many of the calibration data for glucose in the hydrolysate fermentation fell below 0.03 g/L; removing these data provided a more robust model for glucose in the hydrolysate fermentation however this left only 19 reference points for modeling. Additional reference points may increase the robustness of the models; however, more important is reducing the background signal in order to improve the signal-to-noise ratio and reduce prediction errors.

Table 4-1. Prediction model data for both synthetic glucose and switchgrass hydrolysate fermentation partial least squares models. All data calculated with two latent variables.

		R^2	RMSEC*	RMSECV**
Synthetic Glucose Fermentation	Ethanol	0.984	0.010942	0.40995
	Glucose	0.920	0.32228	0.5335
Hydrolysate Fermentation	Ethanol	0.935	0.2009	0.60326
	Glucose	0.513	0.20828	1.0614

*RMSEC is root mean square error of calibration
**RMSECV is root mean square error of covariance

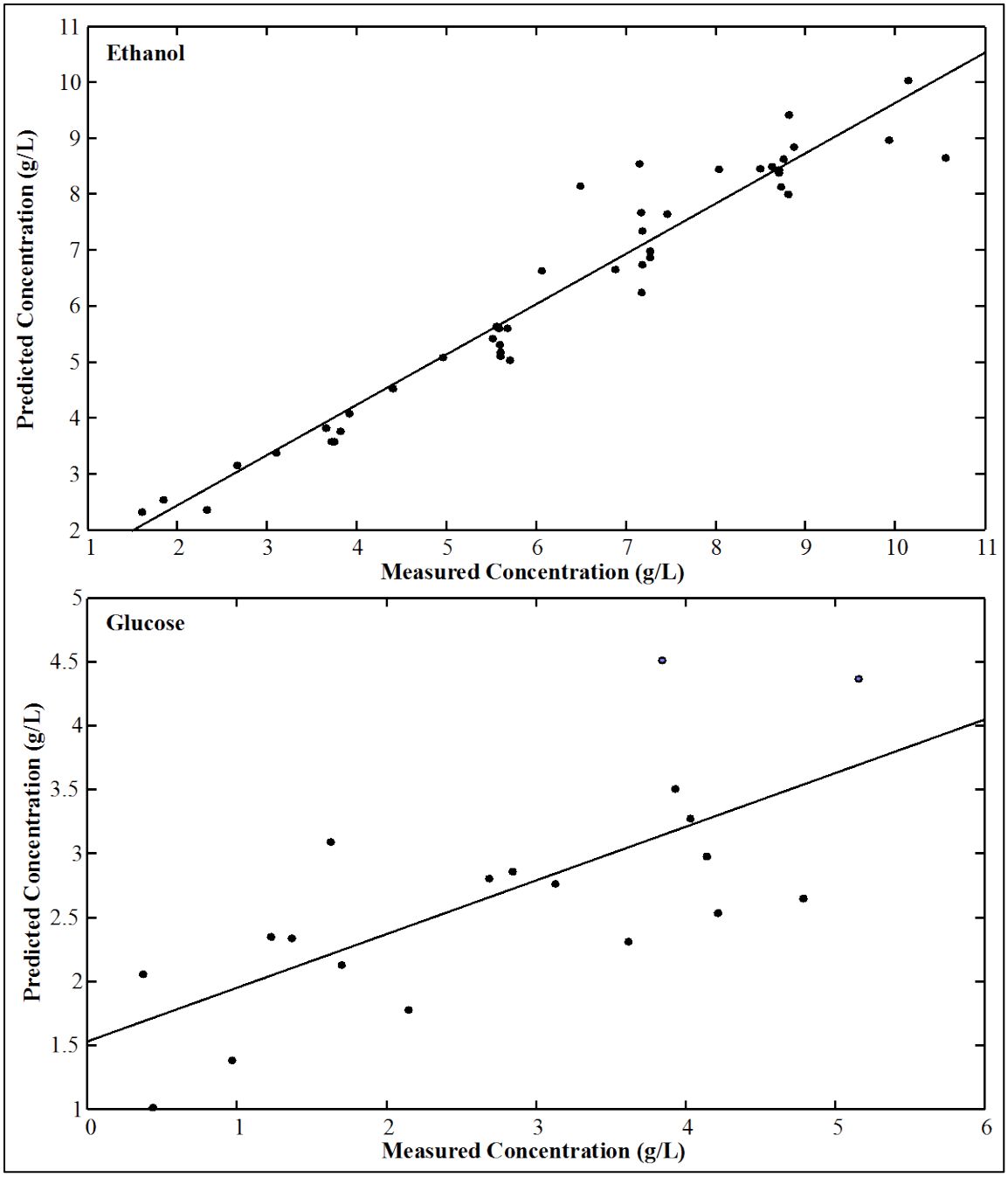

Figure 4-5. Partial Least Squares models generated from hydrolysate fermentation data. Ethanol (top) and glucose (bottom) data were pretreated similarly to previous models. For the glucose model, data below 0.03 g/L were assumed to be below the limit of detection and removed.

We have demonstrated quantitative models for following ethanol production during fermentation and while these models show promising results with a low concentration system, further study is necessary to alleviate the background effects that inhibit monitoring glucose consumption. The glucose concentration in a typical fermentation broth following hydrolysis of polymeric and oligomeric carbohydrates is 20-50 g/L, much higher than the starting glucose concentration in our stepwise fed batch hydrolysate fermentation. In the batch fermentation, the initial glucose was rapidly consumed resulting in low glucose concentrations during much of the fermentation. A real-world fermentation, however, would have a much larger range of concentrations of glucose, which would improve the results from our real time analysis.

4.3 Discussion

Despite widespread use in other areas, Raman spectroscopy has not yet been utilized extensively for continuous analysis of fermentation of lignocellulosic-derived materials. However, small scale ethanol fermentations have been measured by Raman, both through periodic removal and measurement of samples [159] and continuous monitoring of nano-scale reactions with in situ measurement [120]. In addition, Shih et al used a 785 nm Raman microscope to measure offline aliquots of both ethanol and sugar from enzymatic hydrolysis and fermentation of pretreated corn stover [116, 117]. Multiple sugars and ethanol were measured simultaneously, but high background fluorescence was problematic. Attempts to decrease the background and increase the detection limits by extraction of the biomass prior to pretreatment with solvents (ethanol, hexane or water) were successful in reducing the LOD for glucose from 20 g/L to 4 g/L, but such treatments are impractical on a larger scale [117]. An elevated background signal is a persistent issue when dealing with lignocellulosic biomass fermentation processes – lignin is made up of

highly conjugated phenolic groups [19], which can lead to an elevated background signal in the same spectral region as the compounds of interest, potentially masking the Raman features of the spectra [119].

