Improving carbohydrate recovery from uncatalyzed steam pretreated hybrid poplar

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Abstract

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The presence of SO_2 in steam pretreatment creates a series of problems in environmental protection, equipment corrosion, chemical catalysis and waste water treatment. Moreover, SO_2 increases the pretreatment severity, resulting in more sugar degradation and increased inhibitor formation. In this study, hybrid poplar chips were steam exploded using 6 different conditions with or without the addition of SO_2. The steaming temperature ranged from 190 ℃ to 212 ℃, and the residence time ranged from 5 min to 10 min, resulting in a range of fermentation inhibitors, including furfural, 5-hydroxymethyl furfural (HMF), acetic acid and phenolic. It was found that certain concentration of inhibitors, particularly acetic acid, could promote the ethanol yield in hydrolysate fermentation, but always impairs the xylitol yield due to the acetyled xylan in hybrid poplar. At the lowest pretreatment severity condition at 205 ℃, 10 minutes, without SO_2, the best inhibitor concentration for ethanol yield and the highest overall sugar recovery following pretreatment were achieved. However, since no SO_2 was applied, poor digestibility during enzymatic hydrolysis of cellulose reduced the post-hydrolysis sugar recovery. For that reason, mechanical refining was applied to the solid fractions and improved the enzymatic

hydrolysis for solids pretreated at 205 °C for 10 minutes, without SO_2 as much as 23 %. Similar improvements were observed for different enzyme loadings and solid consistencies. However, refining did not improve the hydrolyzability of solids pretreated at five other conditions. Reduced particle sizes were found to be correlated to increased sugar yields in enzymatic hydrolysis. Solids pretreated at 205 °C 10 minutes, without SO_2 exhibited the largest size reduction after refining and correspondingly achieved the highest overall sugar recovery improvement after steam pretreatment and enzymatic hydrolysis. In general, refining can enable a catalyst free, low inhibitor concentration, high overall sugar recovery bioconversion system based on the steam pretreatment and enzymatic hydrolysis method.

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Preface

Chapter 1. Portions of the introductory text are used with permission from Dr. Shannon Ewanick's Ph.D. thesis "Improving the bioconversion yield of carbohydrates and ethanol from lignocellulosic biomass" (2012), Dr. Azra Vajzovic's Ph.D. thesis "Production of xylitol and ethanol from lignocellulosics" (2012) and Dr. Renata Bura's Ph.D. thesis "Bioconversion of corn fibre to ethanol" (2004), the former two were works in Biofuels and Bioproducts Laboratory at the University of Washington, and the latter was completed at the University of British Columbia.

Chapter 2. A version of this material is in preparation and will be submitted for publication in the Journal of Industrial Microbiology and Biotechnology. I prepared the solids fraction of steam exploded hybrid poplar, performed chemical compositional analysis and mass balance calculations, and performed enzymatic hydrolysis. Azra Vajzovic acquired the liquid fractions (hydrolysates) from sugarcane bagasse, hybrid poplar, switchgrass, mixed wood and *Arundo donax* (giant reed), analyzed the inhibitor concentration and performed fermentation. Rodrigo Morales Vera conducted the steam pretreatment and Neethi Nagarajan helped in collecting and analyzing the liquid fraction. Azra Vajzovic, Shannon Ewanick, and I wrote the manuscript of the paper. Renata Bura provided instruction in both experiment and writing.

Chapter 3. The paper is in preparation for submission to Biotechnology for Biofuels. The steam pretreated solid fractions from hybrid poplar were generated as described in Chapter 2. I performed chemical composition analysis, refining, enzymatic hydrolysis, fiber quality analysis and data interpretation. Renata Bura and I conceived the experiments, and Rick Gustafson and William McKean offered valuable insights. I wrote the manuscript, and Shannon Ewanick and Renata Bura provided guidance.

Seattle, July 2013

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献给我的家人，我的导师，我的朋友，我深爱的人。

Chapter 1. Introduction

1.1 Biofuels

Depletion of fossil fuels, growing rates of greenhouse gas emissions and excessive environmental pollutions are forcing people to think about constructing the world future based on the renewable and substantial energy.

To date, first generation biofuel, mostly bioethanol, is mainly produced from carbohydrates in starch or sucrose crops such as corn or sugarcane (RFA, 2012). However, due to the total invested capital, consumed energy, released CO_2 during cultivation, and application of fertilizers and water to grow crops, whether or not first generation biofuel has positive net benefit is still in doubt (Pimentel, 2003). Starch/sucrose based biofuel may not solve the energy and environment problems; on the contrary, it threatens the world's food security and gives rise to other problems (Babcock, 2012).

As one of the world's most abundant and cheap sustainable resources, lignocellulosic biomass has the potential to replace starch/sucrose and become a major source of fermentable sugars for the production of renewable biofuels and biochemicals (Hettenhaus, 2006). It is reported that annually there are 1.3 billion tons of biomass available in U.S. from forestland and agricultural land (Perlack *et al.*, 2005), enough to produce sufficient biofuels to displace one third of the fossil fuel consumption in U.S.. Development of biorefinery technologies is now underway to convert lignocellulosic biomass into fuels and chemicals including ethanol, butanol, methane, and acetic acid which are now mainly generated from petrochemical feedstocks (National Research Council, 2000). Additionally, compared with starch and sucrose crops, lignocellulosic biomass is inedible by humans. In particular, ethanol production from lignocellulosic biomass has a more favorable energy ratio than corn ethanol (Keeney & DeLuca, 1992). So, considering the sustainablity and energy efficiency, lignocellulosic biomass is regarded as one of the most promising alternative replacements of fossil fuel.

1.2 Lignocellulose biomass

In second generation biofuel, many types of lignocellulosic biomass can be utilized to produce fuels and chemicals (Perlack *et al.*, 2005), including woody biomass (*e.g.* hybrid poplar and Douglas fir), herbaceous plants (*e.g.* switchgrass and giant reed) and agricultural residues (*e.g.* corn stover and wheat straw).

Primarily, lignocellulosic biomass is made up of three major components: cellulose, hemicellulose and lignin, and minor components of extractives (small organics) and ash (inorganic compounds) (Balat, 2011). Linked together as microfibrils, cellulose builds the skeleton of lignocellulose. Hemicellulose and lignin encompass and tightly bind around the clusters of microfibrils, forming the nanometer-scale composites that make up the rigid structure of the plant cell (Sjöström, 1993).

The proportions of the above components depend on the feedstock type. As shown in Table 1.1, lignocellulosic biomass of different species varies in their composition ratio. With lignin in particular, softwood has higher lignin composition than hardwood and agricultural residues.

Table 1.1 Composition of various lignocellulosic feedstocks (Ewanick, 2012).

	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

1.2.1 Cellulose

As the major component of lignocellulosic material, cellulose is the target of bioconversion. Linked by β-(1-4) glycosidic bonds, D-glucose units comprise the cellulose chain as a linear polymer backbone (Sjöström, 1993). The number of glucose units in each chain of cellulose is known as degree of polymerization (DP), which varies by feedstocks. The hydrogen bonds connect between cellulose molecules build up the elementary structure of fibrils, including stable crystalline regions and disordered amorphous regions (Sjöström, 1993). Generally, the cellulose

structure determines its resistance to breaking cellulose into glucose by enzymatic, chemical or thermal means, as the crystalline region is more difficult to attack by heat, chemicals and enzymes (Eriksson, 1990).

1.2.2 **Hemicellulose**

Unlike cellulose, hemicellulose has more versatile chemical composition and a branched heterogeneous structure (Ramos, 2003). As the second major polysaccharide, hemicellulose consists of different five and six-carbon sugars, including hexoses (D-glucose, D-mannose and D-galactose) and pentoses (D-xylose and L-arabinose) (Sun *et al.*, 2003). Hardwood and herbaceous residue hemicelluloses are mostly composed of highly acetylated glucuronoxylan, and low amount of glucomannans, while softwoods have higher proportion of galactoglucomannans and partly acetylated arabinoglucuronoxylan (Sjöström, 1993). Owing to the higher acetate group content which can form organic acids during pretreatment, the hardwood and herbaceous hemicelluloses are more labile in pretreatment, undergoing acid autohydrolysis, than softwood hemicellulose (Kong *et al.*, 1992).

1.2.3 **Lignin**

As the second most abundant organic substrate within plant biomass, lignin mainly constituents three phenolic structure compounds, including ρ-hydroxyphenyl (derived from p-coumaryl alcohol), guaiacyl (derived from coniferyl alcohol), and syringyl (derived from sinapyl alcohol). In plant tissue, lignin is generally combined with hemicellulose and its composition and structure varies among species (Fengel, 1989).

1.2.4 **Extractives and ash**

In addition to carbohydrates and lignin, lignocellulosics contain a variety of organic extractives including phenols such as tannins, terpene alcohols, ketones, and resins and inorganic ash such as CA, K, and Na oxides (Sjöström, 1993; Ohlsson, 2000).

1.3 Bioconversion process

The bioconversion of lignocellulosic biomass into fuels and chemicals consists of three main processes (Bura *et al.*, 2009): pretreatment, altering the structure of cellulosic biomass to make cellulose more accessible to enzymes; enzymatic hydrolysis, applying enzymes to catalyze the degradation of cellulose to glucose and of heteroxylans to pentose monomers; and fermentation, converting sugars into desired bioproducts using microorganisms. Several challenges exist in each step of the bioconversion scheme (Himmel *et al.*, 2007). First, pretreatment requires a balanced severity for both good biomass fractionation and low fermentation inhibitor formation. Secondly, cellulytic enzymes are one of the costliest portions of biofuel production; it represents a major barrier to the economic feasibility of biofuel industrialization. Finally, an ideal microorganism and a well-developed process are important to high fermentation productivity. Generally, to achieve the highest fermentation yields, a good overall sugar yield with low inhibitor content from lignocellulosic biomass is the primary objective.

1.3.1 Pretreatment

On the grounds that lignocellulosic biomass exhibits recalcitrant properties, it is difficult to improve the accessibility of enzymes by altering the structure and removing the impediments (Mosier *et al.*, 2005). As shown in Figure 1.1, by breaking the lignin seal, releasing the hemicellulose and breaking the cellulose crystallinity, the lignocellulosic structure is opened up, enabling enzymes to convert carbohydrate polymers to monomeric sugars (Grethlein & Converse, 1991). Regarded as one of the most costly steps in lignocellulosic biomass-to-fermentable sugars conversion process, pretreatment requires large amounts of energy and chemical input (Mosier *et al.*, 2005). From another aspect, this means there is great potential in minimizing energy demands and operational cost of the pretreatment process (Lynd *et al.*, 1996).

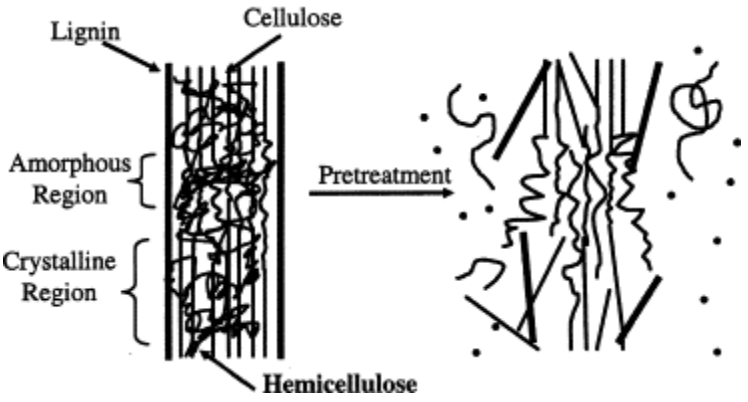

Figure 1.1 Effects of pretreatment on lignocellulosic biomass (Hsu et al., 1980)

Steam pretreatment, sometimes called "steam explosion", is one of the most widely used pretreatment methods in research (Galbe M, 2007) and pilot plants and demo plants (Bacovsky *et al.*, 2013). It is chosen as the pretreatment method in this research because of its versatility in pretreatment conditions and its capability in fractionating various woody biomass and herbaceous residues with different particle sizes (Bura *et al.*, 2009; Ewanick & Bura, 2010, 2011). During steam pretreatment, lignocellulosic biomass is rapidly heated by high-pressure steam for several minutes. After being held for a certain period of time, the pretreated material is rapidly released from the reactor with a fast pressure vent and temperature drop (Ramos, 2003; Mosier *et al.*, 2005).

During uncatalyzed steam pretreatment, high temperature water can act as a catalytic acid (Baugh *et al.*, 1988; Weil *et al.*, 1997) and acetic acids cleaved from acetylated hemicellulose allow the hemicellulose to undergo autohydrolysis (Ramos, 2003), but the pH is not low enough and reaction is too mild and slow for most applications. For that reason, the addition of acid catalysts is required to increase the effectiveness of the steam pretreatment (Ramos, 2003; Schütt *et al.*, 2011; Schütt *et al.*, 2013). Introducing gaseous sulphur dioxide as acid catalyst enables shorter residence time and lower reaction temperature, thus further reducing the energy cost (Bura *et al.*, 2002).

Residence time in the heated reactor, steaming temperature and SO_2 concentration are three factors that determine the severity of steam pretreatment. Overend (1987) developed the reaction severity factor (R_o) based on the well-characterized H-factor theory of the pulp and paper

industry (Vroom, 1957). This factor combines the residence time and steam temperature into a function as follows (where t is time in second and T is temperature in degrees Celsius):

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

Past research indicates that the acid concentration plays a crucial role in pretreatment. Since the equation above only considers the time and temperature but not acid level, Chum et al. (1990) introduced a third parameter, pH, into the equation above to describe the combined severity (CS) (where pH is measured after pretreatment):

$$CS=\log R_o-pH \quad \text{(Equation 1.2)}$$

Typically, a low severity pretreatment may lead to incomplete fractionation of biomass, while a high severity pretreatment may result in a more complete biomass deconstruction with better hemicellulose solubility and further delignification.

1.3.2 **Enzymatic Hydrolysis**

As discussed above, enzymatic hydrolysis is regarded as one of the key processes in bioconversion. To saccharify cellulose into its monomeric units, a host of different glycolytic enzymes are applied to pretreated biomass. Theses enzymes collectively named cellulases are synthesized by several microorganisms. One of the most investigated and utilized strain is the fungi *Trichoderma reesei*, which is the most prominent industrialized microorganism in cellulase production (Durand *et al.*, 1984; Eriksson, 1990; Eriksson *et al.*, 2002).

There are primarily three groups of cellulases necessary in efficient hydrolysis of cellulose into glucose monomers (Eriksson, 1990; Mansfield *et al.*, 1999): endoglucanases randomly attack β-(1-4) glycosidic bonds within amorphous cellulose and generate cellulose chains with free ends; exoglucanases travel along the cellulose chains from both reducing or non-reducing ends, peeling cellobiose units; β-glucosidases hydrolyze the cellobiose to glucose. End-product inhibition is prevalent in all of the above three processes, and reduces the hydrolysis speed when each enzyme is used separately, thus saccharification is carried out by this synergistic enzymatic system (Eriksson *et al.*, 2002).

Structurally, the recalcitrance of lignocellulosic biomass reduces the accessibility of enzymes to cellulose; many factors comprehensively cause this problem: the crystalline structure and the high degree of polymerization of cellulose; cellulose sheathing by hemicellulose and lignin; accessible surface area; and the heterogeneous character of biomass particles (Hsu, 1996; Chang & Holtzapple, 2000; Sun & Cheng, 2002; Mosier *et al.*, 2005; Leu & Zhu, 2013).

1.3.3 **Fermentation**

Fermentation typically refers to the conversion of sugar to biochemicals using microorganisms. In bioconversion, the goal is to convert all the fermentable sugars (*e.g.* glucose, xylose) from lignocellulosic biomass into bioproducts (*e.g.* ethanol, xylitol). In nature, a variety of bacteria and yeast are able to ferment carbohydrates to target products (Lin & Tanaka, 2006). Moreover, compared with bacteria, yeast has faster and higher ethanol yield and better high-ethanol tolerance. Therefore, yeast is more popularly utilized in fermentation (Lin *et al.*, 2006).

The yeast strain utilized in this study, *Candida guilliermondii* (ATCC 201935), exhibits cofermentation of pentoses and hexoses to xylitol and ethanol (Barbosa *et al.*, 1988; Lee *et al.*, 1996). As a pentose fermenting organism, *Candida guilliermondii* is well suited for hardwoods and agricultural residues since these feedstocks typically contain high concentrations of xylose (Lima *et al.*, 2004).

1.3.4 **Inhibitory compounds**

Many compounds formed during steam pretreatment of biomass can be detrimental to yeast metabolism and can reduce the fermentation yield. The majority of these inhibitors from steam pretreatment are present in the water soluble fraction (hydrolysate). Based on their origin, inhibitors are categorized into several groups: 1) compounds naturally released from feedstocks, 2) carbohydrate degradation products, 3) lignin degradation products, 4) compounds from process and equipment (Olsson & Hahn-Hägerdal, 1996).

Compounds naturally released from feedstocks involve extractives and acetic acid. Extractives are small organic compound from wood, including terpenes, alcohols, tannins and resin/fatty acids (Olsson *et al.*, 1996). Acetic acids are liberated from acetyl substituents of hemicellulose

side groups as mentioned previously, and are inhibitory to yeast metabolism and suppress cell growth (Olsson *et al.*, 1996).

