Strategies to increase the sugar concentration and overall sugar recovery from steam pretreated wheat straw and corn stover

by

Carolina Michelle Chanis Morales

BSc, The University of British Columbia, 2009

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Abstract

Wheat straw is available in western Canada and it is a potential feedstock for bio-ethanol production as it can be effectively fractionated into simple sugars using acid catalyzed, steam pretreatment followed by enzymatic hydrolysis. Steam pretreatment is usually a compromise whereby conditions that facilitate effective enzymatic hydrolysis at low enzyme loadings usually sacrifice the recovery of the hemicellulose component. Previous work that tried to optimize the pretreatment to maximize hemicellulose recovery was usually done at the expense of using unacceptably high enzyme loadings to hydrolyze the cellulosic fraction.

The goal in this thesis was to determine the highest possible amount of sugar that could be solubilized after both pretreatment and enzymatic hydrolysis while using low enzyme loadings and high solids concentration. It was anticipated that the optimum conditions for maximizing the total soluble sugar yield would still result in the degradation of a portion of the hemicellulose.

The biomass handling conditions were first investigated to identify the best possible conditions to maximize sugar recovery. An optimized moisture content combined with the explosive decompression resulted in the highest xylose recovery. It was also found that H_2SO_4 could be used at a loading of 1.5% w/w to produce a substrate with similar chemical composition, sugar recovery and ease of enzymatic hydrolysis to what was obtained when using 3% SO_2 as the catalyst.

The pretreatment conditions were then varied to determine the effect of pretreatment severity on the recovery of total soluble sugars. The highest soluble sugar

yield of 75% was obtained after pretreatment at 190°C, 8 min and 1.5% H_2SO_4. This is among the highest sugar yields that have been reported and comparable to those reported when using a three-fold higher enzyme loading. However, at these conditions only 52% of the original xylan was recovered. A less severely treated substrate with 70% xylan recovery achieved a total soluble sugar yield of 72% when the "cellulase mixture" was supplemented with xylanases. Thus, pretreatments at lower severities followed by enzymatic hydrolysis using a "cellulase mixture" with xylanase supplementation may be an effective approach to improve the total soluble sugar yield when processing wheat straw.

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List of Abbreviations

AFEX: Ammonia fiber expansion

AIL: Acid insoluble lignin

ARP: Ammonia recycled percolation

ASL: Acid soluble lignin

ATR: Attenuated total reflectance

Bdl: Beyond detectable limits

CAFI: Consortium for Applied Fundamentals and Innovation

CMP: Chemimechanical pulp

CS: Combined severity factor

CTMP: Chemithermomechanical pulp

Ext: Extractives

FPU: Filter paper units

FT-IR: Fourier transform infrared spectroscopy

GHG: Greenhouse gas emissions

LOI: Lateral order index

LWW: Length weighted average

Mg: Megagram

Mt: Megaton

NREL: National Renewable Energy Laboratory

ODW: Oven-dry weight

PAPTEC: Pulp and Paper Technical Association of Canada

TAPPI: Technical Association of the Pulp and Paper Industry

TSS: Total soluble sugars

TSSY: Total soluble sugar yield

WIF: Water insoluble fraction

WRV: Water retention value

WSF: Water soluble fraction

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Dedication

"Cuando pones la proa visionaria hacia una estrella y tiendes el ala hacia tal excelsitud inasible, afanoso de perfección y rebelde a la mediocridad, llevas en ti el resorte misterioso de un Ideal. Es ascua sagrada, capaz de templarte para grandes acciones. Custódiala; si la dejas apagar no se reenciende jamas."

José Ingenieros, El Hombre Mediocre

A mi familia y a la memoria de Milena. Les dedico este trabajo y estas frases, es por ustedes que existe esta ascua sagrada.