Analysis of ethanol and glucose has also been conducted non-spectroscopically in a number of ways in order to eliminate the need for manual sampling and the associated delay in data procurement. Sequential injection analysis (SIA) with enzyme or amperometric detection [160, 161] can measure both ethanol and glucose in solution. Indirect monitoring of fermentation progress by measurement of headspace CO_2 [111] and electrochemical detection of ethanol by microelectrode array [162] provide information about the progress of the reaction, but cannot pinpoint a cause if the reaction deviates from normal conditions. These methods are an improvement over offline HPLC methods, but still cannot provide information in real time.

The advantages of online Raman spectroscopy over other methods lie mainly in the speed of analysis and the reduction of user interaction. The Raman method was capable of collecting a full spectrum every 30 seconds, from which both ethanol and glucose concentrations could be determined within seconds using multivariate control models. Following the quantities of ethanol and glucose in near real-time provides insight regarding fermentation performance and allows for control decisions to be made in time to effect the quality of the product being formed in the bioprocess.

Many reactions are run for a set period of time depending on their initial sugar concentration, yeast loading, temperature, etc. These parameters are determined based on theoretical values and previous experiments. However, other factors may influence the fermentation rate and so often fermentation processes are run longer than necessary to ensure completion. Raman spectroscopy

allows the user to determine if the cell loading was sufficient, detect possible contamination, determine the rate of fermentation and see exactly when the fermentation has completed. In addition, process upsets or problems can be spotted early, reducing costs and increasing efficiency.

4.4 Conclusions

Fermentation of both synthetic glucose and a lignocellulosic hydrolysate was measured continuously by 785 nm Raman spectroscopy. Despite an elevated background present in the lignocellulosic hydrolysate, effective data pretreatment methods allowed for measurement of ethanol and glucose over the course of the reaction. These results show that Raman has the potential to be an effective tool to improve the efficiency of existing bioconversion processes. With precision sensors continuously monitoring large scale reactions, time and resources can be conserved to help ensure economic sustainability of biomass-based biorefineries in the long term.

4.5 Methods

4.5.1 Steam-pretreated switchgrass hydrolysate

The liquid hydrolysate was prepared as described by Ewanick and Bura [50]. Briefly, SO_2-impregnated switchgrass was pretreated using a 1.5 L batch steam gun (HM^3 Energy Inc, Gresham OR) at 195 °C for 7.5 minutes. The liquid hydrolysate fraction of the resulting slurry was separated by vacuum filtration and stored at 4 °C.

4.5.2 Fermentation

Saccharomyces cerevisiae ATCC 96581 isolated from spent sulphite liquor [72] (obtained from ATCC) was streaked onto YPD agar plates and allowed to grow for 48 hours. Prior to fermentation, preculture cells were grown by adding one colony from the plate to liquid media containing 10 g/L each of glucose, yeast extract and peptone. After 24 hours of growth at 30 °C and 150 rpm orbital shaking, the cells were centrifuged and the spent supernatant removed and replaced with fresh media. The cells were then grown for another 24 hours under the same conditions; the cells were again spun down, washed twice in deionized water, and then resuspended in a small volume of 0.9% sodium chloride. Cell concentration was determined by measuring the optical density of the suspension at 600 nm and comparing to a calibration curve prepared using oven dried cells at varying optical densities.

Synthetic glucose solutions as well as the steam-pretreated hydrolysate were adjusted to pH 6 using dilute NaOH. Nutrients in the form of ammonium phosphate (2 g/L), sodium sulfate (0.2 g/L) and sodium nitrate (2 g/L) were added and the solution was heated to 30 °C in a 1.3 L New Brunswick Scientific BioFlo 115 bioreactor equipped with a water jacket, exhaust condenser and pH probe. The pH was monitored and maintained at pH 6 for the duration of the fermentation with 1 M HCl and 2 M NaOH. The total solution volume was 800 mL with a cell concentration of 5 g/L and the mixture was stirred continuously with a Rushton impellor at 400 rpm. The initial glucose concentration was 5 g/L at time zero, and further 4 g aliquots of glucose were added when the Raman ethanol peak at 883 cm^{-1} reached equilibrium, roughly every 90 minutes. One milliliter samples were removed every 10-15 minutes for HPLC analysis.

4.5.3 Raman data collection and analysis

Real-time analysis data were collected using a RamanRxn1 instrument (Kaiser Optical Systems, Ann Arbor, MI). The excitation wavelength was 785 nm with a power at the sample of 250 mW. Spectra were collected as an average of six, five-second exposures resulting in a collection time of 30 seconds per spectrum. A ballprobe immersion optic (Matrix Solutions, WA) was used for collection of the Raman data. The spherical lens of the ballprobe collects the signal from a small volume very close to the ball surface, providing a constant focal length and greatly enhanced measurement precision. The spherical tip of the probe causes high shear forces as the reaction liquid circulates in the sampling system, preventing accumulation of cells or debris on the probe surface.

A custom sampling loop system that rapidly pumped the fermentation broth out of the fermenter, past the probe and back into the vessel was used with the ballprobe to reduce the possibility of fouling and improve the sampling reproducibility of the fermentation. The slightly increased pressure generated in the sampling loop maintains gases in solution, thus preventing CO_2 bubbles produced during fermentation from interacting with the excitation laser and potentially causing erroneous data points. The sampling loop was designed using NeSSI (New Sampling/Sensor Initiative) sampling blocks that provide a simplified flow path past the ballprobe. NeSSI defines a standard physical format (ANSI/ISA SP76.00.02) to simplify development and installation, and reduce the size of fluid handling systems. The fast loop system was developed using Parker Intraflow (Cleveland, OH) substrates and top mount components and had a volume of approximately 10 ml. The fermentation broth was pumped through the fast loop at 500 mL/min to ensure a rapid sample turnover in the fermenter.

4.5.4 HPLC analysis

Ethanol and glucose were measured using refractive index detection on a Shimadzu Prominence LC. Samples were diluted as appropriate, filtered through 0.22 μm syringe filters and 20 μL of sample were injected onto a Phenomenex Rezex RHM H^+ column at 63 °C with an isocratic mobile phase elution of 0.05 mM H_2SO_4 at 0.6 ml/min. Standards were prepared and used to quantify the unknown samples.

4.5.5 Data analysis

Data models were created and analyzed using Matlab (TheMathWorks, MA) and the PLS_Toolbox (Eigenvector Research, Inc., WA).

4.6 Acknowledgements

We would like to thank the Center for Process Analysis and Control (CPAC) and the Denman Professorship in Bioresource Science and Engineering both at the University of Washington for funding and HM^3 Energy in Gresham OR for the use of their steam gun. The authors would also like to thank Drs. Sergey Mozharov and Thomas Dearing for their insights and useful comments regarding Raman spectroscopy and data analysis.

Chapter 5: Hydrothermal pretreatment of lignocellulosic biomass[3]

Abstract

Lignocellulosic biomass has long been recognized as a potential sustainable source of mixed sugars fermentation to biofuels and other biochemicals. Because of the recalcitrance of the lignocellulosic matrix to enzymatic attack, pretreatment of the material is necessary to enhance the accessibility of the enzymes to substrate. The hydrothermal pretreatments (steam explosion and hot water pretreatment) are the most effective pretreatments for a variety of biomass types and have been shown to work effectively at a commercial scale. Here, we consider the technical maturity of the hydrothermal pretreatments by looking at the process history, describing the mode of hydrothermal reactions and analyzing the influence of pretreatment conditions on the physico-chemical properties of pretreated biomass. Finally, we compare the effectiveness of hydrothermal pretreatments (steam and hydro) and outline the remaining challenges associated with harnessing the pretreatment for production of biochemicals.