The other most influential inhibitors are sugar degradation products, such as furfural from pentoses and 5-hydroxymethyl furfural (HMF) from hexoses. These inhibitors are products dehydrated from sugars when severe acid is present (Dunlop, 1948; Klinke *et al.*, 2004). Furfurals and HMFs weaken the yeasts. But, at the same time, yeasts can metabolize them in low concentrations and reduce the inhibitory effects (Vajzovic *et al.*, 2012).

Including vanillin, syringaldehyde, 4-hydroxybenzaldehyde and phenol, etc. (Clark & Mackie, 1984; Delgenes *et al.*, 1996; Zhang, Geng, *et al.*, 2012), phenolic compounds are derived from degradation of lignin. Some of them, can also be assimilated by the yeast and have a minimal effect at low concentrations (Delgenes *et al.*, 1996).

Finally, inhibitors formed from process and equipment can also negatively affect fermentation. Iron, nickel, metal compounds can be discharged from equipment, especially when the pretreatment is corrosive to the facility (Olsson *et al.*, 1996). Furthermore, compounds like sulphites from introduced SO_2 shows inhibitory effects on the yeast growth (Pilkington & Rose, 1988).

1.4 Refining

Refining, as a traditional process in pulping and paper industry, is one of the most important unit operations when preparing high quality pulp fibers in the papermaking process (Kang & Paulapuro, 2006b). Refining refers to the mechanical action carried out on the fibers by shear stresses between the bars and the grooves and channels of the refiner and can include rolling, twisting and tensional actions (Smook, 1992; Paulapuro & Paper, 2000). This mechanically treats the pulps and simultaneously modifies the fibers with internal fibrillation, external fibrillation, fiber shortening or cutting and fines formation (Smook, 1992; Kang & Paulapuro, 2006a; Kang *et al.*, 2006b).

The major effects of refining on fiber characteristics in the pulp and paper industry are as follows (Smook, 1992; Paulapuro *et al.*, 2000):

- External fibrillation; removal of primary wall; formation of fiber debris and fines.
- Internal fibrillation; delamination, generation of microfibrils on fiber surface.
- Penetration of water into the cell wall (referred to as swelling).
- Breaking of some intra-fiber bonds; replacement by water-fiber hydrogen bonds.
- Increasing fiber flexibility.
- Fiber shortening/cutting.
- Fiber stretching, flattening and compression.
- Curling or straightening of fibers.
- Redistribution of hemicellulose from the interior of the fibers to the exterior.

In order to form strong and smooth paper, the main target of refining is to improve the bonding ability of fibers through some of the mechanisms mentioned above. Also, refining is used to develop pulp into given properties, such as absorbency, porosity and other optical properties. Sometimes, the main purpose of refining is to shorten long fibers to ensure good sheet formation (Paulapuro *et al.*, 2000).

During the refining process, the initial action is to partially peel off the thin primary wall, which exposes the secondary wall; further "internal fibrillation" loosens the internal structure and softens the fiber; then "external fibrillation" creates microfibrils on the fiber surface and largely increases the surface area (Smook, 1992; Kang *et al.*, 2006b; Koo *et al.*, 2011); more inter-fiber bonds are simultaneously exposed on surface area.

Size reduction is described as another effective way to promote the connectivity between pulp fibers during refining (Sehaqui *et al.*, 2011) to a certain extent, as the specific surface area increases during fiber shortening (cutting). However, prolonged refining is often considered undesirable, since fiber over cutting breaks fibers into fragment and removes debris from fiber walls, contributing to slower drainage and loss of strength (Smook, 1992; Paulapuro *et al.*, 2000).

Previous research showed that direct mechanical pretreatment of lignocellulosic biomass was not economically feasible due to the high energy input (Brown, 2003; Alvira *et al.*, 2010). Mechanical pretreatment methods generally acknowledged by academia (Lynd *et al.*, 1996; Yang & Wyman, 2008) and the government (DOE, 2006) only focused on physical modification of biomass prior to chemical pretreatment, which has been shown to be a significantly energy negative approach. However, the literature has rarely mentioned post-chemical pretreatment before 2010.

Refining through use of a disc refiner was first applied by Zhu *et al.* (2010) to improve the sugar yield during enzymatic hydrolysis. The cellulose saccharification of Sulfite Pretreatment to Overcome Recalcitrance of Lignocellulose (SPORL) pretreated lodgepole pine achieved 90% conversion with 15 filter paper units (FPU)/g substrate cellulase loading and 10-20 % solids consistency. Research by Koo *et al.* (2011) reported that PFI refining significantly reduced the enzyme usage by up to 50%. The improvement of sugar conversion was proposed to be an increase in enzyme-accessible surface area and pore volume. And a recent study of Chen *et al.* (2012) demonstrated that mechanical refining (PFI) of steam pretreated corn stover promoted the sugar yield at both low- and high- solid enzymatic hydrolysis with reduced enzyme loadings. The significant particle size reduction to increase the reactive surface area of the biomass was regarded as the factor of enzymatic digestibility improvement. Techno-economic analysis by Tao *et al.* (2012), showed that refining combined with a deacetylation process lowered the minimum ethanol selling price (MESP).

1.5 Objectives

The research presented in this thesis focused on improving the carbohydrate recovery from uncatalyzed steam pretreated hybrid poplar. This was approached in two ways:

- In Chapter 2, hybrid poplar was pretreated using different severity conditions with and without SO_2 to determine which had the lowest inhibitors concentration, the highest ethanol and xylitol yields from the hydrolysate, and the highest sugar recoveries after pretreatment and enzymatic hydrolysis.

- In Chapter 3, refining was applied to solids from hybrid poplar steam pretreated at 6 different severities with and without SO_2 in order to determine whether refining could improve the sugar yield following enzymatic hydrolysis.

Chapter 2. Enhanced xylitol and ethanol yields by fermentation inhibitors in steam pretreated hydrolysates

Abstract

A systematic study of the effects of low concentrations of fermentation inhibitors on the fermentation of xylose to xylitol and hexoses to ethanol by the yeast *Candida guilliermondii* in the steam pretreated hydrolysates from mixed wood, hybrid poplar, *Arundo donax* (giant reed), switchgrass and sugarcane bagasse was conducted. It was shown that fermentation inhibitors are not necessarily harmful compounds. On the contrary, acetic acid, furfural, HMF, and phenolics at certain concentrations can be called enhancers rather than fermentation inhibitors. In the presence of up to 8g/L of acetic acid along with furfural, HMF, and phenolics in sugarcane bagasse hydrolysate, the ethanol and xylitol yields were boosted up to 20% compared to the control. For six different steam pretreatment severities tested for hybrid poplar, the ethanol yield compared to theoretical was enhanced by more than 22% compared to the control, indicating that process-derived and/or biomass-derived inhibitors were enhancing the fermentation. At lower pretreatment severities, the inhibitor concentration in the hydrolysate was reduced and higher ethanol yields were achieved. The overall post-pretreatment sugar recovery was high for hybrid poplar pretreated at the lowest-severity pretreatment (205°C, 10 minutes without SO_2); however, its overall post-hydrolysis sugar recovery was low due to the poor hydrolyzability of the solid fraction, resulting from inadequate fractionation in the absence of SO_2.

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In preparation for publication in Journal of Industrial Microbiology and Biotechnology

2.1 Introduction

A commercial biorefinery requires optimum conversion of biomass feedstock to products in order to provide good economic returns and reduce our dependence on fossil fuels. Various feedstocks such as agricultural and forest residues, food processing and paper waste can be utilized for the production of biofuels and biochemicals (National Research Council, 2000). Many of these feedstocks are available in abundance, and can serve as a low-cost alternative resource for renewable energy production (Kumar *et al.*, 2008; Bura, Ewanick, *et al.*, 2012). Conversion of lignocellulosics to biofuels and biochemicals consists of four major steps (Kumar *et al.*, 2008): pretreatment, hydrolysis, fermentation, and product(s) recovery. Feedstock pretreatment is a key for successful bioconversion of lignocellulosics to biofuels and biochemicals (McMillan, 1994; Mosier *et al.*, 2005; Kumar *et al.*, 2009).

Several different pretreatment methods can be used to facilitate the enzymatic hydrolysis of lignocellulosic material (Mosier *et al.*, 2005; Ewanick *et al.*, 2010). One of the most thoroughly investigated methods is steam explosion pretreatment (Ewanick *et al.*, 2007; Bura *et al.*, 2009; Carrasco *et al.*, 2011). As has been shown previously, SO_2-catalyzed steam explosion at optimized conditions can provide a high recovery of hemicellulosic sugars and minimal production of fermentation inhibitors (Tengborg *et al.*, 1998).

During SO_2-catalyzed steam explosion pretreatment, there are three main process variables: temperature, time and SO_2 concentration. Each feedstock has different processing requirements due to chemical and structural variations, but SO_2-catalyzed steam explosion at optimized conditions can provide a high recovery of hemicellulosic sugars and minimal production of fermentation inhibitors (Tengborg *et al.*, 1998). A low severity can lead to incomplete fractionation of biomass, causing low digestibility of the pretreated solids and subsequently lower hydrolysis yields and overall sugar recovery. Higher severity steam pretreatment results in a more complete biomass fractionation and better hydrolysis conversion, which is desirable, but the inevitable effect of this condition is formation of numerous degradation products which can be inhibitory to fermentative microorganisms (Olsson *et al.*, 1996).

The generation of fermentation inhibitors is one unavoidable consequence of pretreatment at high temperatures and low pH used in the steam explosion process (Olsson *et al.*, 1996). These inhibitory compounds adversely affect microbial growth and fermentation yields (Olsson *et al.*, 1996; Winkelhausen & Kuzmanova, 1998; Kumar *et al.*, 2009). Two major groups of potential inhibitors have been found in the liquid fraction after pretreatment of lignocellulosic feedstocks: naturally-occurring inhibitors from the feedstock (*e.g.* sterols, acetic and uronic acids and resin/fatty acids) and process-derived inhibitors created during pretreatment (*e.g.* lignin and sugar degradation products) (Olsson *et al.*, 1996; Palmqvist *et al.*, 1998), any of which may have adverse effects during fermentation (Tengborg *et al.*, 2001).

Naturally occurring inhibitors are dependent on the chemical composition of the particular feedstock utilized. The most important of this class is acetic acid, which is released from acetylated hemicellulose groups during pretreatment. Certain feedstocks such as softwoods are naturally low in acetate groups, while hardwoods and agricultural biomass contain more acetylated hemicellulose and have the potential to generate more acetic acid.

Process-derived inhibitors are directly related to the severity of pretreatment; among the many compounds generated the three most prevalent are furfural (from dehydration of pentoses), 5-hydroxymethyl furfural (HMF; from hexose dehydration reactions), and phenolic compounds from the degradation of lignin.

In order to improve the fermentability of pretreated hydrolysates, chemical, physical, and biological methods can be used to remove inhibitors prior to the fermentation, which increases the fermentability of the solution (Olsson *et al.*, 1996). Some of these detoxification methods include pH adjustment, pre-growing-adaptation of microorganisms to the unfavorable environment, steam stripping, ion-exchange or ion-exclusion chromatography, overliming, and organic solvents extraction (Maddox & Murray, 1983; van Zyl *et al.*, 1991; Larsson *et al.*, 1999; Hyne, 2001). However, detoxification may not be necessary when low and unique concentrations of fermentation inhibitors are present in hydrolysates and when a fermenting organism with high inhibitor tolerance is used (Amartey & Jeffries, 1996; Palmqvist & Hahn-Hägerdal, 2000). For instance, complete fermentation of an acid hydrolysate of spruce, which was strongly inhibiting in batch fermentation has been achieved in fed-batch fermentation without any detoxification

treatment (Taherzadeh *et al.*, 1999). Fermentation inhibitors present at low concentrations, as in the fed-batch fermentation, instead can impose a stimulatory effect on final products yield and therefore their presence may be somewhat desirable (Palmqvist *et al.*, 1999; Bura, Vajzovic, *et al.*, 2012; Schmitt *et al.*, 2012; Vajzovic *et al.*, 2012). These compounds may even lead to increased ethanol yield and productivity due to the presence of weak acids (Palmqvist *et al.*, 1999; Taherzadeh *et al.*, 1999), or due to decreased glycerol production in the presence of furfural (Palmqvist *et al.*, 1999). Therefore, pretreatment degradation products are not necessarily always inhibitory compounds as long as their concentration is lower than a threshold concentration that negatively affects the process (Larsson *et al.*, 1999).

In lignocellulosic hydrolysates, the concentration of sugars as well as the concentration of pretreatment by-products depends on the pretreatment conditions and the feedstock used (Larsson *et al.*, 1999). An increased understanding of the influence of both biomass type and pretreatment conditions on sugar release and production of degradation products can aid in selecting a feedstock and optimizing the pretreatment conditions for enhanced final products yields.

The first part of this study investigated the contribution of the biomass source in determining the concentration of fermentation inhibitors. Hydrolysates originating from five different steam pretreated feedstocks were fermented by *Candida guilliermondii* to see what effect their unique inhibitor profiles had on xylitol and ethanol yields (Figure 2.1). Secondly, hybrid poplar was pretreated at six different conditions and the resulting ethanol and xylitol yields measured (Figure 2.2) to determine the effect of increased pretreatment severity on the fermentability. In addition, the overall sugar recovery of the pretreated biomass was measured after enzymatic hydrolysis to assess the digestibility of the solids.

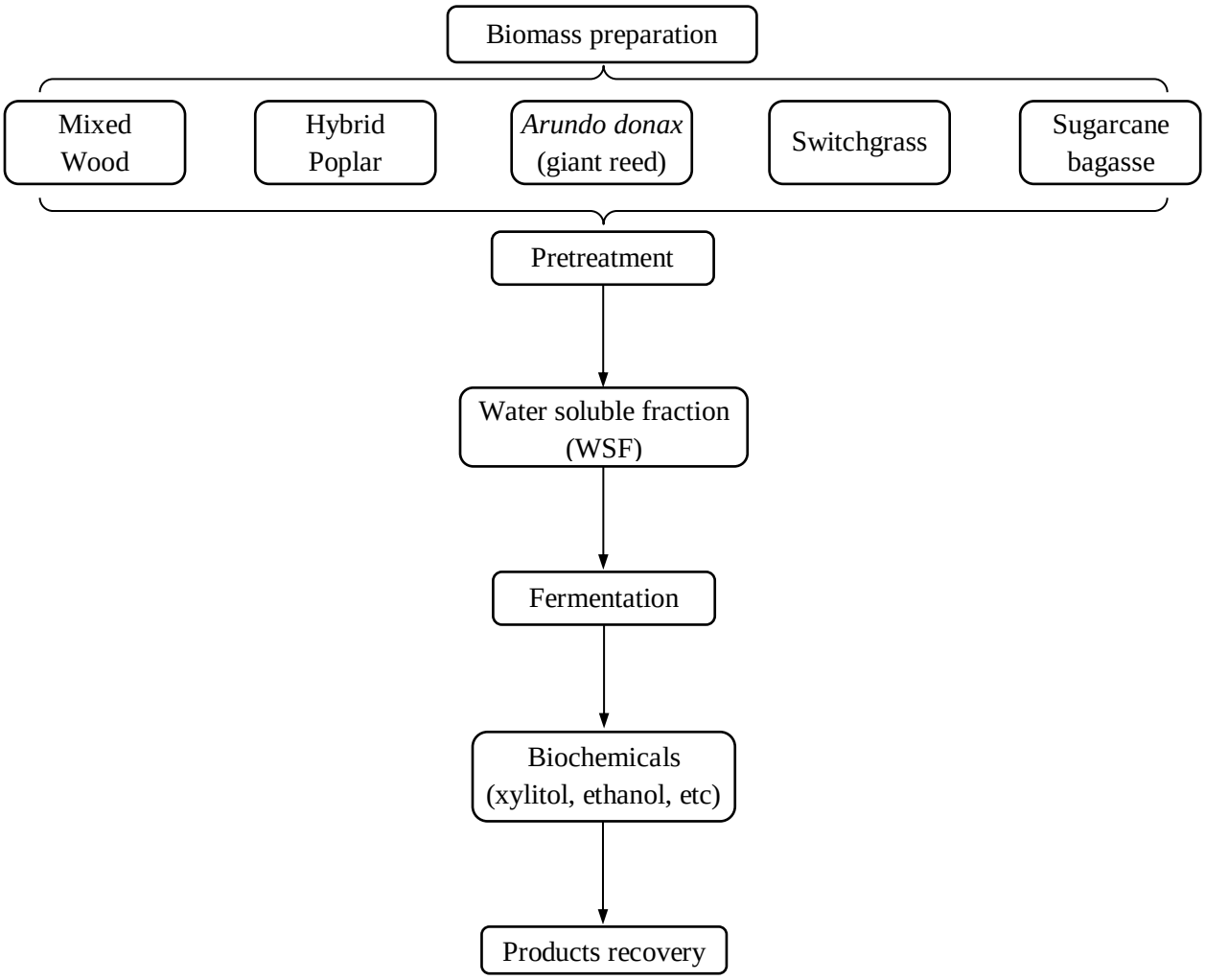

Figure 2.1. Simplified process flow diagram for the bioconversion of five lignocellulosic biomass types (mixed wood, hybrid poplar, giant reed, switchgrass, sugarcane bagasse) into bioproducts (ethanol and xylitol). This process flow illustrates bioconversion of liquid phase only via fermentation process.