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5.1 Introduction

Processing of lignocellulosic biomass to ethanol consists of four major unit operations: pretreatment, hydrolysis, fermentation and product separation/purification. Pretreatment, disruption or fractionation is an important tool in the biomass to ethanol conversion process and is required to alter the structure of lignocellulosic biomass to make cellulose more accessible to the enzymes that convert the carbohydrate polymers into fermentable sugars. The goal is to break the lignin seal and disrupt the crystalline structure of cellulose. Regardless of biomass type, the pretreatment has to separate the biomass into cellulose, hemicellulose and lignin with high recovery of all components in pure form to allow for economical feasibility, *i.e.*, through the separation of individual cells or destructuration of the cell wall to loosen up complexes and allow for further separation of main polymers.

It is apparent that an effective pretreatment method should be efficient on different types of lignocellulosic biomass, inexpensive (for both operating and capital costs), require a minimum of pre-pretreatment (preparation/handling) steps and affect a maximum recovery of all lignocellulosic components in usable form. In addition, if the ethanol is the final product of biomass to ethanol conversion, the effective pretreatment should ensure the maximum hemicellulose and cellulose recovery in hydrolysable and fermentable form. Although many pretreatment processes have been studied (biological, physical, chemical and the combination of these approaches) no process currently available can provide all of these desired outcomes on all lignocellulosic materials. However, hydrothermal pretreatment is the most effective pretreatment for a variety of biomass types and has been shown to work effectively at a commercial scale. In this review, we will first analyze the technical maturity of the hydrothermal pretreatment process

by looking at the history and description of the process conditions. Then, we will describe the mode of hydrothermal reactions and the influence of pretreatment conditions on the physico-chemical properties of pretreated biomass. The final section offers a comparison of the hydrothermal pretreatments (steam and hydro) and outlines the remaining challenges associated with harnessing the pretreatment for production of biochemicals.

5.2 Physical comminution

Size reduction of lignocellulosic biomass is an important factor in any pretreatment process. Mechanical means can be used to reduce particle size sufficiently so that no further pretreatment is required prior to enzymatic hydrolysis, obviating usage of chemicals and associated concerns such as corrosion, recycling, neutralization and storage. However, high energy requirements for these processes mean that they are typically not economically feasible. As particle size decreases, crystallinity is reduced, which increases enzymatic digestibility. To significantly improve hydrolysis, treatments must reduce particle size to less than 50 μm [163]. However, due to the high energy cost of mechanical size reduction, comminution past 200 μm is not generally economically feasible [163].

Milling processes include dry, wet and vibratory ball milling [164–166]. Compression, hammer and disc milling are also used [167]. These vary widely in terms of particle size distribution, energy usage and efficacy on different feedstock types. For example, far more energy is required to mill hardwoods to a given size than agricultural residues using both hammer and knife milling [168]. In starch-to-ethanol bioconversion, wet and dry milling are the most cost effective pretreatments [169]. However, for lignocellulosic biomass, the energy demands of any physical size reduction process are high. Comminution is consequently limited to pre-pretreatment, to be

followed by a chemical or thermal pretreatment process.

5.3 Hydrothermal pretreatment (liquid hot water and steam)

Physical pretreatment of lignocellulosic biomass is often inadequate in providing complete fractionation to a readily digestible and fermentable product. The cell structure of lignocellulosic biomass is by nature complex and difficult to penetrate, so fractionation requires chemical reactions in addition to physical restructuring. Pretreatments utilizing primarily steam or liquid water at high temperatures can efficiently convert biomass to a form which can be easily digested by enzymes by facilitating autohydrolysis reactions within the biomass. Processes utilizing hot water or steam as the primary chemical are known as hydrothermal pretreatments. The two forms of hydrothermal pretreatment utilize steam (steam explosion) and aqueous water (liquid hot water pretreatment). These processes are advantageous compared to chemical methods as they are regarded as safer – equipment corrosion is reduced - and more "environmentally friendly", as often, no chemicals are required [170, 171]. Although process residence times and temperatures are similar, liquid hot water pretreatment differs from steam explosion in that water is present as a liquid instead of a gas during pretreatment. As a result, there are differences in reactor configurations, solids consistency during and after pretreatment and concentration of reaction products. In the last 80 years, there has been great progress in the development of aqueous processes to break down all types of lignocellulosic biomass. From agricultural residues to hardwoods to softwoods, hydrothermal pretreatments have the potential to sustainably generate material which can be readily converted to ethanol.

5.3.1 Process history and description

Steam explosion

Steam explosion has long been used as a means of deconstructing biomass for many purposes, from structural materials to paper to biochemicals. The first use of steam explosion to produce a commercial product was the masonite process. Developed in the 1920's, the process was used to produce a fiberboard building material [172] with very high yields and minimal energy usage [54, 173]. However, the coarse, dark substrate, while suitable for fiberboard, was unsuitable for paper products. Asplund used a similar high temperature and pressure process with the addition of mechanical refining to produce fiber for board manufacturing [174]. Refining at high temperatures allowed fibers to be fully separated with very high yields, although there was no delignification. By addition of ammonia and SO_2, it was possible to use the Asplund process to produce fibers for papermaking but the cost associated with use and recycling of these chemicals was prohibitive [173].

Incorporating defibrillation with steam explosion, siropulping was developed as a batch process using a masonite gun equipped with a defibrillation nozzle aimed at a metal grating. To increase the force of biomass contacting the grate, pressure in the gun was increased to 4.8 MPa using CO_2. Addition of nitrogen enabled a pressure of up to 13.8 MPa, far higher than the 1.6 MPa obtained using pure steam at 200 °C [175, 176]. Although costly to run, results from siropulping were promising. Increasing the pressure resulted in increased digestibility of the solids, indicating a mechanical disruption effect. However, attempts to reduce temperature and cooking time without affecting digestibility were unsuccessful, demonstrating the importance of chemical processes in the pretreatment [177].

The Masonite process was revisited in the 1980's as a means of fractionating lignocellulosic biomass for animal feed and for biofuel production. Process configurations ranged from application of the original batch method by the IOGEN Corporation to a continuous digester developed by STAKE Technology. Both methods are used currently for bioethanol production at pilot scale. At lab scale, steam explosion has been shown to be effective in pretreating a wide variety of biomass types, including agricultural residues (corn fiber, corn stover), hardwoods (poplar, willow) and softwoods (Douglas-fir, pine, spruce) [46, 139, 178–182].

The STAKE continuous process utilizes a coaxial feeder to move a plug of biomass through a steam-injected reactor chamber for a set time period. It then exits the digester through a discharge screw and blow valve into a cyclone [183, 184]. The IOGEN batch method involves loading of biomass inside a cylindrical digester injected with steam. The temperature is held at a constant value (from 150- 280 °C) for anywhere from 10 seconds to 15 minutes. After the time has elapsed, a valve at the bottom of the digester is opened and the biomass released to a cyclonic collection vessel [185, 186]. For both batch and continuous processes, as the pressure drops to atmospheric, water and steam inside the biomass expand and cause the "explosion" of the cell structure. Following pretreatment, substrates undergo hydrolysis, fermentation and distillation to ethanol. The IOGEN demonstration plant in Ottawa, ON, Canada is designed to produce 3 million liters of ethanol annually from wheat, barley and oat straw [187].

Steam explosion fractionates biomass to yield a liquid fraction rich in monomeric and oligomeric sugars and solid fraction made up of digestible cellulose and lignin. Under optimized conditions, relatively pure products in high yields can be achieved, such as highly digestible cellulose or high yields of solubilized hemicellulosic sugars. Following enzymatic hydrolysis of the solid

fraction, the majority of the cellulose is commonly converted to glucose, leaving behind lignin. These solids have a high heating value and can be burned for process energy or converted to pellets, which can be sold to improve process economics [187]. The hemicellulosic sugars contained in the water-soluble fraction have value as a fermentation substrate for ethanol production or a starting reactant for other products [188]. The process demonstrates versatility and robustness on many types of biomass. While softwoods were formerly thought to be not suitable for steam explosion [189], it has been shown in recent years that softwoods including spruce and pine can be pretreated using steam explosion to provide high ethanol yields (Table 5-1) [46, 190, 191]. Since steam explosion can be used to pretreat the widest range of lignocellulosic biomass, it has been shown to be a robust process capable of generating a variety of products based on the pretreatment conditions and feedstock chosen.

Table 5-1. Pretreatment conditions and results for different feedstocks undergoing steam explosion pretreatment. Xylose recovery is determined based on xylose in original material. Ethanol yields are calculated based on the theoretical yield of 100% conversion of all fermentable sugars in the raw biomass.

Biomass	Conditions	Xylose recovery	Ethanol yield (% of theoretical)	Reference
Wheat straw	180 °C, 10 min, 0.9% w/w H_2SO_4	85% [a]	70%	[192]
Corn stover	190 °C, 1.5 min, 1% w/w H_2SO_4	90%	85%	[193]
Corn fiber	190 °C, 5 min, 3% SO_2 (w/w)	50%[a]	89%	[194]
Willow	200 °C, 4 min, 0.5% H_2SO_4 (w/w)	72%	79%	[195]
Poplar	205 °C, 3 min, 1% SO_2 (w/w)	65%	64%	[196]
Lodgepole pine	200 °C, 5 min, 4% SO_2 (w/w)	73%	77%	[46]
Spruce	215 °C, 5 min, 2.4% SO_2 (w/w)	68%	68%	[197]

[a] **total hemicellulosic sugar recovery**

Liquid hot water

Liquid hot water was used in the pulp and paper industry as early as the 1930's as an extraction method to remove hemicelluloses from wood prior to pulping [198]. This was most commonly used for production of dissolving pulp, where very pure cellulose (>90%) was desired [199]. Hot water pretreatment for subsequent enzymatic hydrolysis of cellulose was first developed to provide a carbon source for fermentative protein production [200, 201]. The process was further developed under the name of hydrothermolysis [200, 201], aquasolv [202], aqueous/steam aqueous fractionation [183] and uncatalyzed solvolysis [203].

Depending on the circulation direction of hot water relative to the biomass, three different process configurations are commonly employed. In co-current or batch reactors, biomass and water are heated and held at temperature together for the desired time. Counter current reactors move water and biomass in opposite directions, while in flow-through reactors hot water flows over a stationary bed of biomass. In each configuration, the hot water dissolves biomass components, particularly hemicelluloses [204]. Flow-through and counter current systems generally provide higher hemicellulose sugar yields and cellulose digestibility, while batch systems require less water and energy to operate [205]. Operating conditions for all configurations range from 140-240 °C for 0-20 minutes [203].

Operating at pilot scale in Denmark, the Integrated Biomass Utilization System (IBUS) is a continuous counter-current liquid hot water pretreatment system currently used to produce ethanol from wheat straw [206]. Optimum conditions for maximum yield of ethanol from the solid material were found by Petersen et al. to be 195C for 6-12 minutes. At these conditions, 70% of hemicellulose is recovered, and of the 93-94% of recovered cellulose in the solids, 89%

is converted to ethanol. At present, only the pretreated solids are converted to ethanol. The pentose content in the liquid stream is such that a pentose-fermenting organism is required before use of this stream is economical [207]. Prior to hydrolysis and fermentation, the pretreated cellulose is washed to remove inhibitors and residual hemicellulosic sugars [206]. The IBUS and numerous recent lab and pilot scale hot water studies utilize primarily hardwoods and agricultural residues including wheat straw, aspen, sugar cane bagasse and corn fiber [170, 171, 208, 209].

5.3.2 Feedstock characteristics

Pretreatment conditions and raw biomass species greatly affect the final product of pretreatment. However, other factors play an important role as well, though they are much less well understood. Within a given type of biomass, differences include seasonal changes in chemical composition, ash content, and age. For wood, younger trees are more easily fractionated than older ones [29, 189], as is material derived from the more permeable sapwood compared to denser heartwood [210]. Particle size, as well as the timing of harvest and storage prior to pretreatment, can have a major role in determining the efficacy of pretreatment. The moisture content in any type of biomass is naturally highest immediately after harvest and can dramatically decrease during storage. Particle moisture content affects both steam consumption and pretreatment efficacy. Raw biomass at a high moisture content (above 50%) requires a longer reaction time in order to heat the additional water inside the cells, and consumes up to 50% more steam compared to air dried biomass [107]. This is especially evident when particle sizes are large, as the outside of the particle may cook faster than the inside. As particle sizes decrease, pretreatment conditions may become too severe as the biomass heats quickly without

the buffering effect of a slower heating, increasing hemicellulose degradation [106, 211]. The "pre-pretreatment" of raw biomass (storage conditions, moisture content and particle size) is important to the final product of pretreatment, and becomes increasingly important in commercial processes where large amounts of biomass are required. Further research in this area is required in order to determine the optimum storage and pre-pretreatment conditions to maximize yields for a given feedstock.

5.3.3 Method of action

Hydrothermal (steam explosion and liquid hot water) pretreatments utilize acid liberated from hemicellulose side chains and high temperatures to hydrolyze hemicellulose, cellulose and lignin. Some biomass requires the addition of mineral acids (SO_2 or H_2SO_4) to achieve the same level of pretreatment, but the method of action in both cases is similar. In both catalyzed and autohydrolysis chemical reactions, physical rearrangements govern the breakdown of biomass from polymers to oligomers to monomers. In the context of bioethanol production, the benefit of any pretreatment is increased cellulose surface area available to cellulytic enzymes. This occurs by dissociation of lignin and hemicellulose from cellulose and reductions in crystallinity and particle size. Enzyme accessibility has been extensively studied and is governed by pore size, surface area, and a number of other factors that will not be covered in this chapter [154, 212–214].