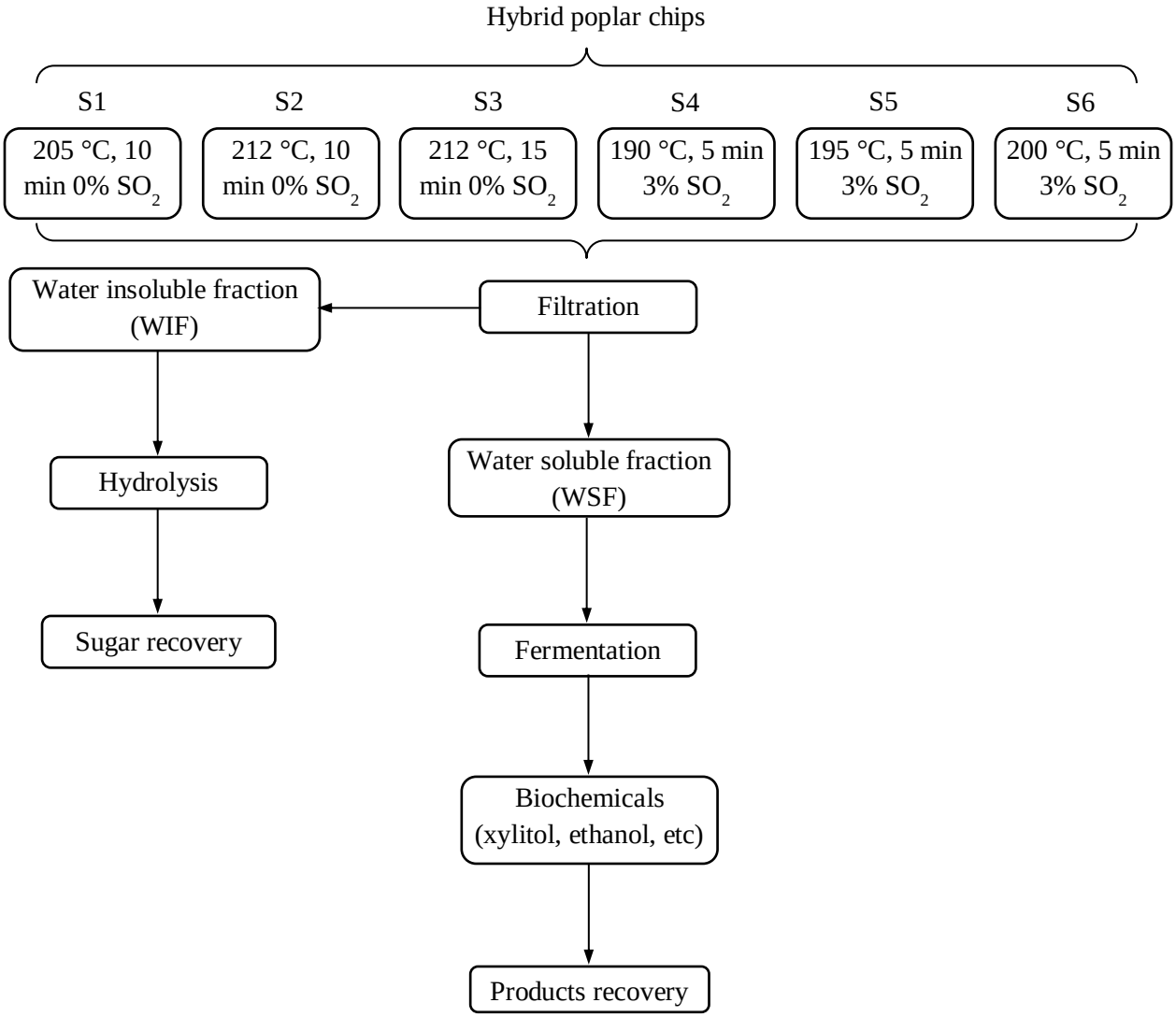

Figure 2.2. Simplified process flow diagram for the bioconversion of hybrid poplar into bioproducts (ethanol and xylitol) testing six different steam explosion conditions. This process flow illustrates the separate hydrolysis and fermentation (SHF) process.

2.2 Materials and methods

2.2.1 Yeast strain

Candida guilliermondii FTI-20037 (NRC 5578) was obtained from the ATCC, a nonprofit biological resource center (BRC), Manassas, Virginia. This strain was taken from -80°C stocks and maintained on YPG solid medium (10g/L yeast extract, 20g/L peptone, 20g/L glucose, and 18g/L agar, Difco, Becton Dickinson, MD) at 4°C and transferred to fresh plates on a weekly basis.

2.2.2 Culture media conditions

Cells were grown to high cell density in foam-plugged 1L Erlenmeyer flasks containing 500ml YP-sugar liquid media (10g/L yeast extract and 10g/L peptone, supplemented with 10g/L xylose) in an orbital shaker for 2 days at 30°C and 150 rpm, with concurrent transfer to fresh medium performed every 24 h. After 48 hours of growth, cell cultures were harvested, centrifuged, and decanted to yield cell pellets. Pellets were then washed three times with sterile distilled water and subsequently adjusted with sterile distilled water to a calculated concentration of 5g dry cell weight (DCW) per liter on a spectrophotometer (Shimadzu UV-1700, Columbia, MD) via standard curves relating 600nm absorbance to $DCWL^{-1}$ (dry cell weight (DCW) per liter) concentration.

2.2.3 Pretreatment

In the first part of this study, lignocellulosic hydrolysates from steam pretreated mixed wood (mix of hardwood and softwood), hybrid poplar, giant reed, switchgrass and sugarcane bagasse were obtained from previous experiments, as detailed in the associated publications listed in Table 2.1.

Table 2.1. Pretreatment conditions used to generate the five lignocellulosic hydrolysates used in this study.

Feedstock	***Conditions***	***Reference***
Mixed wood	210°C, 10 min, 3% SO_2	(Schmitt *et al.*, 2012)
Hybrid poplar	200°C, 5 min, 3% SO_2	(Bura *et al.*, 2009)
Giant reed (*Arundo donax*)	190°C, 5 min, 3% SO_2	(Bura, Ewanick, *et al.*, 2012)

Switchgrass	195°C, 7.5 min, 3% SO_2	(Ewanick *et al.*, 2011)
Sugarcane bagasse	205°C, 10 min, 3% SO_2	(Ewanick *et al.*, 2011)

In the second part of this study, steam pretreatment of hybrid poplar utilized chips (screened to approximately 5mm thickness and 1-3 cm length and width) without bark (50% moisture content) obtained from Forest Concepts (Auburn WA) and stored at -20°C until use. Half of samples of 800g oven-dried weight (ODW) hybrid poplar chips were impregnated overnight with anhydrous SO_2 in plastic bags. The samples were then loaded, in 400g batches, into a preheated, 2.7 L batch steam pretreatment reactor, manufactured by Aurora Technical, Savona BC Canada, and pretreated as follows: three samples impregnated with 3% (w/w) SO_2 concentration were exploded for 5 minutes at temperature of 190°C, 195°C, and 200°C. The other three samples were exploded without SO_2 at 205°C and 212°C for 10 min, and 212°C for 15 min reaction time. The water soluble fractions (hydrolysates) from steam explosion of the hybrid poplar chips at six different conditions were recovered by filtration and kept at -20°C until use. Water insoluble fractions (solids) were separated and washed with water until the wash water was free of sugar.

Severity factor

Pretreated SO_2-impregnated as well as uncatalyzed biomass were attributed a severity factor , used for the evaluation of the explosion process and to describe the lignin reduction and xylan solubilization (Overend *et al.*, 1987). Equation 2.1 describes the severity factor of the pretreatment which increases as a function of time *t* (min) and temperature *T* (ºC):

$$R_o = te^{(T-100)/14.75} \quad \text{(Equation 2.1)}$$

The combined severity (CS) factor is calculated based on the severity factor $\log(R_o)$, and the pH of the liquid hydrolysate after pretreatment, shown in Equation 2.2 (Chum *et al.*, 1990; Rabelo *et al.*, 2012).

$$CS = \log R_o - pH \quad \text{(Equation 2.2)}$$

2.2.4 **Compositional analysis**

Carbohydrates, alcohols, acetic acid, furfural, and HMF

The liquid fractions, along with the wash fraction, were analyzed for monomeric, and oligomeric carbohydrates, acetic acid, HMF and furfural. Monomeric and oligomeric soluble carbohydrates were determined as previously described (Ewanick *et al.*, 2011). Oligomeric sugars were calculated by subtracting monomeric sugars content from total sugars content. Synthetic sugars (glucose, xylose, galactose, mannose, and arabinose) were obtained from Supelco (Bellefonte, PA). Ethanol 4mg/ml, xylitol 5mg/ml, furfural, HMF and acetic acid were obtained from Sigma–Aldrich (St. Louis, MO).

Compositional analysis of solid fraction

A modified Tappi method T-222 om-98 (TAPPI, 1998) was used to determine the chemical composition of original material and steam exploded solids, as previous described (Bura *et al.*). Briefly, 0.2 g of finely ground oven dried sample was treated with 3 ml of 72 % H_2SO_4 for 120 min at 20 °C, then diluted into 120ml total volume and autoclaved at 121°C for 60 min. After filtration through a tared sintered-glass crucible, the acid insoluble lignin was determined by weighing the oven dried crucibles, and acid soluble lignin in filtrate was analyzed by UV at 205nm. Carbohydrate composition of the filtrate was analyzed by HPLC as described in the *HPLC analysis* section. The acetate groups in solid fraction were analyzed by HPLC as previously described (Vajzovic *et al.*, 2012).

Phenolics assay

The Folin–Ciocalteu (F–C) assay was used as a standardized method for approximating the total phenolics concentrations in the hydrolysates, using gallic acid as a standard (Ainsworth & Gillespie, 2007). Folin Ciocalteu reagent and gallic acid were purchased from Sigma. The samples were analyzed by determining the absorbance of each solution at 765 nm against the blank and absorbance vs. concentration were plotted.

2.2.5 **HPLC analysis**

Monomeric sugars

The concentration of monomeric sugars (arabinose, galactose, glucose, xylose and mannose) was measured on a Dionex (Sunnyvale, CA) HPLC (ICS-3000) system equipped with an AS autosampler, ED electrochemical detector, dual pumps, and anion exchange column (Dionex, CarboPac PA1). Deionized water at 1 ml/min was used as an eluent, and post-column addition of 0.2 M NaOH at a flow rate of 0.5 ml/min ensured optimization of baseline stability and detector sensitivity. After each analysis, the column was reconditioned with 0.25 M NaOH. Twenty microliters of each sample were injected after filtration through a 0.22µm syringe filter (Restek Corp., Bellefonte, PA). Standards were prepared containing sufficient arabinose, galactose, glucose, xylose and mannose to encompass the same range of concentrations as the samples. Fucose (0.2g/L) was added to all samples and standards as an internal standard.

Ethanol, xylitol, acetic acid, furfural, HMF, and phenolics analysis

Ethanol and xylitol were measured using refractive index detection on a Shimadzu Prominence LC. Separation of these compounds was achieved by an anion exchange column (REZEX RHM-Mono saccharide H^+ (8 %), Phenomenex, Inc., Torrance, CA) with an isocratic mobile phase that consisted of 5mM H_2SO_4 at a flow rate of 0.6 ml/min. The column oven temperature was maintained at a constant temperature of 63 °C. Twenty microliters of each sample were injected after being appropriately diluted in deionized water and filtered through a 0.22 µm syringe filter (Restek Corp., Bellefonte, PA). Standards were prepared and used to quantify the unknown samples.

2.2.6 **Water soluble fraction (hydrolysate) fermentation**

Liquid hydrolysates produced by steam pretreatment were fermented by *C. guilliermondii*. The initial concentration of sugars (glucose and xylose) present in the hydrolysate was brought up to 30 g/L each. Solutions with sugars were filter-sterilized separately, and appropriate quantities added aseptically to obtain the desired concentration in the hydrolysate fermentation media. 0.1 % (w/v) yeast extract, 0.17 % (w/v) yeast nitrogen base without amino acids and 5 % (w/v) urea were added to the hydrolysates. The initial pH of the hydrolysates was adjusted to 6 prior to fermentation. The controls consisted of synthetic sugars and nutrients dissolved in pure water at the same concentration as measured in the hydrolysates.

The theoretical yield for ethanol production from glucose is 0.51 g ethanol g^{-1} glucose (Olsson *et al.*, 1996). Ethanol yields and percent theoretical yields were calculated using the equations formulated by Keating (2004). The theoretical yield for xylitol production from glucose used was 0.91 g xylitol g^{-1} xylose (Winkelhausen *et al.*, 1998). It was assumed that all xylitol formed during the growth phase of the mixed sugar fermentations was derived from xylose. Cumulative xylitol ($Y_{xylitol}$; g xylitol produced g^{-1} total xylose consumed) yields were calculated during and at the end-point of the fermentations.

The specific consumption and production rates were calculated based on the log-mean cell density (Equation 2.3), where S is the substrate or product, X is dry cell weight, and t is time (Kastner & Roberts, 1990). Within each experiment, tests were conducted in triplicate in separate flasks and the standard deviation was calculated between three samples.

$$q_s = \frac{(S_0 - S)\ln\left(\frac{X}{X_0}\right)}{(X - X_0)\Delta t} \quad \text{(Equation 2.3)}$$

2.2.7 Water insoluble fractions (solids) enzymatic hydrolysis

Enzymatic hydrolysis of washed solids was carried out in 5 % (w/v) consistency with a total volume of 50 ml in 125 ml Erlenmeyer flasks. 50 mM citric acid buffer was used to maintain the pH at 4.8 and the flasks were incubated at 50 °C and 175 rpm in an orbital shaker (New Brunswick). Cellulase (Celluclast 1.5 L from Novozym) at 5 FPU/g cellulose and β-glucosidase (Novozym 188) at 10 CBU/g cellulose were loaded into each flask. 1 ml samples were taken periodically over 96 hours, boiled for 10 min to denature enzymes, and stored at -20 °C for HPLC analysis.

2.3 Results and Discussion

2.3.1 Fermentation of the water soluble fractions (hydrolysates) obtained after steam pretreatment of five different feedstocks

Five lignocellulosic hydrolysates obtained after steam pretreatment of mixed wood, hybrid poplar, giant reed, switchgrass and sugarcane bagasse were fermented by *Candida guilliermondii*. Two major groups of potential inhibitors were identified in all of the liquid fractions after

pretreatment: feedstock-inherited (acetic acid) and process-derived (furfural, HMF, and phenolics). As shown in Table 2.2, the concentration of acetic acid ranged from 1 g/L to 8.2 g/L and was the highest in giant reed hydrolysate. The concentration of furfural ranged from 0.3 g/L to 3.4 g/L, HMF from 0.1 g/L to 0.5 g/L, and phenolics from 1.3 g/L to 3.3 g/L. All the highest inhibitors concentrations measured were noted in giant reed hydrolysate. Following fermentation, the ethanol and xylitol yields were measured as high as 120 % and 88 % of theoretical compared to the controls, 100 % and 67 %, respectively.

The concentrations of fermentation inhibitors were dependent on applied pretreatment condition and the type of feedstock used. For example, in mixed wood hydrolysate, the concentrations of all the inhibitors were the lowest in comparison to the other hydrolysates (Table 2.2). Since all five hydrolysates originated from different feedstock and were pretreated at different conditions, the chemical composition of the hydrolysates has shown that acetic acid concentrations up to 5 g/L measured in lignocellulosic hydrolysate resulted in higher xylitol yield of theoretical produced as compared to the control (Table 2.2 and Figure 2.3).

Table 2.2. Process variables, xylitol and ethanol yields, and chemical composition in hydrolysates obtained by steam pretreatment of mixed wood, hybrid poplar, giant reed, switchgrass, and sugarcane bagasse.