Hydrothermal reactions

Biomass added to the steam or hot water reactor first undergoes hydrolysis of hemicellulose, the most labile of the three primary components of lignocellulose [215]. Saturated steam or hot water condenses any water present in the cells of the biomass. As a result, organic acids (acetic

acid and uronic acid) are liberated by saponification of hemicellulosic uronic and acetyl groups. Water itself acts as an acid at high temperatures; at 220 ˚C, water has a pH of 5.6 and an ion product of 10^{-11} [216]. In fact, at these conditions, it is thought that water has a greater affect than acetic or formic acid [17]. Liberated acids hydrolyze the hemicellulose glycosidic bonds to form low to intermediate weight water soluble oligomers. At increased residence time and temperature, these oligomers undergo further hydrolysis to yield monomers. Monosaccharides, particularly pentoses, are highly unstable in high temperature, acidic environments. Under these conditions, pentoses and hexoses undergo dehydration reactions to form furfural, hydroxymethylfurfural, levulinic acid, formic acid and other degradation products known to be inhibitory to fermentation [38] and enzymatic hydrolysis [217]. Fortunately, the rate of formation of these compounds is slower than the depolymerization of hemicellulose, and can often be controlled by use of lower temperature and shorter residence time [190] which preserve oligomeric sugars and prevent depolymerization to monomers [171]. Liquid hot water pretreatments control the degradation of oligomers by maintaining a pH close to 4 [204]. Removal of sugars as they are solubilized by means of a flow-through or countercurrent liquid hot water process also controls the amount of degradation. In addition to hemicellulose, cellulose is also modified during hydrothermal pretreatment. Glycosidic bonds are hydrolyzed, albeit at a lower rate than hemicellulosic acidolysis. As cellulose molecules are randomly hydrolyzed, the degree of polymerization (DP) decreases [218].

As hemicellulose and cellulose are degraded, lignin is depolymerized simultaneously, though at a somewhat slower rate. Acid hydrolysis of primarily β-O-4 ether bonds gives rise to a high free phenolic count in the lignin [219–221]. Almost immediately after acidolysis, the lignin repolymerizes by acid catalyzed condensation between the aromatic C_6 or C_5 and a carbonium

ion [219]. While a small portion of the newly condensed lignin is soluble in the aqueous media, the majority is hydrophobic and migrates to the middle lamella and lumen. At higher temperatures, lignin becomes highly fluid and forms spherical droplets visible in the lumen [222] and on the surface of fibers [223].

The full effect of the chemical reactions and rearrangements that occur during hydrothermal pretreatment depends on the severity of pretreatment as well as the original feedstock characteristics. The resulting unique physical and chemical characteristics of pretreated biomass determine enzymatic digestibility, ease of fermentation and subsequent ethanol yields.

5.3.4 Pretreatment severity

Severity is determined by the relationship between three factors: residence time, temperature and acid concentration. Time and temperature are easily measured, whereas the acid concentration is much more difficult to quantify. In autohydrolysis reactions with no added acid, many researchers make use of a severity factor calculated using time and temperature to determine relative severity of different combinations (Equation 5.1) [54]. This severity factor was developed based on the assumption of first order kinetics and on earlier factors such as the H-factor [224] and P factor [225].

$$Log\,(R_o) = Log\left(t \cdot \exp^{\frac{T - T_{ref}}{14.75}} \right) \qquad \textbf{[Equation 5.1]}$$

R_o is the severity parameter, time is t, T_{ref} is a reference temperature for a base case (often 100 °C) and T is the reaction temperature. This factor is a good estimate of pretreatment severity for uncatalyzed samples, but does not take into account the effect of acid. Chum *et al.* devised a

combined severity factor, adding the effect of the pH of the biomass prior to pretreatment to the equation (Equation 5.2) [55].

$$Log\,(R_o) = Log\left(t \cdot \exp^{\frac{T-T_{ref}}{14.75}}\right) - pH \qquad \textbf{[Equation 5.2]}$$

The assumption of first order kinetics in steam explosion is of limited utility, particularly at high severity. Neither severity parameter takes into account that the cellulose fraction produced under severe conditions is much more readily digestible, while the liquid hemicellulosic fraction produced under mild conditions is much more fermentable. However, both reaction ordinates are useful as a guide to compare the effect of different pretreatment conditions.

Steam explosion

When it was first developed, steam explosion of wood for fiberboard was an uncatalyzed process. Since the purpose was to break down wood into a structure which could be formed into a sheet, temperatures as high as 280 °C were employed [226]. Use of similar conditions to pretreat biomass for bioethanol production would generate unacceptably high levels of inhibitory compounds. The use of milder pretreatment conditions is necessary to minimize the formation of these compounds. With some types of biomass, simply reducing the time and temperature of pretreatment is enough to generate a substrate which is readily digestible and fermentable. Hardwoods and agricultural residues typically have highly acetylated hemicellulose (4-O-methylglucuronoxylans) which allows the biomass to undergo “autohydrolysis” under steam explosion conditions and obviates the need for an acid catalyst [24]. More recalcitrant feedstocks, such as softwood, lack acetylated hemicellulose and instead have primarily

minimally acetylated glucomannans and galactoglucomannans [38] and require addition of an acid catalyst prior to pretreatment. Acid addition lowers the severity of the conditions required to achieve optimal substrate characteristics and minimizes inhibitor formation [38]. To maximize ethanol yields, two-stage steam pretreatments utilizing low severity conditions to hydrolyze hemicellulose followed by higher severity to generate digestible cellulose have been employed [58, 227]. While marginally higher ethanol yields were obtained compared to an equivalent one-stage process, the additional cost of a second steam pretreatment step would likely prevent adoption of this technique.

SO_2 and H_2SO_4 are the most commonly used acid catalysts. Catalysis with SO_2 has been shown to provide a higher glucose yield after hydrolysis and lower inhibition of fermentation, while use of H_2SO_4 has been shown to enhance the recovery of hemicellulosic sugars [45]. SO_2 is introduced in gaseous form prior to steam pretreatment, preventing the addition of extra moisture required by soaking in H_2SO_4. At low temperatures, it is converted to sulfuric acid by oxidation in the presence of oxygen. At elevated temperature in the absence of oxygen, disproportionation reactions convert a third to a half of the SO_2 to reduction products including elemental sulfur or thiosulfate [228]. In addition, some SO_2 is lost to the vapour phase. As a result of these reactions, it is difficult to determine exactly how much acid contributes to the pretreatment. It has been shown that impregnation of SO_2 at concentrations higher than 3% have no additional beneficial effect [229, 230]. H_2SO_4 is introduced by soaking or spraying biomass in a dilute acid solution prior to pretreatment. While sugar recovery after pretreatment is higher than when SO_2 is used, generation of inhibitors is higher [45]. Overall ethanol yields are therefore higher when SO_2 is used, despite slightly lower sugar yields after pretreatment.