Sample	***Acetic acid (g/L)***	***Furfural (g/L)***	***HMF (g/L)***	***Phenolics (g/L)***	***[XOH] Y%T (%)***[1]	***[EOH] Y%T (%)***[1]
Mixed wood	1.0	0.3	0.1	1.3	86 ± 0.8	117 ± 1.1
Hybrid poplar	4.9	0.8	0.4	2.6	74 ± 0.9	100 ± 1.0
Giant reed	8.2	3.4	0.5	3.3	41 ± 1.0	100 ± 1.1
Switchgrass	1.0	0.3	0.1	1.5	77 ± 0.5	112 ± 1.0
Sugarcane bagasse	2.1	0.8	0.3	1.7	88 ± 0.6	120 ± 0.8
Control	0.0	0.0	0.0	0.0	67 ± 1.0	100 ± 0.5

The reported results are the average of triplicate studies with a deviation of ≤ 2%.
[1]Y%T (%) denotes the percent of theoretical yield of xylitol (XOH) and ethanol (EOH)

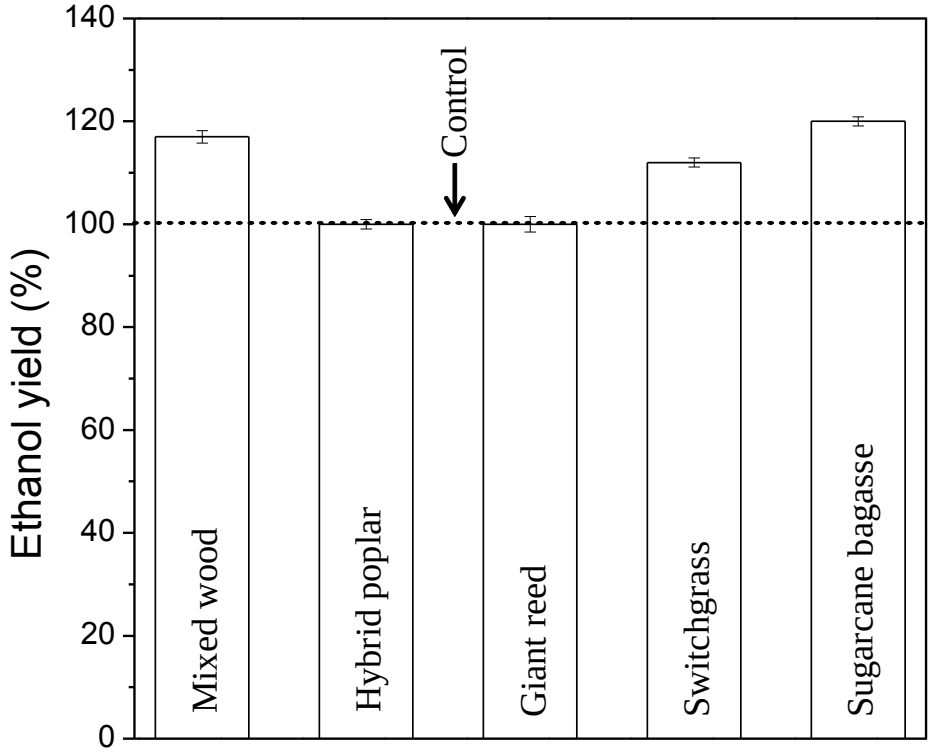

Figure 2.3. Ethanol production expressed as percentage of theoretical yield from five steam pretreated lignocellulosic hydrolysates by *C. guilliermondi*.

Vajzovic (2012) and Bura (2012) previously reported that unique concentrations of fermentation inhibitors appeared to have a stimulatory effect on xylitol and ethanol yields by the *Rhodotorula mucilaginosa* strain PTD3. The ethanol yield of theoretical was up to 20 % (sugarcane bagasse) higher compared to the control (100 %) (Figure 2.3). Ethanol production in excess of the stoichiometry [48 xylose + 21 H_2O → 42 xylitol + 3 ethanol + 24 CO_2] was ascribed to enhanced xylose fermentation in presence of acetic acid (Sene *et al.*, 2001).

As the hydrolysates contain more inhibitors, the increased glucose and xylose uptaken by *C. guilliermondii* possibly reflects the extra amount of energy required for proton transport through the plasma membrane. The intracellular pH becomes unbalanced due to the heterogeneous acetate/acetic acid distribution between the inside and the outside of the yeast cell (Silva *et al.*,

2004; Sampaio *et al.*, 2007). The data presented in this work leads to the hypothesis that the presence of acetic acid in the culture medium can favor xylose metabolism of *C. guilliermondii* through an increase in the activities of xylose reductase [XR] and xylitol dehydrogenase [XDH]. The driving force for this phenomenon could be the result of an increase in ATP concentration inside the cell, which would favor cross membrane proton transport. As a consequence, the internal cell pH would be maintained near neutrality (Lima *et al.*, 2004; Vajzovic *et al.*, 2012).

Table 2.3. The specific rates of glucose and xylose consumption and xylitol and ethanol production during fermentation of different hydrolysates by *Candida guilliermondii*.

	Consumption*[1]*/ Production*[2] *($gg^{-1}h^{-1}$)					
Feedstock	**Control**	**Mixed wood**	**Hybrid poplar**	**Giant reed**	**Switchgrass**	**Sugarcane bagasse**
Xylose	0.05	0.11	0.07	0.04	0.10	0.11
Xylitol	0.03	0.09	0.03	0.02	0.08	0.09
Glucose	0.13	0.43	0.13	0.07	0.23	0.25
Ethanol	0.06	0.08	0.03	0.04	0.06	0.06

The reported results are the average of triplicate studies with a deviation of ≤ 2%.
[1]The specific rates of sugar consumption were calculated based on the log-mean dry cell density and the Δsubstrate and Δtime.
[2]The specific rates of xylitol from xylose and ethanol from glucose, production were calculated based on the log-mean dry cell density and the product concentration and Δtime.

In attempts to explain the conversion of xylose to ethanol by known reactions, it is generally accepted that the initial steps involve sequential reduction to xylitol and oxidation to convert xylitol to xylulose (Lee *et al.*, 1996; Winkelhausen *et al.*, 1998). Xylulose kinase then catalyzes the formation of xylulose-5-phosphate, which undergoes rearrangements catalyzed by transketolase and transaldolase to form hexose phosphate (Lee *et al.*, 1996; Granström *et al.*, 2007). Finally, hexose phosphate is converted to ethanol by the glycolytic pathway (Lee *et al.*, 1996; Winkelhausen *et al.*, 1998). Considering that ethanol yield increased by 20 % in sugarcane bagasse compared to the control (Table 2.2), we speculate that a fraction of xylitol is converted into xylulose due to the simultaneous increase of XR and XDH activities. In giant reed hydrolysate, the ethanol yield by *C. guilliermondii* was not enhanced at acetic acid concentrations of 8.2 g/L and was similar to the control (100 % of theoretical) (Figure 2.3, Table

2.2, 2.3). This signals that the concentration of acetic acid was approaching a threshold concentration point, beyond which, the lag-phase in growth would occur and ultimately lower ethanol yields.

Similarly, in the presence of acetic acid, fermentation of xylitol was enhanced up to 20 % for concentrations up to 5 g/L of acetic acid (Table 2.2 and Figure 2.4). However, fermentation of xylose to xylitol from the hydrolysate originating from pretreated giant reed was negatively affected by the presence of 8 g/L of acetic acid (Figure 2.4). Vajzovic (2012) reported that the ethanol yield by *Rhodotorula mucilaginosa* strain PTD3 was enhanced even in the presence of the highest concentration of 20 g/L of acetic acid tested while xylitol yield was lowered even at the lowest concentration of 5 g/L of acetic acid tested. In the current study the highest furfural (3.4 g/L), HMF (0.5 g/L), and phenolics (3.3 g/L) were measured in the giant reed hydrolysate (Table 2.1) and contributed to the lowered xylitol yield due to the synergism and cumulative concentration effects of these compounds. Indicative of the negative effect of higher concentrations of acetic acid, furfural, HMF, and phenolics, the xylitol yield in giant reed hydrolysate was reduced to 41 % of theoretical compared to inhibitor-free control (67 %) (Figure 2.4, Table 2.1). The specific xylitol and ethanol production rates as well as the specific xylose and glucose consumption rate dropped (Table 2.3). So far, the effects of inhibitors on xylose to xylitol bioconversion have not been deeply investigated (Pereira *et al.*, 2011).

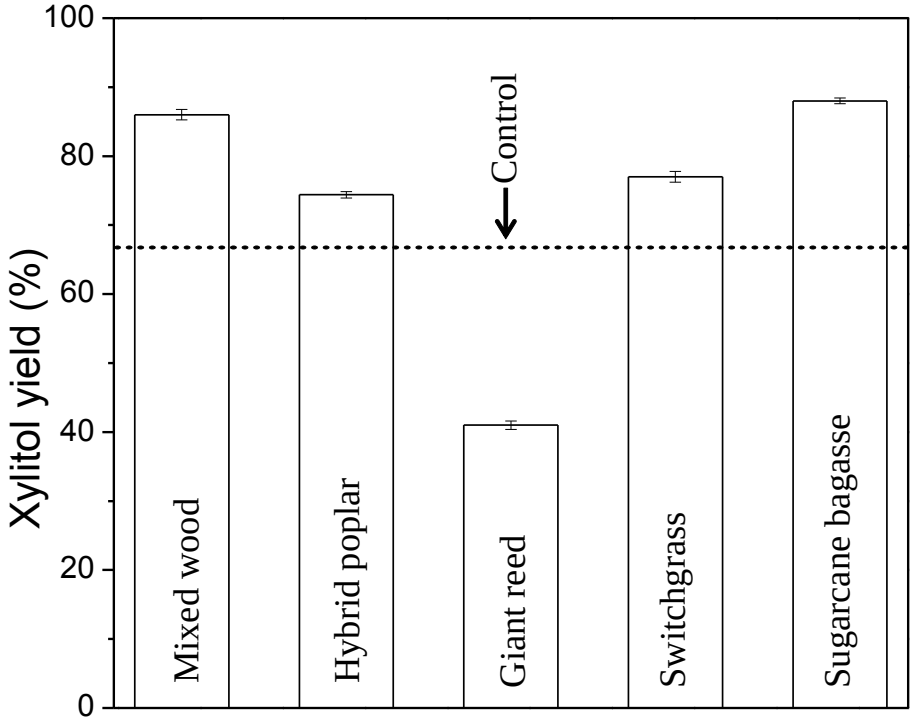

Figure 2.4. Xylitol production expressed as percentage of theoretical yield from five steam pretreated lignocellulosic hydrolysates by *C. guilliermondi.*

It is understood that yeast, during bioconversion of six carbon sugars to ethanol, metabolize furfural to furfural alcohol (Palmqvist *et al.*, 2000). NADH-dependent alcohol dehydrogenase is thought to be responsible for this reduction, which could be responsible for causing the reduced xylitol and ethanol yields in our study. Since all NADH generated is used for furfural reduction, the glucose to ethanol and also the xylose to xylitol processes are greatly affected. This is due to an increased acetaldehyde accumulation inside the cell caused by an insufficient amount of NADH-dependent alcohol dehydrogenase available in order to reduce acetaldehyde to ethanol (Palmqvist *et al.*, 2000; Kelly *et al.*, 2008). Intracellular acetaldehyde accumulation is therefore considered to be the reason for the lag-phase in growth and ultimately resulting in lower yields at the higher concentration of this inhibitor in giant reed hydrolysate. Similarly to the inhibition

mechanism by furfural, yeast metabolizes HMF to HMF-alcohol (Palmqvist *et al.*, 2000; Kelly *et al.*, 2008). Likewise, NADPH-dependent alcohol dehydrogenase is understood to be responsible for this reduction. As such, the reduction of HMF does not regenerate NAD^+, and thus carbon is allocated to glycerol production (to produce NAD^+ and thus maintain the overall redox balance) (Palmqvist *et al.*, 2000).

In spite of the presence of all four inhibitory compounds in the five hydrolysates (mixed wood, hybrid poplar, switchgrass, and sugarcane bagasse) there was no synergistic effect observed by combining inhibitors, and in contrast, the combinations were enhancing both, xylitol and ethanol yields. This is likely because the total concentration of all the inhibitors was below the inhibitory threshold concentration by which the cell growth rate and the kinetics of product formation by *C. guilliermondii* were not affected (Figure 2.3, Figure 2.4, Table 2.2, Table 2.3). Hence, in this study certain concentrations of acetic acid, furfural, HMF, and phenolics have been shown to exert a stimulating effect on the xylitol and ethanol yields by *C. guilliermondii* up to 20 % in sugarcane bagasse.

2.3.2 Fermentation of the water soluble fractions (hydrolysates) obtained after steam pretreatment of hybrid poplar at six conditions

To further examine the role of process-derived inhibitors while removing the variation in feedstock-derived inhibitors, hybrid poplar was selected as a feedstock for the systematic screening of six different steam pretreatment conditions. The liquid fractions obtained after pretreatment were characterized and fermented with *C. guilliermondii* (Table 2.4). As the combined severity (CS) factor increased, concentrations of acetic acid and phenolics increased sharply for conditions P1-P3 and plateaued for conditions P4-P6 with higher CS factors. As the CS factors increased, the concentration of furfural and HMF increased linearly for both uncatalyzed and SO_2-catalyzed pretreatments. Ethanol yields (up to 47 % more, as compared to the control) by *C. guilliermondii* were enhanced by the presence of the fermentation inhibitors for all the concentrations tested (Table 2.4). Clearly, xylitol yield was impaired by the inhibitory compounds for all the conditions tested. The drop in xylitol yields was especially pronounced in hydrolysates collected after SO_2-catalyzed steam pretreatment (P4-P6) with the lowest xylitol

yields (22 % of theoretical) compared to the control (67 % of theoretical). As the CS factors increased, the specific sugar (glucose and xylose) consumption and specific production (xylitol and ethanol) rates decreased for all the pretreatment conditions tested (Table 2.5).

Table 2.4. Process variables, xylitol and ethanol yields, combined severity, and chemical composition in hydrolysates obtained by steam pretreatment of hybrid poplar chips at 6 different pretreatment severities.

Sample	*Temperature (°C)*	*Time (min)*	*SO_2 (%)*	*CS*[1]	*Acetic acid (g/100g)*[2]	*Furfural (g/100g)*[2]	*HMF (g/100g)*[2]	*Phenolics (g/100g)*[2]	*[XOH] Y%T (%)*[3]	*[EOH] Y%T (%)*[3]
P1	205	10	0	0.6	1.6	0.7	0.2	0.8	57 ± 0.5	147 ± 1.1
P2	212	10	0	1.0	2.6	1.2	0.3	1.0	51 ± 0.9	142 ± 1.0
P3	212	15	0	1.3	3.5	2.0	0.6	1.1	34 ± 0.5	122 ± 1.2
P4	190	5	3	1.9	3.6	0.9	0.2	1.4	42 ± 0.7	138 ± 1.0
P5	195	5	3	2.0	3.7	1.3	0.3	1.4	24 ± 0.8	132 ± 1.3
P6	200	5	3	2.1	3.8	1.7	0.4	1.5	22 ± 0.4	128 ± 1.0
Control	*NA*	*NA*	*NA*	*NA*	0.0	0.0	0.0	0.0	67 ± 1.0	100 ± 0.5

The reported results are the average of triplicate studies with a deviation of ≤ 3%.
[1]The combined severity factor is calculated based on the severity factor log(R_o) (Equation 1), and the pH after pretreatment, through Equation 2 where the pH is measured after the pretreatment.
[2] g/100g denotes g of product per 100 g raw biomass
[3]Y%T (%) denotes the percent of theoretical yield of xylitol (XOH) and ethanol (EOH)

Table 2.5. The specific rates of glucose and xylose consumption and xylitol and ethanol production during fermentation of hybrid poplar hydrolysates by *Candida guilliermondii*

	Consumption*[1]*/ Production*[2] *($gg^{-1}h^{-1}$)						
Condition	**Control**	**P1**	**P2**	**P3**	**P4**	**P5**	**P6**
Xylose	0.05	0.08	0.06	0.04	0.1	0.06	0.06
Xylitol	0.03	0.04	0.04	0.02	0.04	0.01	0.01
Glucose	0.13	0.36	0.22	0.09	0.27	0.11	0.11
Ethanol	0.06	0.12	0.06	0.04	0.09	0.06	0.05

The reported results are the average of triplicate studies with a deviation of ≤ 2%.
[1]The specific rates of sugar consumption were calculated based on the log-mean dry cell density and the Δsubstrate and Δtime.
[2]The specific rates of xylitol from xylose and ethanol from glucose, production were calculated based on the log-mean dry cell density and the product concentration and Δtime.

In addition, it was observed that as the CS factor increased, the ethanol and xylitol yields in the hydrolysates obtained from uncatalyzed steam pretreatment (conditions P1-P3) decreased (Table 2.4). Similarly, the impregnation of the material with SO_2 resulted in increased formation of inhibitors and thus lowered ethanol and xylitol yields, when compared to a not-catalyzed pretreatment (conditions P4-P6) (Table 2.4). However, although the specific production rate of ethanol decreased as the CS factor increased, the ethanol yield was still stimulated by the presence of fermentation inhibitors and was higher compared to the control (Table 2.4, Table 2.5). The lowest ethanol yield observed was 22 % higher compared to the control (100 % of theoretical). As seen in the previous section, this behavior can be explained by xylose contributing to the ethanol yield in the presence of the fermentation inhibitors. Nevertheless, the concentration of fermentation inhibitors matters.

At lower pretreatment severities, the recovery of hemicellulosic sugars is high and the least amount of inhibitory compounds are produced (Excoffier *et al.*, 1991; Mosier *et al.*, 2005). However, within the high-range of pretreatment severities, soluble sugars are converted to inhibitors (Olsson *et al.*, 1996). In general, the overall carbohydrate yield decreases sharply when temperature increases, while at longer reaction time, higher yields of lignin condensation and pentose dehydration products are observed (Overend *et al.*, 1987; Excoffier *et al.*, 1991; Ramos, 2003; Kumar *et al.*, 2009). In addition, the use of acid catalyst in steam pretreatment can elevate the production of process derived inhibitors due to the improved recovery of the hemicellulose-derived sugars (Overend *et al.*, 1987; Mosier *et al.*, 2005). According to Garrote and Parajó (2002) even at lower pretreatment severities a portion of hemicellulosic acetyl esters are removed as acetic acid, catalyzing xylan depolymerization. Our study showed that acetyl bonds were completely hydrolyzed through SO_2 catalyzed pretreatment under the highest severity condition (P6), resulting in the highest concentration of acetic acid in the liquid stream (Table 2.4) and no remaining acetate in the pretreated solids (data not shown). Nonetheless, some acetic acid was detected in the liquid stream under low severity conditions both with and without an SO_2 catalyst (Table 2.4).

The major chemical and physical changes to lignocellulosic biomass following catalyzed steam explosion are often attributed to the elevated removal of hemicellulose. This is due in part to the

SO_2 penetrating into the fibers, removing more acetyl groups and causing more sugar release (Overend *et al.*, 1987), thereby improving the accessibility of the enzymes to the cellulose fibrils (Mosier *et al.*, 2005). Due to the increased sugar release, impregnation of the material with SO_2 resulted in increased formation of fermentation inhibitors, when compared to non-catalyzed pretreatment of the same feedstock (Table 2.4). Based on these data sets we can conclude that the severity of the pretreatment influenced the concentration of process-derived fermentation inhibitors and those unique concentrations of the inhibitors positively influenced ethanol and negatively affected xylitol yields by *C. guilliermondii*.

2.3.3 Analysis of water insoluble fractions (solids) obtained after steam pretreatment of hybrid poplar at six conditions

In addition to the fermentability of the liquid stream, the overall ethanol and xylitol yield is also determined by the amount of fermentable sugars generated from the water insoluble (solid) fraction following pretreatment and enzymatic hydrolysis. To determine the maximum possible yield of ethanol and xylitol from hybrid poplar pretreated at 6 different conditions, washed solid fractions were subjected to enzymatic hydrolysis. The cellulose to glucose conversions for the solids were investigated over 96 hours of enzymatic hydrolysis (Figure 2.5). Clearly, all the three solids from SO_2-catalyzed pretreatment (P4-P6) have higher glucose yield in enzymatic hydrolysis, compared with uncatalyzed ones (P1-P3). As the CS factors increased, the cellulose to glucose conversion increased significantly from 45 % to 82 %. And the lowest cellulose conversion was obtained in the solids pretreated under 205 °C, 10 min, 0 % SO_2. The relationship between the pretreatment condition and the enzymatic hydrolysis is that the higher the severity, the better biomass fractionation, and the better solids digestibility.

Table 2.6. Process variables, glucose, xylose and overall sugar yields in solid/liquid fraction from steam exploded hybrid poplar, and glucose, xylose and overall sugar yields in enzymatic hydrolyzed solid and hydrolysates obtained by steam pretreatment of poplar.

Sample	*CS*	*Initial sugar recovery after pretreatment (%)*				*Sugar recovery after pretreatment and hydrolysis (%)*			
		Glucose[1]	*Xylose*[1]	*Glucose & Xylose*[1]	*Overall Sugar*[3]	*Glucose*[2]	*Xylose*[2]	*Glucose & Xylose*[2]	*Overall Sugar*[3]
P1	0.6	109.8	57.7	96.3	96.6	50.8	53.1	51.4	53.3
P2	1.0	101.0	33.3	83.5	83.6	63.9	31.0	55.4	56.5
P3	1.3	87.2	14.9	68.5	67.6	55.7	14.0	44.9	44.8
P4	1.9	104.3	60.7	93.0	93.7	80.4	59.3	74.9	76.2
P5	2.0	109.4	56.9	95.8	97.2	88.0	55.7	79.6	81.6
P6	2.1	109.7	46.6	93.4	93.8	93.4	45.5	81.0	81.8

The reported results are the average of triplicate studies with a deviation of ≤ 3%.
[1] The glucose recovery is calculated by summation of glucose (including hydrolysates, wash and unhydrolyzed solid fraction) and divided by glucose content in raw biomass, so as to xylose recovery and glucose & xylose recovery.
[2]The glucose recovery is calculated by summation of glucose (including hydrolysates, wash and enzymatic hydrolyzed solid fraction) and divided by glucose content in raw biomass, so as to xylose recovery and glucose & xylose recovery.
[3]The overall sugar is the summation of arabinose, galactose, mannose, xylose and glucose in hydrolysates, wash and enzymatic hydrolyzed solid fraction.

Sugar yields (glucose and xylose) from the 6 different steam pretreated hybrid poplar samples were studied after pretreatment and enzymatic hydrolysis (Table 2.6). The post-pretreatment sugar recovery determines how much of the sugars in the raw material are recovered in liquid fraction and solid fraction after pretreatment. Interestingly, the CS does not show a universal correlation with the glucose, xylose, glucose & xylose recovered after steam explosion (Table 2.6). To the samples P1-P3, which were pretreated without SO_2, the glucose, xylose, overall sugar yield decreases with the increasing severity. Under higher pretreatment severity without SO_2 more sugars are degraded into inhibitors. These results correspond with increased furfural and HMF concentrations for sample P1-P3 in the liquid fraction (Table 2.4). Generally, the pretreatments with SO_2 have higher glucose & xylose yields and post-pretreatment sugar recoveries compared with those pretreatments without SO_2. Among all samples, sample P1

(205 °C, 10 min, 0% SO_2) and sample P5 (195 °C, 5 min, 3 % SO_2) achieve the highest overall sugar recovery (97 %).

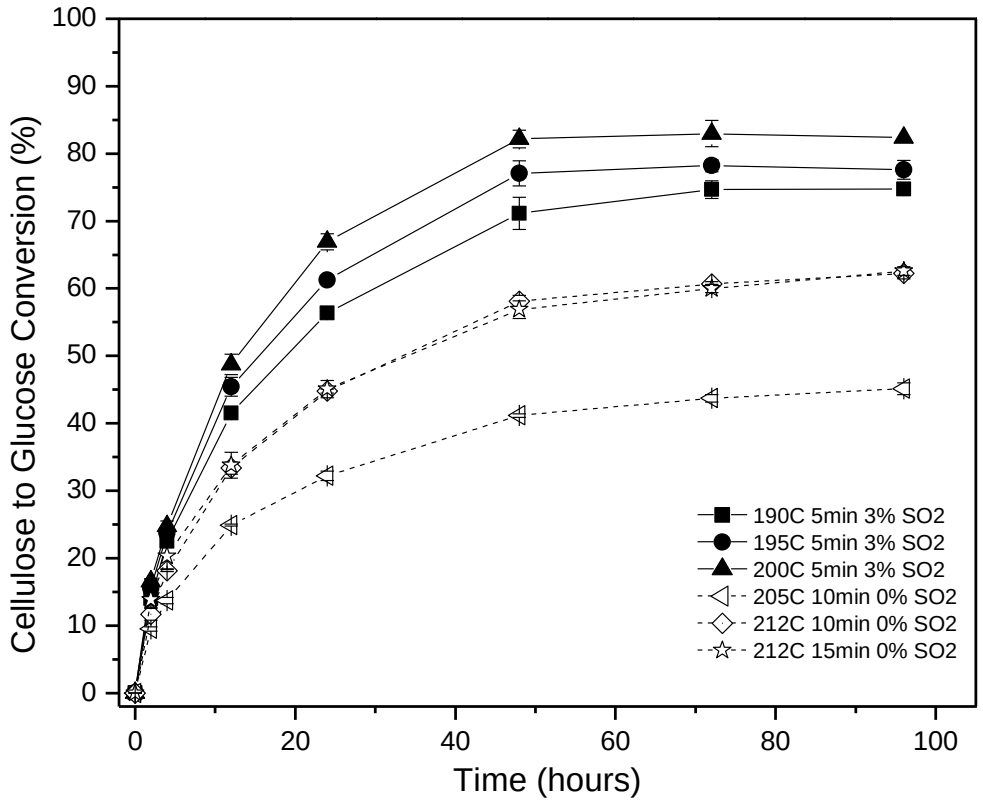

Figure 2.5. Cellulose conversion to glucose of pretreated hybrid poplar in 6 different pretreatment conditions during enzymatic hydrolysis at 5 % solids consistency and 5 PFU/g cellulose cellulase loading.

The post-hydrolysis sugar recovery defines how much of the sugars in the raw material are recovered in liquid fraction after pretreatment and from the solid fraction after enzymatic hydrolysis (Table 2.6). Due to the low cellulose to glucose conversion of uncatalyzed samples shown in Figure 2.5, the glucose recovery of uncatalyzed pretreated solids are much lower after considering the enzymatic hydrolysis. Compared with catalyzed pretreated solids, the uncatalyzed samples have a lower overall sugar recovery.

2.4 Conclusions

In this study we investigated the effect of inhibitors on the production of xylitol and ethanol by *Candida guilliermondii*. Contrary to previous observations, it was shown that certain concentrations of acetic acid, furfural, HMF, and phenolics boosted the xylitol and ethanol yields in hydrolysates from five different steam pretreated feedstocks. In the presence of up to 2.1 g/L acetic acid enhanced ethanol yields up to 20 % of theoretical in mixed wood, switchgrass and sugarcane bagasse hydrolysates compared to the control (67 %). This work demonstrated that acetic acid boosted the xylitol yields at up to 5 g/L of this inhibitor with constant xylitol production and xylose consumption rates.

Varying the steam pretreatment conditions of hybrid poplar generated a range of process-derived fermentation inhibitors that resulted in enhanced ethanol yields but not enhanced xylitol yield Ethanol yields were up to 47 % higher compared to the control and were enhanced by the presence of acetic acid and other fermentation inhibitors for all the concentrations tested. Nevertheless, a 10 % drop in xylitol yield was noticed even at the lowest acetic acid concentration (1.6 g/100g) tested compared to the control (67 % of theoretical). Generally the highest ethanol and xylitol yields were achieved from the hydrolysate obtained from the lowest-severity steam pretreatment (205 °C, 10 minutes, without SO_2), which had the lowest inhibitor concentration. Further characterization of the solid fractions resulting from the six pretreatment conditions showed that although the overall post-pretreatment sugar recovery was high for low-severity steam pretreatment without SO_2, the hydrolyzability was poor compared with high-severity ones, rendering the post-hydrolysis sugar recovery low.

Based on the fermentation and sugar recovery results, it appears that lowest severity steam pretreatment has the lowest inhibitor formation, which is likely responsible for the increased ethanol yield during hydrolysate fermentation and overall post-pretreatment sugar recovery. However, due to the poor digestibility of low-severity pretreated solid fraction, the overall post-hydrolysis sugar recovery was inadequate. In that case, more work must be done to improve the hydrolyzability of low-severity pretreated solids to improve the sugar yield of enzymatic hydrolysis and maximize both hydrolysate fermentation and overall sugar recovery.

Chapter 3. Post-treatment refining to improve cellulose conversion during enzymatic hydrolysis of uncatalyzed steam pretreated hybrid poplar

Abstract

The presence of SO_2 in steam pretreatment creates a series of problems in regards to environmental protection, process complexity, equipment corrosion, chemical catalysis and waste water treatment. However, when no SO_2 is applied, the poor digestibility of cellulose reduces the sugar yield during enzymatic hydrolysis. In this study, hybrid poplar chips were steam exploded using 6 different conditions with or without the addition of SO_2. The steaming temperature ranged from 190 °C to 212 °C, and the residence time ranged from 5 min to 10 min. Mechanical refining was applied to the solid fractions and was shown to improve the enzymatic hydrolysis yields for solids pretreated at 205 °C for 10 minutes without SO_2 (sample S1) by as much as 23 %. The same improvement was evident at different enzyme loadings and solid consistencies. However, refining did not improve the hydrolyzability of solids pretreated at the five more severe conditions. Our results also showed that increased sugar yields in enzymatic hydrolysis are correlated with reduced particle sizes. The refining process reduced the particle size of all the samples. Among them S1 showed the largest size reduction and correspondingly achieved the most significant improvement in sugar recovery. This research demonstrates the possibility of using post-treatment refining to achieve high yields from an uncatalyzed steam pretreatment process.

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3.1 Introduction

The bioconversion of lignocellulosic biomass to fuels consists of four main processes which include pretreatment, hydrolysis, fermentation and product separation (Mosier *et al.*, 2005). Pretreatment is the fractionation of the tight structure of lignocellulosic biomass; hydrolysis involves enzymes accessing exposed cellulose in the lignocellulosic biomass and hydrolyzing it into fermentable sugars; fermentation utilizes microorganisms to convert sugars into fuels; and product separation recovers fuels from the fermentation broth by distillation and purification. An ideal lignocellulosic biofuel system should have economically feasible pretreatment process (Kumar *et al.*, 2009; Zhu, Pan, *et al.*, 2010), low-input enzyme cost (Jorgensen *et al.*, 2007; Meyer *et al.*, 2009) and productive fermentation yield (Cysewski & Wilke, 1978; Duff & Murray, 1996). To achieve this goal, the foremost target is to reach a cost-effective high overall sugar recovery (Lynd *et al.*, 2008).

Hybrid poplar is well recognized as one energy crop for biofuel. As highlighted in the literature, it's a short rotation, fast growing wood species with high biomass production (Esteghlalian *et al.*, 1997; Dinus, 2001); it's suitable to marginal land cultivation with lower fertilizer requirement (Sannigrahi *et al.*, 2010); and it's easy to propagate with a broad genetic base (Dinus *et al.*, 2001). More importantly, hybrid poplar presents high cellulose and contents, moderate lignin and hemicellulose content and low ash and extractive amounts (Esteghlalian *et al.*, 1997; Sannigrahi *et al.*, 2010) which are the prerequisites of effective sugar conversion.

Steam explosion has been proposed as a cost effective pretreatment method for a wide variety of biomass types, from softwood to hardwood to agricultural residues (Ewanick *et al.*, 2007; Ewanick *et al.*, 2010). It is able to efficiently fractionate lignocellulosic materials with limited use of chemicals, low energy consumption and short reaction time (Chandra *et al.*, 2007; Bura *et al.*, 2009). The process involves heating the biomass with high pressure steam to the target temperature for certain time period and followed by a rapid decompression at the end of the pretreatment. By rapidly reducing the temperature, the pressure release terminates/quenches the reaction, opens up the lignocellulosic biomass from the inside out and decomposes the main components, namely, cellulose, hemicellulose and lignin (Ramos, 2003; Mosier *et al.*, 2005). Three variables control the severity of steam explosion; time, temperature and pH (controlled by

the addition of acid catalyst) (Overend *et al.*, 1987; Chum *et al.*, 1990). Generally, a higher-severity pretreatment fractionates the lignocellulosic biomass more deeply, improving the digestibility of pretreated biomass (Jorgensen *et al.*, 2007). However, higher-severity pretreatment have more sugar degradation (Schwald *et al.*, 1987; Bura *et al.*, 2002), which causes lower overall sugar recovery and higher degradation product-derived inhibitor formation which can hinder the enzymatic hydrolysis and fermentation (Jorgensen *et al.*, 2007; Franden *et al.*, 2009). Nevertheless, lower severity pretreatment, although responsible for smaller sugar losses, is not as able to open up the lignocellulose structure, resulting in a poor hydrolyzability of pretreated biomass (Ramos, 2003). Considering the overall sugar recovery, biomass hydrolyzability and the drawback of inhibitors, simply increasing or decreasing the pretreatment severity cannot achieve the maximum sugar yield. Therefore, most of pretreatment optimization research in the past decades has focused on making a compromise between these two opposite trends.

SO_2 impregnation before steam explosion pretreatment has been shown to facilitate lower reaction temperatures and shorter reaction times (Excoffier *et al.*, 1991; Bura *et al.*, 2002), and has been prove to successfully pretreat softwood (Schwald *et al.*, 1987; Boussaid *et al.*, 2000) and hardwood (Sassner *et al.*, 2005; Schütt *et al.*, 2013) prior to enzymatic scarification.

However, there are a number of issues when adding SO_2 as a catalyst in steam explosion pretreatment:

- As a significant air pollutant currently derived mainly from fossil fuel combustion, SO_2 has significant impacts upon human health (EPA, 2010).
- When working with hazardous SO_2, the impregnation needs a sealed condition with cautious manipulation. The risks of operation and complexity of the process might explain why SO_2 as a catalyst has not been well reported in pilot-scale or demonstration-scale steam pretreatment facilities, although popular in bench-scale research.
- SO_2 is readily soluble in water and easily forms sulfurous acid (H_2SO_3) (Swaddle, 1990). SO_2 is also oxidized into sulfuric acid (H_2SO_3) in the presence of oxygen or converted into elemental sulfur or thiosulfate in the absence of oxygen (Brownell *et al.*, 1988), forming a strong acidic environment, which inevitably requires neutralization prior to

enzymatic hydrolysis and fermentation. That means more expense on neutralizing reagent and the subsequent waste water treatment in the downstream process. In general, the more complicated SO_2-catalyzed steam pretreatment needs especial facilities and extra steps, representing to a potential increase in cost.

- Sulphurous compounds generated from introduced SO_2 can also have effect on the pH and composition of pretreated hydrolysate and solids, which influence the microorganism during fermentation. These include sulphites, which showed inhibitory effect on the growth of S. cerevisiae (Pilkington *et al.*, 1988; Gardner *et al.*, 1993). Moreover, sulphide can also be toxic to many microbes (Chen *et al.*, 2008).
- Reactor corrosion is a serious problem in acid catalyzed steam pretreatment. Metal ions generated from equipment corrosion are toxic to subsequent enzyme hydrolysis and fermentation (Wang *et al.*, 2009). To mitigate this, exotic metal alloys must be applied as the manufacturing materials, which significantly increases the capital cost (Chen *et al.*, 2012).
- Sulfur species are unfriendly to the chemical catalytic activity of most transition metals involved reactions (Oudar, 1980; Bartholomew *et al.*, 1982). Sulfur-containing compounds, *e.g.* H_2S, are considered as major problems in many catalytic reactions, as they can drastically reduce the transition metal catalysts (Oudar, 1980). Such poisonous compounds will probably jeopardize the catalytic processes employing reduced metals (Dunleavy, 2006) in any downstream products catalytically produced from sugars or biochemicals (Bailie *et al.*, 1998; Zhang *et al.*, 2008).