The required severity for different types of biomass is highly variable. Table 5-1 lists a select number of feedstocks along with their respective pretreatment conditions and yields. Xylose recovery is a good indicator of overall sugar recovery, as xylose is one of the most labile carbohydrates present in lignocellulosics [215]. Ethanol yields are typically stated as a percentage of the theoretical value. Depending on the fermentative organism, only certain sugars can be converted to ethanol and at a certain rate. For example, *Saccharomyces cerevisiae* converts 6-carbon sugars and produces ethanol at a ratio of 0.51 g ethanol per gram of sugar. In general, increased severity is required to go from agricultural residues to hardwoods to softwoods, with a concurrent decrease in xylose recovery. Ethanol yields do not exhibit such a clear trend. As shown in Figure 5-1, as severity increases, the concentration of hexoses and pentoses increase in solution, but subsequently drop off as degradation reactions begin to dominate.

Figure 5-2 shows the effect of increased severity on the composition of the water insoluble fraction. At high severity, much of the hemicellulose is hydrolyzed, increasing the relative concentrations of cellulose and lignin. As hemicellulose is removed and cellulose and lignin undergo chemical reactions, the cellulose becomes more readily digested by cellulases, generating more fermentable sugars. The final ethanol yield, therefore, depends on the fractionation of fermentable sugars between liquid and solid fractions along with the digestibility of the solids and level of sugar degradation in the liquid.

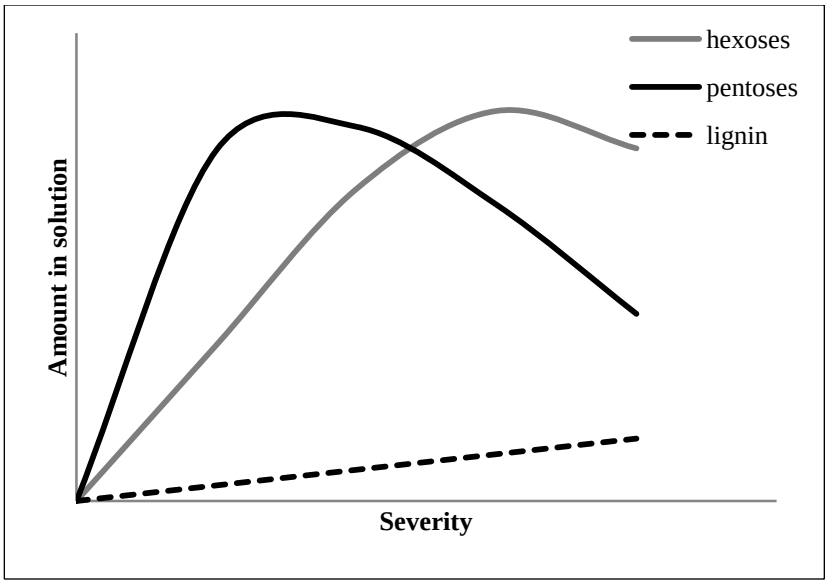

Figure 5-1. Schematic representation of the effect of pretreatment severity on concentrations of soluble lignin, pentoses and hexoses in the water insoluble fraction.

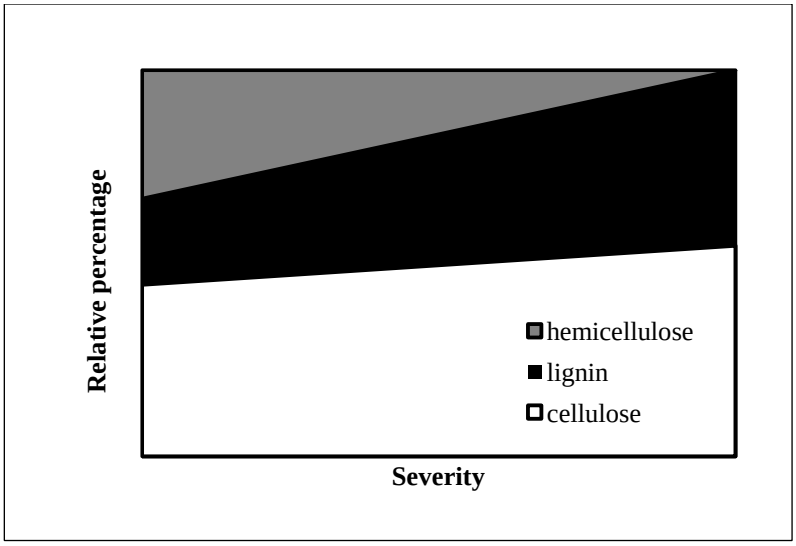

Figure 5-2. Schematic representation of the relative amount of hemicellulose, lignin and cellulose in the water insoluble fraction as severity increases.

Liquid hot water

While steam explosion is highly sensitive to pretreatment severity, liquid hot water is much less affected by changes to time and temperature. With many feedstocks and process configurations, final sugar recoveries and subsequent ethanol yields are independent of pretreatment severity [203]. Particularly at low consistency, time and temperature have minimal effect on sugar recovery and overall ethanol yield. An approximate representation of the relative polymer degradation as severity increases is shown in Figure 5-1 for hemicellulose, lignin and cellulose. Increasing severity leads to an increase in monomeric subunits in solution, then a decrease as monomers are degraded. The rate of both production and degradation of monomers is highly dependent on the process conditions as well as the feedstock and determine the resulting physical and chemical characteristics of the substrate.

5.3.5 Physical and chemical characteristics of pretreated biomass

Steam explosion

The characteristics of steam exploded biomass are highly dependent on the severity of the pretreatment conditions. Different levels of time, temperature and catalyst concentration on the same raw feedstock will result in radically different pretreated substrates. In addition, the rate of decompression of the reactor at the end of the residence time can vary, depending on how the contents of the reactor are brought to atmospheric pressure - whether by a gradual bleeding of the pressure or a sudden release of pressure resulting in an “explosion”. Both methods have the same macroscopic effect – saturated water present inside the cells vaporizes and expands, separating the fibers. The microscopic effect of decompression is dependent upon the temperature differential. Even at relatively low process temperatures (180-200 °C), lignin is past its glass

transition temperature and exhibits thermoplastic behavior. Fibers separate at the middle lamella to yield a large number of bundled fibers, some individual fibers and minimal broken fibers. At moderate temperatures (200 °C) free fibers dominate, while at higher temperatures (above 230 °C) fiber damage and lignin repolymerization reactions result in mostly fiber fragments fused together by lignin [231, 232]. Temperatures required for optimal fiber separation vary by feedstock, with hardwoods requiring lower temperatures for fiber separation than softwoods.