In the case of uncatalyzed (no SO_2) steam pretreatment, hemicelluloses are degraded by the acetic acids cleaved from acetyl groups, which function as a self-catalyst leading to autohydrolysis (Ramos, 2003). However, owing to the limited acetic acid formation, the pH drop is restricted such that the autohydrolysis is mild and slow. For that reason, uncatalyzed steam pretreatment usually suffers from a lower cellulose digestibility compared with acid catalyzed steam pretreatment (Schütt *et al.*, 2013).

High severity or low severity, SO_2 or no SO_2? Instead of struggling with such a dilemma, refining can be utilized as a substitute for SO_2 at low severity steam pretreatment. A mature

technology in the pulp and paper industry, refining is commonly applied to develop pulp fiber qualities and improve the final paper properties (Gil *et al.*, 2009; Zhang, Song, *et al.*, 2012). The main target of refining is to improve the bonding ability of fibers such that they form strong and smooth paper sheet with good printing properties. During refining, shearing action exposes microfibrils on the fiber surface and shortens the fiber with mechanical cutting (Kang *et al.*, 2006a, b).

Due to its fibrillation and size reduction, refining increases the specific surface area of fibers and, therefore, positively influences the cellulose to glucose conversion during enzymatic hydrolysis (Wu *et al.*, 2010). Size reduction of lignocellulosic biomass is an important factor during pretreatment (Dasari & Berson, 2007; Vidal *et al.*, 2011; Zhu, 2011). Particle size is related to cellulase-accessible surface area has been described as a major factor in cellulose to glucose conversion during enzymatic hydrolysis (Jeoh *et al.*, 2007; Leu *et al.*, 2013).

Although previous reports demonstrated that direct mechanical treatment was not economically feasible because of high energy requirements (Brown, 2003; Alvira *et al.*, 2010), post-pretreatments, such as disc milling, can significantly offset energy consumption from the whole bioconversion approach (Zhu & Pan, 2010). To date, refining has been newly introduced into the biofuel process to improve the sugar yield with reduced enzyme dosage and higher solids consistency (Koo *et al.*, 2011; Chen *et al.*, 2012), and has been proven to be a promising post-pretreatment process to achieve a cost-effective process (Tao *et al.*, 2012).

The goal of this research is to investigate the mechanism of refining in changing the fiber structure and evaluate the possibility of replacing SO_2 impregnation in steam pretreatment by the refining process. The specific objectives of this work were to 1) determine whether refining changes chemical composition of steam pretreated hybrid poplar; 2) study the influence of refining on cellulose to glucose conversion for steam pretreated poplar with and without SO_2; 3) investigate the refining effects on hydrolyzability of steam pretreated hybrid poplar at different enzyme loadings and solid consistencies; 4) analyze the mechanism of refining process in changing the physical characteristics of steam pretreated hybrid poplar, and its relationship with enzymatic hydrolysis improvement.

To achieve this, hybrid poplar chips were steam pretreated using 6 different conditions with or without SO_2 catalysis. The pretreatment conditions ranged from 190 °C to 212 °C, and 5 to 15 minutes. After each pretreatment, half of the solid fractions were refined with a valley beater refiner for 30 minutes. After separation of the water insoluble fraction, all 12 samples, including both refined and unrefined solids, were enzymatically hydrolyzed at 5 % consistency and 5 FPU/g cellulose enzyme loading. Further enzymatic hydrolysis was carried out at three enzyme loadings (1 to 5 FPU/g cellulose) and three solids consistencies (5 % to 15 % w/v) for one sample (S1, pretreated at 212 °C, 10 min, 0 % SO_2). Then, this sample was refined for different durations (5 to 30 minutes) and hydrolyzed at 5 % consistency and 5 FPU/g cellulose. In addition, the fiber length and width for all the solids were measured.

3.2 Materials and methods

3.2.1 Feedstock

Hybrid poplar chips used in this research were from fresh 18-year-old hybrid poplar (*Populus deltoides* × *Populus nigra*) obtained from Forest Concepts (Auburn, WA) in Puyallup, WA. Trees were debarked and chipped using equipment provided by Acrowood (Everett, WA) and screened into to 5mm thickness and 1.0 cm ×2 cm. Chips (50% moisture content) were stored at -20°C until use.

Figure 3.1 shows the conditions and processes utilized in this research.

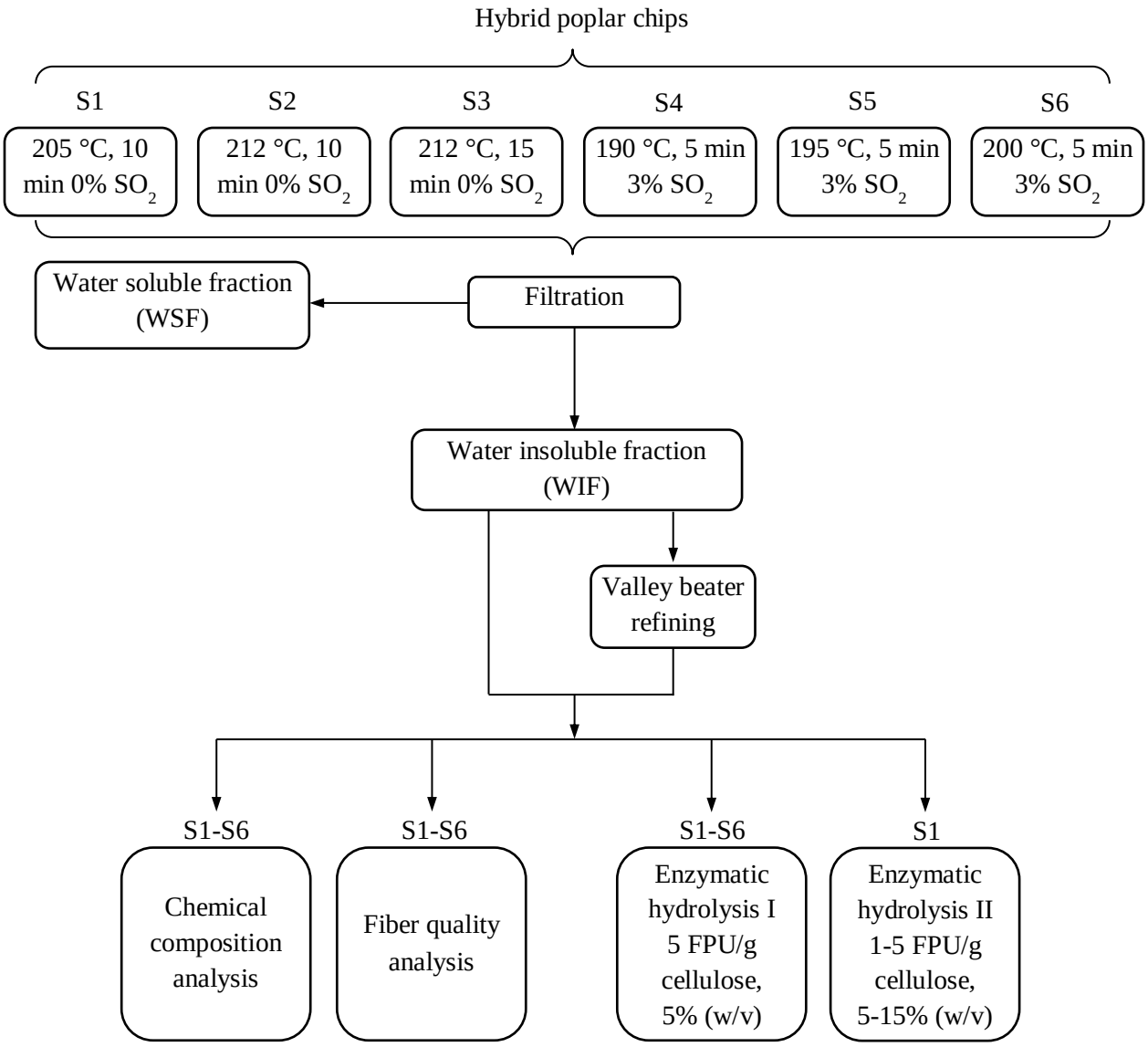

Figure 3.1 Process flow diagram of steam pretreatment, refining and enzymatic hydrolysis for hybrid poplar.

3.2.2 **Steam explosion pretreatment**

In experiments with the SO_2 catalyst, samples of 800 g oven-dried weight (ODW) hybrid poplar were pre-impregnated overnight with anhydrous SO_2 in plastic bags at atmospheric pressure. The amount of SO_2 added to the bag corresponded to 3% by ODW of the samples, and was determined by weighing the bag before and after the addition of gas.

Steam pretreatment was performed in a 2.7 liter batch reactor (Aurora Technical, Savona, BC, Canada). Briefly, samples were loaded, in 400 g ODW batches, into the preheated reactor. As shown in Table 3.1, the samples were successively heated at temperatures ranging from 190 to 212 °C for 5 to 15 min periods.

Table 3.1 Steam pretreatment conditions for hybrid poplar

Sample	***Temperature (°C)***	***Time (minutes)***	***SO_2 (% w/w)***	***Combined severity factor***
S1	205	10	0	0.6
S2	212	10	0	1.0
S3	212	15	0	1.3
S4	190	5	3	1.9
S5	195	5	3	2.0
S6	200	5	3	2.1

After steam explosion, the pretreated biomass slurry was separated into water soluble fraction (WSF) and water insoluble fraction (WIF) using vacuum filtration. To remove residual sugars, the WIF was then washed with a volume of deionized water equivalent to 20 times the dry weight of the sample.

For each sample, severity was calculated using the combined severity factor CS = log $te^{(T-100)/14.75}$ - pH (Equation 1.1, Equation 1.2) based on time (t, min), temperature (T, °C) and pH (measured from the water insoluble fraction following pretreatment). The pretreatment severity ranged from 0.6 to 2.1 (Table 3.1).

3.2.3 **Mechanical refining**

A 23 liter laboratory scale Valley Beater refiner was used to refine the washed WIF using adapted TAPPI Standard Method T-200 (TAPPI, 2001) with 47 kg of weight on bedplate lever arm. 360 g ODW of each sample was refined in a single batch at 1.57 % consistency for 5 to 30 min at room temperature. After refining, solids were collected and washed with water.

3.2.4 **Enzymatic hydrolysis**

Enzymatic hydrolysis was carried out using cellulase (Celluclast 1.5 L) and β-glucosidase (Novozym 188) from Novozymes. 50 mM citrate buffer was used to maintain the pH at 4.8 and the flasks were incubated at 50°C and 175 rpm in an orbital shaker. Additionally, tetracycline (40 mg/mL) and cycloheximide (30 mg/mL) were used to inhibit microbial contamination (Ewanick *et al.*, 2007).

In the first set of experiments, all 12 samples, including 6 refined and 6 unrefined solids, were hydrolyzed at 5% (w/v) consistency in a total volume of 50 ml in 125 ml Erlenmeyer flasks. Cellulase at 5 filter paper units (FPU)/g cellulose and at β-glucosidase at 10 cellobiase units (CBU)/g cellulose were added to each flask.

For the second set of experiments, an orthogonal design was performed with different enzyme loadings and solid consistencies. Specifically, refined and unrefined solids from 212 °C, 10 min without SO_2 pretreatment were hydrolyzed at 5%, 10% and 15% (w/v) consistencies with three enzyme loadings (1 FPU, 2 FPU and 5 FPU/g cellulose).

For the third set of experiment, sample one was refined for 5, 10, 20, 30 minutes in valley beater, and enzymatically hydrolyzed at 5 % consistency and 5 FPU/g cellulose.

For all hydrolysis experiments, 1 ml samples were taken periodically over 72 h, boiled for 10 min to denature enzymes, and stored at -20°C for HPLC analysis.

3.2.5 **Chemical compositional analysis**

The chemical composition of both refined and unrefined solids was determined as previously described (Ewanick *et al.*, 2011) according to a modified method derived from TAPPI Standard

Method T-222 om-98(TAPPI, 1998), "Acid-insoluble lignin in wood and pulp". Briefly, 0.2 g of finely ground oven dried sample is treated with 3 ml 72% H_2SO_4 for 120 min at room temperature, then diluted into 120 ml total volume and autoclaved at 121°C for 60 min. After filtration through tared sintered-glass crucibles, the acid insoluble lignin is determined by weighing the oven dried crucibles, and acid soluble lignin in the filtrate is analyzed by UV at 205 nm. Carbohydrate composition of the filtrate is analyzed by HPLC.

The concentration of monomeric sugars (arabinose, galactose, glucose, xylose and mannose) from chemical composition analysis and enzymatic hydrolysis was measured on a Dionex (Sunnyvale, CA) HPLC (ICS-3000) system equipped with an AS autosampler, ED electrochemical detector, dual pumps, and anion exchange column (Dionex, CarboPac PA1). Deionized water at 1 ml/min was used as an eluent, and post-column addition of 0.2 M NaOH at a flow rate of 0.5 ml/min ensured optimization of baseline stability and detector sensitivity. After each analysis, the column was reconditioned with 0.25 M NaOH. Twenty microliters of each sample were injected after filtration through a 0.22 µm syringe filter (Restek Corp., Bellefonte, PA, U.S.). Standards were prepared containing sufficient arabinose, galactose, glucose, xylose, and mannose to encompass the same range of concentrations as the samples. Fucose (0.2 g/L) was added to all samples and standards as an internal standard.

3.2.6 **Fiber quality analysis**

Both unrefined and refined samples from steam pretreatment are small fragmented particles, and differ from long pulp fibers. In this case, the solids from steam pretreated hybrid poplar are referred to as "particles" instead of "fibers". Basically "particles" and "fibers" are interchangeable, and in this paper, "fibers" are only used when mentioning about pulp fibers.

Pretreated particle length, width and size distribution was measured using a Fiber Quality Analyzer (FQA-360 LDA12, OpTest Equipment, Inc., Hawkesbury, ON, Canada). As described on the FQA-360 operation manual (OpTest-Equipment-Inc., 2012), all samples were diluted with deionized water to 0.75 mg/L ODW (7.5×10^{-5} %) to reach a fiber frequency of 20 - 40 events per second (EPS). Since the large particles in the solids block the feed tube of the FQA and

interrupt the analysis, samples were disintegrated with a Hamilton Drink Mixer (Southern Pines, NC) by treating the samples for 60 seconds at 10% (w/v) solid consistency (Del Rio, 2012).

As shown in Equation 3.1, the most common particle length measurements of FQA is length weighted mean length (L_w) (Ai & Tschirner, 2010; Li, Bandekar, *et al.*, 2011; OpTest-Equipment-Inc., 2012). This value is most often used to compare differences between fibers in the pulp and paper industry. L_w is preferred since it places more weight on to the fibers and reduces the impact of fines. By de-emphasizing the fine fraction, it is easier to compare the fiber size on this result (Li, Bandekar, *et al.*, 2011; OpTest-Equipment-Inc., 2012). The particle width reported by FQA is the arithmetic mean width based on the pulp and paper industry standards (OpTest-Equipment-Inc., 2012).

$$L_w = \frac{\sum_i n_i l_i^2}{\sum_i n_i} \quad \text{(Equation 3.1)}$$

(n_i is the number of fibers in the specified length class l_i)

3.2.7 **Statistical analysis**

The results were subjected to one way analysis of variance (ANOVA) analysis followed by a Tukey test. Chemical composition (glucan, xylan and lignin), physical characteristics (length and width) and sugar yield in enzymatic hydrolysis were analyzed based on 5 % alpha level every null hypothesis. Judged by the p-value ($p < 0.05$), the chemical composition, physical characteristics and sugar yield were claimed whether there is statistically significant difference or not. Data were analyzed using R (version 3.0.1) procedures.

3.3 Results and discussion

3.3.1 **Compositional analysis**

Hybrid poplar chips were steam pretreated at temperatures from 190 to 212 °C for 5 to 15 minutes with or without SO_2. Considering the pH of the liquid hydrolysate after pretreatment, the combined severity factors of SO_2 catalyzed samples (S1, S2 and S3) were higher (from 1.9 to 2.1) than uncatalyzed samples (S4, S5 and S6) with factors ranging from 0.6 to 1.3 (Table 3.2). Since

arabinose, galactose, and mannose made up only a minor contribution to total sugars, only glucan and xylan were considered in chemical composition (Table 3.2). Consistent with previous work on hybrid poplar (Ewanick, 2012; Morales *et al.*, 2013), the glucan, xylan and lignin (including acid soluble and insoluble) content ranged from 61.3 % to 65.6 %, from 0.2 % to 2.6 % and from 27.1 % to 35.6 %, respectively.

After steam pretreatment, no statistically significant differences in glucan and xylan content were found among S1, S2 and S3 or among S4, S5 and S6, whereas, statistical significant differences in glucan and xylan content were found between catalyzed pretreated solids (S1, S2 and S3) and uncatalyzed pretreated solids (S4, S5 and S6). It is thought that SO_2 is mainly responsible for the difference; when SO_2 is applied as acidic catalyst, more cellulose and hemicellulose are fractionated during the reaction (Ewanick *et al.*, 2010). Similar to previous work using SO_2 catalyzed steam pretreated sugarcane bagasse (Ewanick *et al.*, 2011), corn stover (Bura *et al.*, 2009), switchgrass (Ewanick *et al.*, 2011) and willow (Sassner *et al.*, 2005), the majority of the hemicellulose was solubilized into the liquid fraction due to its labile structure. Additionally, SO_2 in some degree increased the combined severity factor such that cellulose were partially hydrolyzed during the steam pretreatment (Ramos, 2003; Sassner *et al.*, 2005; Bura *et al.*, 2009). Thus, sugar content, especially the xylan content, of SO_2-pretreated solids was lower than those pretreated without SO_2. Of all the samples, there was no statistical significant difference in lignin content.

Comparing chemical composition before and after refining, no statistically significant difference was discovered in the glucan, xylan and lignin content. That is to say, refining is purely a mechanical process and does not modify the chemical compositions of the substrates which was also found in refining of organosolv-pretreated lodgepole pine (Del Rio, 2012). Therefore, the remainder of this paper will mainly focus on the modification of physical characteristics during refining.

Table 3.2 Chemical compositions of refined and unrefined steam pretreated hybrid poplar chips, as percentages of the solid weight.

Sample	*Temperature (°C)*	*Time (min)*	*SO_2 (% w/w)*	*Combined Severity*	*Refining Conditions*	*Glucan (%)*	*Xylan (%)*	*Lignin (%)*
S1	205	10	0	0.6	Unrefined	62.2	2.4	34.5
					Refined	63.5	2.6	32.6
S2	212	10	0	1.0	Unrefined	62.2	2.0	28.9
					Refined	62.9	2.1	33.5
S3	212	15	0	1.3	Unrefined	61.4	1.7	32.3
					Refined	61.3	1.7	35.6
S4	190	5	3	1.9	Unrefined	65.3	0.5	32.7
					Refined	65.2	0.5	27.1
S5	195	5	3	2.0	Unrefined	65.6	0.3	31.1
					Refined	64.3	0.3	31.3
S6	200	5	3	2.1	Unrefined	64.3	0.2	30.3
					Refined	64.6	0.2	32.9

Standard deviations were determined to be less than 3 %.

3.3.2 **Solid particle size analysis**

The length and width of the pretreated solids particles before and after refining were measured by fiber quality analysis (Figure 3.2). The lengths reported are the length weighted mean length, and widths are the arithmetic mean width. For unrefined samples, with increasing pretreatment severity, the particle length and width decreased from 0.72 mm to 0.19 mm and from 25 µm to 21 µm, respectively. Meanwhile, the same trends were observed for particle length and width for the refined samples, i.e., the length decreased from 0.31 mm to 0.17 mm and width decreased from 23 µm to 20 µm. Generally, the uncatalyzed particles were longer and wider than catalyzed particles.

After refining with the valley beater, particle lengths of S1-S5 samples were shortened to a statistically significant extent, ranging from 0.17 mm to 0.31 mm (Figure 3.2A). Among them, S1 was notable with a 57 % length reduction from 0.72 mm to 0.31 mm. Particle shortening were also statistically significant for S2 to S5 with length reduction of 46 % to 26 %. The length reduction of S6 from 0.19 mm to 0.17 mm was not statistically significant. All the samples width slightly decreased after refining, distributing from 20 µm to 23 µm (Figure 3.2B). However, only

the width reduction of S1 was statistically significant. Typically, refining reduces both particle length and width, resulting in the overall size reduction (Chen *et al.*, 2012). Refining had greater size reduction effects at lower severity pretreated samples. The largest size reduction was observed on S1, likely because S1 was not well pretreated and had the greatest potential to reduce in size, compared with other particles (S2-S6) which were already well pretreated and small.

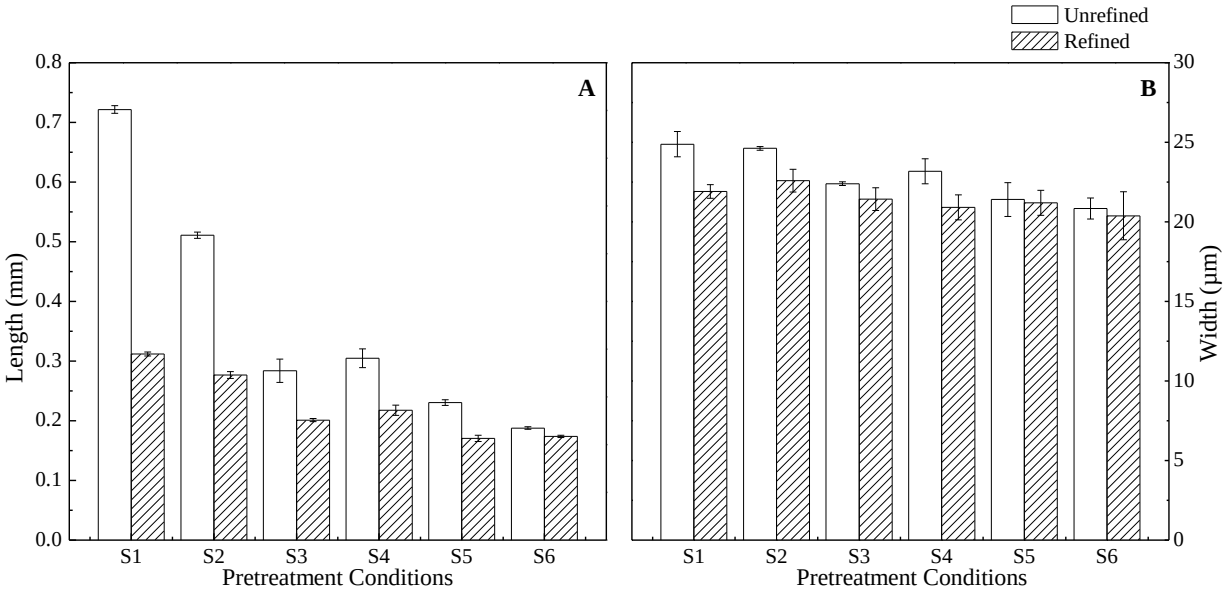

Figure 3.2 Fiber length (A) and width (B) of refined and unrefined steam pretreated hybrid poplar

3.3.3 **Enzymatic hydrolysis**

The effects of pretreatment and refining were assessed by the glucose yields after enzymatic hydrolysis. Figure 3.3 demonstrates the cellulose-to-glucose conversion for refined and unrefined solids at 5 FPU/g cellulose and 5 % (w/v) solid consistency. After 72 hours of enzymatic hydrolysis, the glucose conversion of unrefined samples ranged from 48 % to 73 %. For uncatalyzed samples, glucose yield improved when pretreatment severity increased; for catalyzed samples S4 to S6, all the glucose yield plateaued around 69 % conversion.

Consistent with earlier work on steam pretreated willow (Sassner *et al.*, 2005; Horn *et al.*, 2011) and birch (Vivekanand *et al.*, 2013), the enzymatic release of glucose increased with

pretreatment severity to a certain extent. It has been generally recognized this trend is explained by particle size reduction (Figure 3.2) and hemicellulose dissolution (Table 3.2). Particle size reduction enhances the specific area of biomass (Chagaev, 2007; Li, Li, *et al.*, 2011; Mou *et al.*, 2013), and hemicellulose removal unveils more surface area of cellulose for enzyme access (Jeoh *et al.*, 2007; Bura *et al.*, 2009), both of which promote sugar yield by introducing more accessible surface area to the enzyme (Grethlein *et al.*, 1991; Leu *et al.*, 2013).

Refining improved the cellulose-to-glucose conversion for all samples, resulting in cellulose conversion of around 70 %. S1 achieved a 23 % improvement in enzymatic hydrolysis, which corresponded to the most significant particle size reduction as mentioned above (Figure 3.2). Contrarily, other samples did not achieve significant sugar yield improvement, possibly since their particle size reduction was far less than S1 during refining process (Figure 3.2). Smaller particles have larger surface area per unit volume, therefore more cellulose is accessible for enzyme to reach (Dasari *et al.*, 2007; Zhu *et al.*, 2009; Vidal *et al.*, 2011; Leu *et al.*, 2013). For that reason, glucose yields were significantly improved for S1 through the refining process. Improvements in other samples, although visually observed, were not significant following statistical analysis.

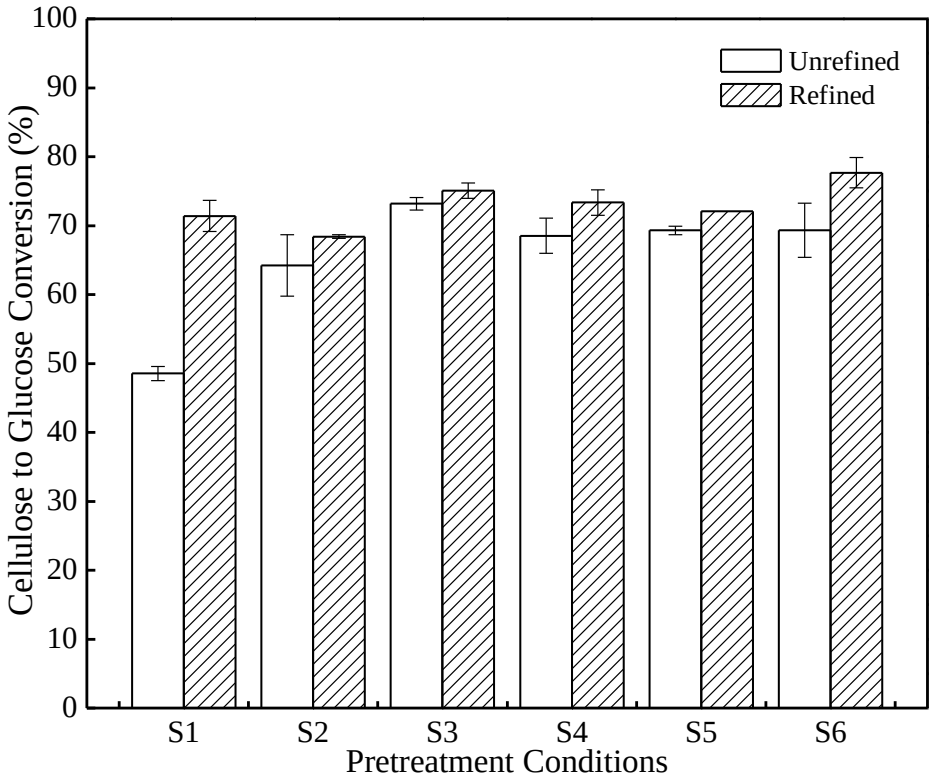

Figure 3.3 Cellulose to glucose conversion of refined and unrefined steam pretreated hybrid poplar with cellulase at 5 FPU/g cellulose and 5 % (w/v) solid consistency in 72 hours.

Considering the non-SO_2 condition and hydrolyzability improvement, S1 was chosen as a prototype for studying the effect of refining on enzymatic hydrolysis at different enzyme loadings and solids consistencies. Three enzyme loadings, 1 FPU, 2 FPU and 5 FPU/g cellulose, and three consistencies, 5 %, 10 % and 15 % (w/v) were orthogonally performed. As expected, for unrefined samples, when applying higher enzyme loading from 1 FPU to 5 FPU/g cellulose, the cellulose-to-glucose conversion enhanced significantly from 11.8 % to 47.6 %. Similar trends were also observed in other solid consistencies (Figure 3.4). However, all hydrolysis yields were 12 % to 23 % lower at the highest solids consistency, which is potentially due to the limited flowing water available for enzyme diffusion (Chen *et al.*, 2012).

The effects of enzyme loading and solids consistency of refined solids were similar to the effects shown in unrefined solids. Particularly, by refining, the cellulose-to-glucose conversion at 5 %

solids consistency were significantly promoted from 11.8 % to 27.8 %, from 26.6 % to 48.1 % and from 47.6 % to 70.8 % at 1 FPU, 2.5 FPU and 5 FPU/g cellulose, respectively. Similar improvements exist in all the other enzyme loadings and solid consistencies. Specifically, by saving half of enzyme loading from 5 to 2.5 FPU/g cellulose, refining achieved the same cellulose-to-glucose conversion at 5 % consistency for 48 %. Considering the enzyme cost, refining demonstrated the benefit of saving enzyme dosage and reached the same sugar yield (Koo *et al.*, 2011). Refining also helped to recover the sugar effectively at higher consistency. For example, with the same enzyme loading (5 FPU/g cellulose), refined solids at 15 % consistency reached the similar cellulose conversion (48 %) with unrefined solids at 5 % consistency. Refining improves the solids consistency without depressing the sugar yield, and could eventually decrease the capital cost in enzymatic hydrolysis and energy requirement in product recovery (Humbird *et al.*, 2010).

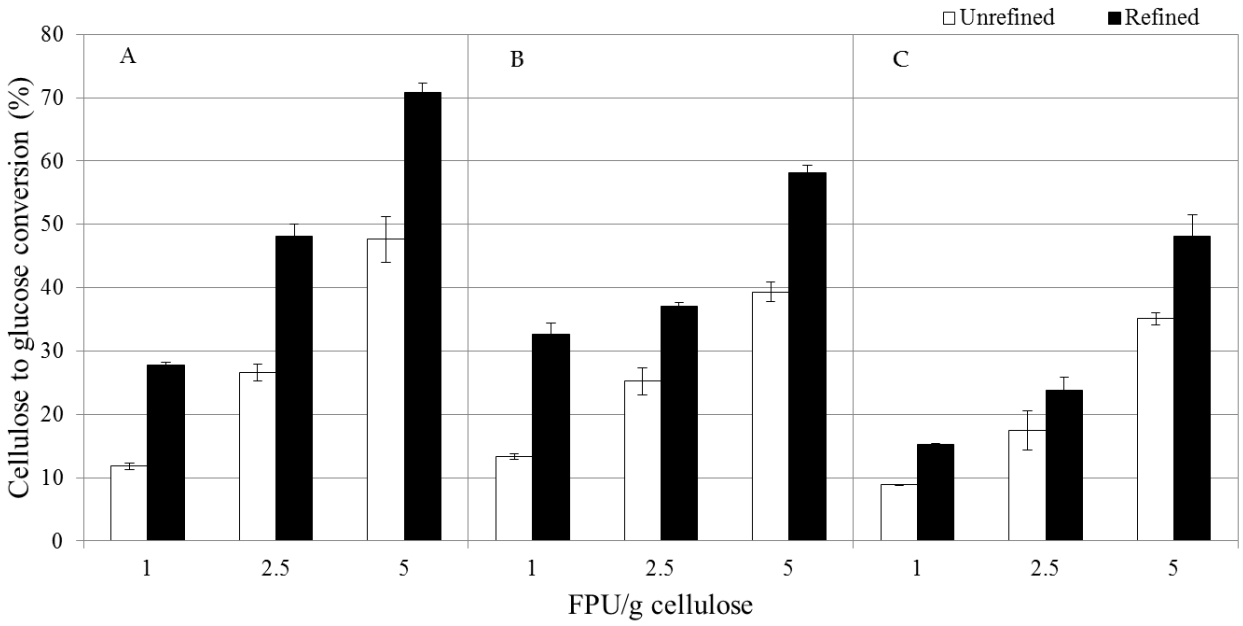

Figure 3.4. Cellulose to glucose conversion in 72 hours of different enzyme loading and solids consistency for steam pretreated hybrid poplar pretreated at 205 °C, 10 min, without SO_2 impregnation. A: 5 % consistency; B: 10 % consistency; C: 15 % consistency.

3.3.4 **Effects of refining time on particle size and hydrolysis**

In order to understand the function of refining in modifying particle size and improving enzymatic hydrolysis, sample S1 was refined in valley beater for 5 different durations. As shown

in Figure 3.5, both length and width were reduced during the process of refining. Specifically, there was a very good linear fit ($R^2 = 1$) between the length and refining duration, indicating refining shortened the particle length at a constant rate. However, the particle width decreased very slightly through the time with a poor regression line ($R^2 = 0.38$).

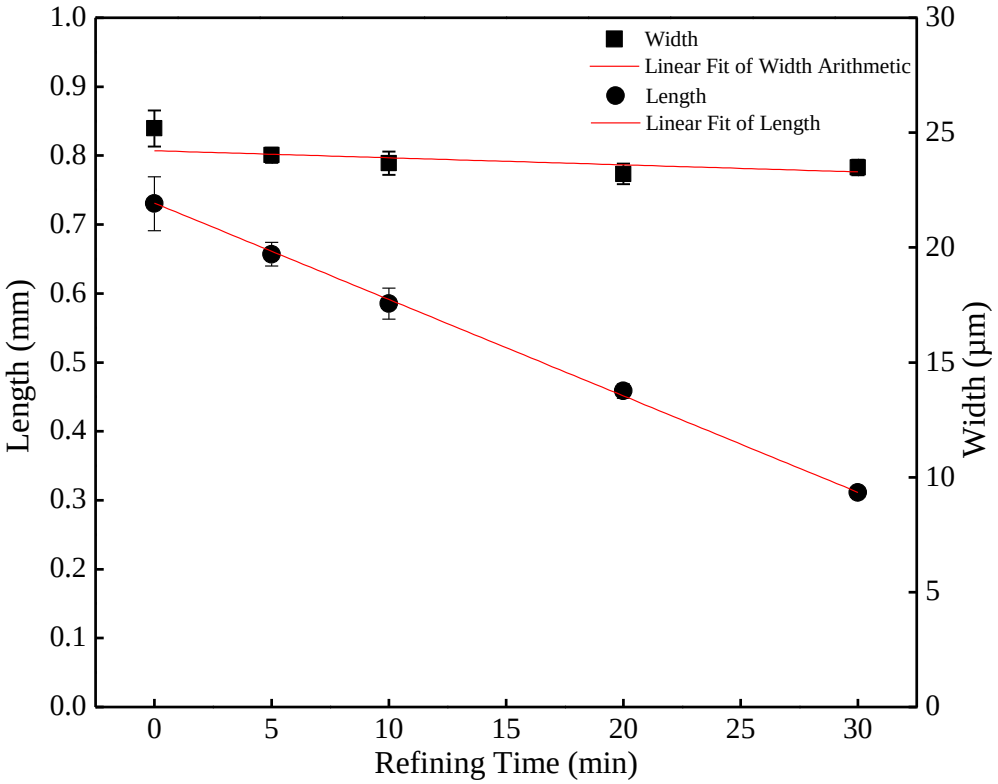

Figure 3.5 Fiber length and width of steam pretreated hybrid poplar (205°C, 10 min, without SO_2) with different refining times and their linear regression lines. (R^2_{length} = 0.38, R^2_{width} = 1.00).