The effect of the rate of pressure drop on fiber properties has been shown to be significant but varies depending on the type of biomass and severity of the pretreatment. Brownell and Saddler [210] showed that the overall ethanol yield of aspen steam pretreated with a gradual bleeding of pressure yielded the same amount of ethanol as material which underwent explosive decompression. This effect may be biomass dependent – studies on eucalyptus have shown that explosive decompression prevents the steam pretreated solids from drying up when steam is slowly released [233]. Another potential benefit of bleeding off excess pressure is a reduction in volatile inhibitors [186].

Cellulose crystallinity also changes as severity increases. As lignin is reorganized and hemicellulose solubilized, the molecular tensions holding crystalline cellulose together are weakened. This allows formerly amorphous cellulose to be incorporated into the crystalline structure, leading to an increase in the overall crystallinity [220, 234, 235]. However, this change may be dependent on process parameters, as other researchers have found that there is no change in crystallinity [236]. Cellulose degree of polymerization (DP) also decreases; with the DP decreasing more rapidly after most of the hemicellulose is depolymerized. The DP tends towards the limiting degree of polymerization (LODP) of approximately 150-250 DP. Once the limit is

reached the average cellulose molecule will not reduce further in size [105].

The colour of both insoluble and soluble fractions darkens from light to dark brown as pretreatment severity increases [237, 238]. This is thought to be related to the breakdown of lignin and wood extractives. Lignin condensation could activate tannins and flavonoids towards condensation by lysing of protecting groups (sugars). Reaction with furfural and hydroxymethyl furfural, may also be responsible for these colour changes [238]. While colour is not important for bioethanol production, it can be a qualitative measure of pretreatment severity.

As severity increases, relative lignin content in the solid fraction increases as cellulose is solubilized. The lignin generated from repolymerization reactions is intermediate between native lignin in raw biomass and heavily condensed Klason lignin formed from acid pretreatment [239]. Furfural and other degradation products react with lignin to form new C-C bonds, giving rise to condensation products known as pseudolignin [240]. Addition of supplemental acid is thought to increase the molecular weight of condensation products and reduce solubility in organic solvents [241]. Increased condensed lignin in the solid fraction can also reduce enzymatic digestibility by adsorbing proteins and inhibiting hydrolysis [242].

Insoluble hemicellulose decreases dramatically as severity increases. The amount of hemicellulosic monomers and oligomers in the water soluble fraction concurrently increases as solubilization of the hemicellulose increases, then decreases as these sugars are degraded [56, 218]. Pentose sugars (arabinose and xylose) degrade by dehydration reactions to furfural, hexoses (glucose, mannose and galactose) to 5-hydroxymethyl furfural (HMF). Furfural and HMF are further degraded to formic and levulinic acids [217]. These compounds, along with lignin degradation products and acetic acid, are inhibitory to fermentative microorganisms.

Liquid hot water

Liquid hot water processes are often run as a flow-through process where liquid is continually passed over biomass. This allows for continuous removal and cooling of the dissolved components, but results in a very dilute sugar stream (0.6-5.8 g/L) [209]. Co-current or batch processes result in a higher concentration of sugar in the liquid stream but are more sensitive to process time and temperature. Mok and Antal found that for 10 different herbaceous and hardwood species treated using a batch process for 0-15 minutes at 200-230 °C, 100% of hemicellulose was dissolved with an average of 90% recovery of monomeric sugars [203]. Solids concentration influences the final product, particularly in a batch system where increasing solids increases the acid concentration and subsequent degradation reactions. Laser et al. found that an increase from 1% to 5% solids resulted in a 97% reduction in ethanol yield after SSF although xylan yields remained high (81%) [171].

5.3.6 Conclusions: comparison of steam and liquid hot water pretreatment

Steam explosion pretreatment is a robust means of fractionating lignocellulosic biomass. However, maximization of ethanol yield from enzymatic hydrolysis and microbial fermentation requires a compromise. Conditions yielding highly digestible cellulose will generate high levels of fermentation inhibitors, while milder conditions improve yields of fermentable sugars but fail to generate digestible cellulose. It is nearly impossible to maximize both enzymatic digestibility and fermentability. For a low value product like ethanol, the mixed stream resulting from a compromise severity is acceptable. The process can be adapted to fractionate highly pure components as well, such as furfural and HMF, or a clean hemicellulosic sugar solution.

Liquid hot water pretreatments, particularly at high consistency, are capable of producing

hydrolysates with low levels of inhibitors and highly digestible solids, but dilute liquid streams prevent efficient fermentation. Since acid catalysts are not required, problems with corrosion and chemical recycling and disposal are eliminated. In addition, while the process is highly effective on agricultural residues and some hardwoods, it has not yet been shown to be effective on softwood feedstocks.

Table 5-2 compares the two processes of steam and hot water pretreatments. Both are carried out at similar temperatures and reaction times, so using the reaction ordinate in Equation 5.1, the relative severity of pretreatments carried out using the same conditions with different amounts of water present would be the same. It has been shown, however, that sugar recoveries and overall ethanol yields differ [243]. The consistency during the reaction plays an important role. At higher consistency, as in steam explosion (>50%), the relative acid concentration is much higher, leading to increased sugar degradation. At the lower consistencies (1-10%) used in liquid hot water treatments, the pH is higher and sugar recovery and solids yields are higher while the production of fermentation inhibitors is greatly reduced [170, 171, 243].

Table 5-2. Comparison of steam and liquid hot water pretreatments

Pretreatment characteristic	Steam explosion	Liquid hot water
Solids consistency during pretreatment	High	Low
Readily digestible fiber	Yes	Yes
Sugar concentration in water soluble fraction	High	Low
Pentose recovery	Low	High
Fermentative inhibitor formation	High	Low
Works on softwood	Yes	No
Effective on hardwoods, agricultural residues	Yes	Yes
Water usage	Low	High

5.4 Future work

At its present stage of development, the technology for processes based on steam treatment is mature enough at pilot plant and demonstration level, if the final product is ethanol. Several laboratory scale steam guns are operational at the national or university research labs such as National Renewable Energy Laboratories (NREL), University of British Columbia, Lund University, Virginia Tech University, and University of Washington. Currently, SunOpta Inc. (www.sunopta.com) is producing steam guns for commercial purposes.

However, one of the problems associated with commercialization of the biomass to ethanol process is feedstock availability and cost. A future bioethanol facility able to utilize multiple feedstock sources would be at an advantage in that it would have more consistent supplies of raw material and would be better positioned to find this raw material at lower cost. In addition, it is likely that a future conversion facility will process biomass with impurities from harvesting and chipping units, such as soil, branches and needles. Although considerable research has been done in converting uniform, homogeneous feedstocks to ethanol (corn stover, wheat straw and hybrid poplar, pine among others) not much attention has been paid to the "pre-pretreatment" of lignocellulosics and the effects of biomass physical characteristics on pretreatment and ultimately, the overall ethanol yield. Additional efforts are required in understanding the effects of particle size, thickness, moisture content, biomass "freshness" and "purity", and in wood, for example, the effect on bark, needles or branches on the fractionation process. In addition, for the lignocellulosic biomass to ethanol process to be economically feasible, it has to produce high value co-products. The process itself should be relatively simple.

Although the phenomena involved in the steam-aqueous pretreatment of biomass to ethanol is

well understood, not much research has been done in the fractionation of cellulose, hemicellulose and lignin into pure fractions for the green polymers industry such as polyols (xylitol and arabitol), ethylene, and propylene glycols, furfural, levulinic acid, among others [244]. Ultimately, a greater fundamental understanding of the chemical and physical effects that occur during hydrothermal pretreatment, along with improved understanding of the physico-chemical structure of pretreated biomass, are essential for utilizing steam-aqueous pretreatment as a pretreatment process for biochemicals production.

Chapter 6: Conclusions, future work and references

6.1 Summary and conclusions

Improving the efficiency of lignocellulosic ethanol production is of the utmost importance if cellulosic bioethanol is to be competitive with fossil fuels and first generation bioethanol from starch and sucrose. Improvements in individual processes (pretreatment, saccharification, fermentation) have been ongoing, but few researchers have considered the effect that the incoming raw biomass can have on the process. There are considerable differences in the way that biomass is chosen, harvested, comminuted and stored depending on the type of biomass (eg. woody or herbaceous), type of crop (eg. agricultural residue or dedicated energy crop), time of year (eg. fall or spring) and geographical area (eg. pine in the South or poplar in the North). Rather than designing a biorefinery around a particular source of a given feedstock, it is preferable to understand how biomass can be altered to provide the maximum yield of hydrolysable and fermentable sugars from whatever is available. Since the moisture content is highly variable and easily altered, the effect of drying and rewetting on bioconversion was studied on switchgrass, sugarcane bagasse and hybrid poplar.

The moisture content of lignocellulosic biomass is of particular importance when using SO_2 catalyzed steam explosion. Unlike aqueous catalysts, SO_2 is applied as a gas and its diffusion within the biomass particle determines both the severity of pretreatment and the uniform cooking of the material. SO_2 can both be absorbed more readily and penetrate more deeply in biomass that is close to saturation with water, even if it has been previously air dried. For switchgrass and sugarcane bagasse, the ethanol yield after simultaneous saccharification and fermentation was improved 18-24% by increasing the moisture content by soaking prior to pretreatment. It was

also found that soaking had no effect when the samples were not catalyzed with SO_2, confirming that the effect of moisture content is directly related to SO_2 uptake and diffusion into the biomass. In hybrid poplar, the results were similar to herbaceous biomass for chips with less than 2% absorbed SO_2. However, when the SO_2 uptake was increased to 3% even the air dried chips exhibited high digestibility, indicating that increased SO_2 uptake can overcome the poor diffusion in dried biomass.

Alongside controlling the biomass moisture content, improving control and knowledge of the processes can also increase efficiency and product yields. Avoiding process upsets and contamination could be the difference between an economically viable biorefinery and one that struggles to compete. By monitoring reactions with accurate, on-line sensors, operators can detect when reactions deviate from the norm, and when they are complete. These sensors must be robust and provide continuous, real-time information about the process. Raman spectroscopy, a vibrational spectroscopy capable of rapidly measuring aqueous reactions without any sample preparation is one such method.

Real time, continuous Raman spectroscopy was used to continuously monitor both a synthetic glucose and a lignocellulosic fermentation and measure glucose and ethanol. Models developed using offline HPLC validation samples had extremely high correlation between predicted and observed values for ethanol in both fermentations (R^2 = 0.98 and 0.94 for synthetic and hydrolysate, respectively) while glucose proved more difficult to detect in the hydrolysate fermentation (R^2 = 0.92 and 0.51). This work showed that it is possible to monitor the ethanol and glucose in a hydrolysate with a high fluorescent background by using a fastloop sampling system to take actual measurements and chemometrics to process the data.

6.2 Future work

The work outlined in this thesis forms a basis of understanding on how to improve process efficiency through feedstock alteration and process sensors. Future work should expand on this research in the following ways:

6.2.1 Biomass moisture content

The effect of moisture content on bioconversion was determined for switchgrass, sugarcane bagasse and hybrid poplar. The same experiments could be carried out on locally relevant softwoods, such as the hemlock and Douglas-fir available from Pack Forest, and on the wheat straw and giant reed available in the Pacific Northwest. It would also be useful to obtain and compare never-dried agricultural residues and how they compare to the results from their dried counterparts.

It would also be useful to examine the effect of drying on a microscopic level by observing the physical changes in the fiber structure. How do different re-wetting regimes change the structure? Utilize Simons stain, microscopy and other methods to examine the differences in accessibility and pore size between dried and never dried biomass.

Another question that has not been satisfactorily answered in the literature is the role of SO_2 impregnation in subsequent pretreatment, hydrolysis and fermentation. How important is the impregnation time, and is it dependent on particle size? What is the maximum amount of SO_2 a given type of biomass will absorb, and how does the amount of SO_2 absorbed affect downstream processes? What role does SO_2 play in the formation of rejects, and how can rejects be more accurately quantified and characterized?

Modeling of the process would be very useful; for example, developing a relationship between the pretreatment severity and biomass moisture content to predict solids digestibility and ethanol yield. Is there a moisture threshold above which the yields are unaffected? What effect does increasing moisture content have on the economics of the process in terms of water use and heating costs? What effect does reducing moisture content have on shipping, storage and comminution costs?

6.2.2 Application of Raman spectroscopy to bioconversion

The detection and quantification of ethanol and glucose using Raman has now been shown. However, the presence and amount of many other compounds would be useful and should also be modeled. These include xylose and xylitol (of particular importance for xylose-utilizing organisms), glycerol (an indicator of cell stress), and acetic acid, furfural and 5-hydroxymethyl furfural (potential inhibitors).

A portion of the background fluorescence present in the fermentation was due to cell biomass fluorescence. By separating the cell biomass from the liquid being measured, a cleaner signal might be obtained. This could be done by adding a filter to the intake for the fastloop, but such a filter could become easily fouled. A sweeping mechanism could be added that would take advantage of the stirring velocity of the reactor. Similarly, measuring production of glucose from cellulose necessitates measuring a solution of pretreated solid particles – filtration prior to analysis would prevent their interference with the laser and improve the data.

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Vita

Shannon Melinda Ewanick was born in Williams Lake, British Columbia, Canada, on November 14, 1980, the daughter of Gladys and Ken Ewanick. After completing high school at Mount Baker High School in Cranbrook BC, she went on to the University of British Columbia in Vancouver BC where she studied Biochemistry and received her Bachelor of Science in May 2003. For the next year she traveled and worked in Europe and in May 2004 started her Masters in Jack Saddler's lab at the University of British Columbia. She finished her MSc in September 2006 and worked in the Saddler lab for a year, after which time she began her doctoral studies in Renata Bura's lab at the University of Washington, Seattle in January 2008. She married her longtime love, Dr. Adam Warner, in July 2009 and received her PhD in December 2012.

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