Enzymatic hydrolysis of the solids refined for 5 different durations showed a parallel to size reduction, with cellulose-to-glucose conversion increasing as refining time increased (Figure 3.6). At 72 hours, the most refined (30 min) solids achieved sugar conversion as much as 71 %, while the unrefined solids (0 min) had merely 49 % conversion of cellulose to glucose.

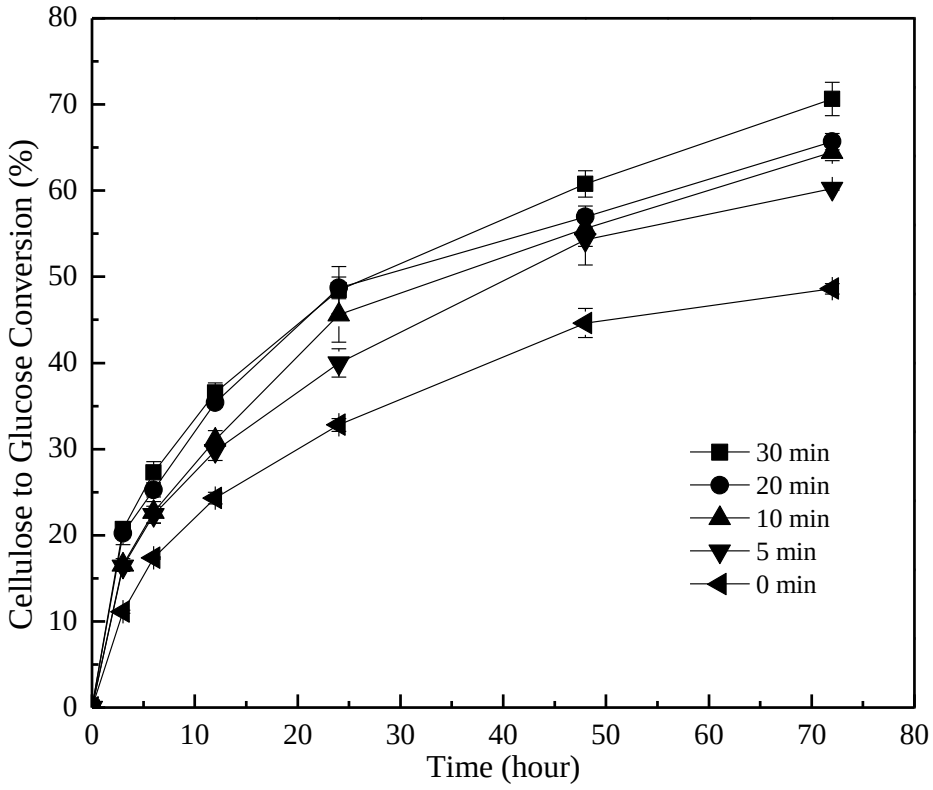

Figure 3.6. Cellulose to glucose conversion of steam pretreated hybrid poplar (205 °C, 10 min, without SO_2) with different refining time at 5 FPU/g cellulose and 5 % (w/v) solid consistency.

Interestingly, it was observed that the cellulose-to-glucose conversion of S1 in the first 5 minutes refining increased by 11.6 %, which was a greater improvement than that achieved with an additional 25 minutes of refining (10.4 %) (Figure 3.6). As shown in Figure 3.7, the relationship between cellulose-to-glucose conversion and refining time doesn't have a perfect linear fit (R^2=0.82) when considering all refining times. However, if the 0 min to 5 min time interval is eliminated and only the refining time from 5 min to 30 min is considered, the cellulose-to-glucose correlates well (R^2=0.95) with the time (Figure 3.7). That is to say, the size reduction improved enzymatic hydrolysis at a constant rate only after 5 minutes. Based on that phenomenon, particle size reduction may not be the only mechanism of refining that improved the sugar yield.

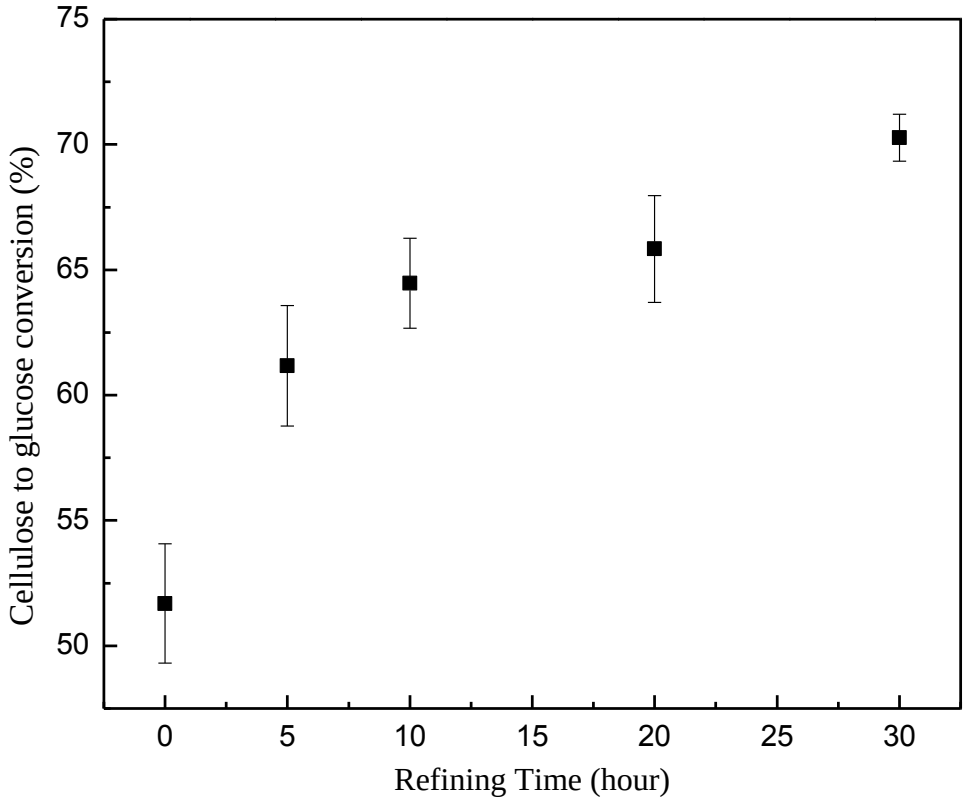

Figure 3.7 Cellulose to glucose conversion to different refining time (0 min to 30 min) in 5 FPU/g cellulose and 5 % (w/v) solid consistency. Linear regression lines not shown (R^2 = 0.82 for samples refined from 0 min to 30 min, R^2 = 0.95 for samples refined from 5 min to 30 min).

Figure 3.8 shows the relationship between enzymatic conversion and particle length for all of the samples studied in this research (n= 17). Putting all the particle lengths and corresponding cellulose-to-glucose conversions together, the overall particle size reduction matches well with the enzymatic hydrolysis conversion (Figure 3.8). The goodness of fit of the regression line (R^2 = 0.82) indicates that the hydrolysis yield was improved as particle size decreased. It is therefore proposed that enzymatic hydrolysis is generally influenced by the particle size reduction in refining, since smaller particle provides higher specific surface area, more enzymes will be able to access the particles (Chen *et al.*, 2012).

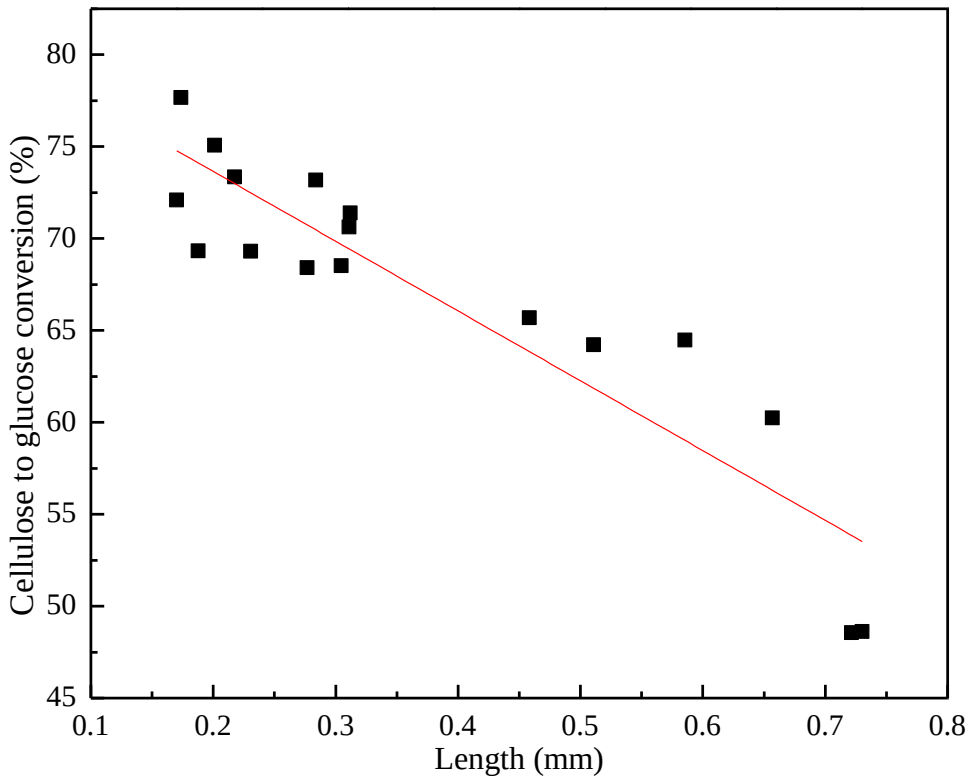

Figure 3.8 Cellulose to glucose conversion of refined and unrefined steam pretreated hybrid poplar to particle length with cellulase at 5 FPU/g cellulose and 5 % (w/v) solid consistency in 72 hours and their linear regression line (R^2 = 0.82).

The reason why the first 5 minutes of enzymatic hydrolysis was the most improved with limited particle size reduction is conjectured to be due to the surface modification. Refining, besides shortening fibers, also makes other morphological and structural modifications (Page, 1989; Zhu *et al.*, 2009). Effects include, for example, external/internal fibrillation (Gil *et al.*, 2009; Koo *et al.*, 2011) and capillary (or porous) structure formation (Zhang, Song, *et al.*, 2012). All those mechanisms presumably provide more surface area accessible to the enzymes.

Also, it can be explained by the two class size reduction theory from Leu et al (2013): Class I size reduction merely increases the external surface area by producing fibers or fiber bundles without significant breakup of fiber cell walls. Class II completely breaks down fibers to expose

cell walls fully accessible to enzymes. Hypothetically, during the first 5 minutes, Class I and Class II synergistically affected the solids by opening up the structure and trimming the particles, generating the largest improvement in enzymatic hydrolysis. After 5 minutes, most of the Class I had occurred, and only Class II reductions were happening, resulting in a constant improvement of hydrolyzability.

There is also possibility that refining modifies the particle structure. As mentioned earlier (Mou *et al.*, 2013), pulp fiber shape was changed from tubular to flat due to the beating effect during the refining in paper making process. Since valley beater works as a bedplate beater, the refining (beating) process fractures the particle and flattens the shape (Touzinsky *et al.*, 1977), which may soften the fiber internal structure and improve the digestibility.

In general, refining enhanced the sugar yield during enzymatic hydrolysis of low severity steam pretreated hybrid poplar without the use of SO_2. Compared with SO_2 catalyzed steam pretreatment, refining achieves similar enzymatic hydrolysis yield, meanwhile it might mitigate the problems of environmental pollution, operation complexity, extra operation process, waste water treatment, reactor corrosion and chemical catalysis toxicity. Refining therefore presents a sulfur free pretreatment strategy which increases the sugar conversion while reducing required enzyme dosage.

3.4 Conclusions

Refining of hybrid poplar steam pretreated at conditions ranging from 190 °C to 212 °C for 5 min to 10 min with or without SO_2 did not change the chemical composition, but reduced the particle size. Not all samples achieved statistically significant improvement of refining in enzymatic hydrolysis. However, sample S1 that had undergone pretreatment of 205 °C 10 min, with no SO_2 showed the most significant improvement in sugar yield during enzymatic hydrolysis --- as much as 23%. Refining improvements were also obtained at different enzyme loadings and solid consistencies during enzymatic hydrolysis of S1. For instance, by reducing the enzyme loading from 5 to 2.5 FPU/g cellulose, refining achieved the same cellulose conversion (48 %) as unrefined samples. By increasing two-fold the enzymatic hydrolysis solids consistency

from 5 % to 15 %, refined solids reached a similar cellulose conversion (48 %) as unrefined solids. It was observed that particle size reduction was positively correlated with the refining time, which was shown to promote the digestibility of the solid fraction. As a potential substitute for SO_2, refining demonstrates a method to achieve good sugar recovery after low-severity, uncatalyzed steam pretreatment.

Chapter 4. Conclusions and future work

4.1 Conclusions

The steam pretreatment is regarded as one of the most cost effective and efficient pretreatment method for lignocellulosic biomass. The addition of SO_2 as acid catalyst makes the steam pretreatment more effective with shorter residence time and lower temperature condition. However, few researchers have considered the adverse impacts of the incoming SO_2. The SO_2-catalyzed steam pretreatment may create a series of problems in environmental protection, process complexity, equipment corrosion, chemical catalysis and waste water treatment. Moreover, since SO_2 increases the pretreatment severity, it may result in more sugar degradation and inhibitor formation. Steam pretreatment without SO_2-catalyst, however, has a poor hydrolyzability of solid fraction and results in a low overall post-hydrolysis sugar recovery. This research mainly focuses on developing a catalyst free and high sugar yield bioconversion system based on the steam pretreatment and enzymatic hydrolysis.

In this study, hybrid poplar chips were steam exploded using 6 different conditions with or without the addition of SO_2. The steaming temperature ranged from 190 ℃ to 212 ℃, and the residence time ranged from 5 min to 10 min. Liquid fractions (hydrolysate) were investigated for the fermentability and analyzed for inhibitors concentration; the solid fractions were refined by a valley beater to test the hydrolyzability and the physical characteristic of particles before and after refining were assessed. Based on the research I was able to draw the following conclusions:

- Different concentrations of inhibitors were formed in different pretreatment conditions. As the severity of the pretreatment increased more inhibitors were produced.
- Certain concentration of inhibitors, particularly acetic acid, increased the ethanol yield in hydrolysate fermentation, but decreased the xylitol yield.
- The hydrolysate from steam pretreated hybrid poplar obtained at 205°C, 10 minutes without SO_2 had the lowest inhibitors concentration, thus produced the highest ethanol yields. Without SO_2 impregnation, solids from steam pretreated hybrid poplar at 205°C, 10 minutes had the lowest cellulose to glucose conversion, although the overall sugar

recovery following pretreatment was the highest for that condition. This resulted in the lowest overall sugar recovery after enzymatic hydrolysis.

- Mechanical refining had the largest improvement of 23 % in enzymatic hydrolysis for solids pretreated at 205°C, 10 minutes without SO_2, which significantly enhanced the overall post-hydrolysis sugar recovery.
- Similar improvements were observed for different enzyme loadings and solid consistencies for solids pretreated at 205°C, 10 minutes without SO_2.However, refining did not improve the hydrolyzability of solids pretreated at five other conditions.
- Reduced particle sizes were found to be correlated to increased sugar yields in enzymatic hydrolysis. Solids pretreated at 205 °C 10 minutes, without SO_2 exhibited the largest size reduction after refining and correspondingly achieved the highest sugar recovery improvement after steam pretreatment and enzymatic hydrolysis.
- In general, refining can enable a catalyst free, low inhibitor concentration, high overall sugar recovery bioconversion system based on the steam pretreatment and enzymatic hydrolysis method.

4.2 Future work

The work outlined in this thesis explores a basic understanding on improving sugar recovery through low-severity pretreatment and refining process. Future work should expand on this research in the following ways:

- from 180 °C to 200 °C for 10 min without SO_2, measure the production of inhibitors, hydrolysability before and after refining and the overall ethanol and xylitol yields. To understand the mode of action of mechanical refining, further study the changes in the hemicellulose and lignin distribution, available surface area, porous level and cellulose crystallinity before and after refining.
- To investigate the low water usage during refining increase the refining consistency to 10 or 20 % (w/v) solids. To evaluate techno-economic and environmental feasibility of

steam pretreatment with/without SO_2 and with/without refining by using Aspen models and life cycle assessment (LCA).

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