1. Introduction

1.1. The future of energy

Ever since the Industrial Revolution, there has been an accelerated increase in the emissions of carbon dioxide and other greenhouse gases (GHG) into the atmosphere. In this short period of time carbon dioxide concentration in the atmosphere has increased by 35% (Hindrichs & Kleinbach, 2013). These emissions are mostly due to anthropogenic activity and it has resulted in an increase in global air temperatures of 0.4-0.8°C (National Research Council, 2001) over a period of years. If they are not reduced, it is very likely that global temperatures will rise by approximately 4°C by 2100 (Berrang-Ford et al., 2011). It has been projected that global warming will lead to changes in water levels, weather and rainfall patterns which will have devastating effects on ecosystems, agriculture, water availability and biodiversity (Gosling et al., 2011).

Carbon dioxide emissions contribute to greenhouse gas emissions (Lashof & Ahuja, 1990) and the primary source of these emissions is fossil fuel burning for heat, energy and transportation as well as land use change due to deforestation and agriculture (Berndes, 2010; National Research Council, 2001; Sims et al., 2007). About 80% of the world's energy use comes from fossil fuels, which adds 8 billion tons of carbon to the atmosphere each year and this number keeps increasing as developing countries become more industrialized (Hindrichs & Kleinbach, 2013). Climate change has made fossil fuel consumption environmentally unsustainable, but there is another economic driver to reduce our dependency on oil. As a non-renewable energy source, we are bound to deplete our oil reserves and it is estimated that we have or will reach "peak oil" during

this century (Bardi, 2009). As demand and the price of oil increases, extraction of unconventional deposits becomes feasible, which brings about additional environmental challenges (Rogner, 2012). Our society and economic system has always relied on cheap energy to drive its progress and development. The challenge of the 21st century will be to procure an energy supply that is secure and also meets our greenhouse gas reduction goals without compromising our livelihoods.

Biofuels from biomass represent an alternative source of fuel that is expected to have an important role in the development of a low-carbon energy society that can also meet the demand for energy (Beringer et al., 2011). Ethanol is a clean burning, biodegradable fuel that is used as a replacement for methyl tert-butyl ether (MTBE) to reduce emissions from gasoline (Vasudevan et al., 2010). In 2010, global biofuel production rose to 100 billion litres, of which Brazil and the USA are the major producers (International Energy Agency, 2011). Brazilian ethanol is derived from sugarcane while the USA produces ethanol from corn (International Energy Agency, 2011; Kim & Dale, 2004). However, these sources of biomass are a cause of concern since they could compromise our food supply (Ragauskas et al., 2006). A viable option that would not compromise our food supply and that could also offset carbon dioxide emissions if managed properly is ethanol produced from lignocellulosics. Lignocellulosics consists of the non-edible parts of crops such as the leaves and stalk as well as woody material from trees.

The transition to biofuels from lignocellulosics still faces many economic challenges due to the cost of converting the cellulosic components into fermentable sugars (Viikari et al., 2012). However, it is the best transition technology available at the

moment to reduce GHGs emissions and its success is dependent on the efficient utilization of all types of biomass. Among the different types of lignocellulosic material available, residues from agricultural crops are the most abundant sources and they will likely become the first sources of lignocellulosic ethanol production (Mabee & Saddler, 2010). Even after taking into account sustainability guidelines to conserve nature and the constraints of land use, it is estimated that there is enough biomass to supply 15-25% of the world's energy demand in 2050 (Beringer et al., 2011). Although there seems to be a substantial amount of agricultural residues for fuel production, efficient utilization of these resources is crucial to develop an industry that is economically sustainable. Regardless of the efficiency of the bioconversion technologies available, feedstock cost can constitute up to 40% of the production cost (Hamelinck et al., 2005). Thus, it is imperative to reduce wastage of material, from the moment it is shipped to the facility, up to its conversion into fuel. Among the agricultural residues available for production of cellulosic ethanol, wheat straw is one of the most abundant cereal straws available in Canada. The following section will cover the availability of wheat straw worldwide and in Canada, and its suitability for bioconversion into ethanol.

1.2.Availability of wheat straw for ethanol production

Annual global production of wheat is estimated to be 647 Mt (Kim & Dale, 2004; Phillips & Norton, 2012). Its abundance makes it a potential as a feedstock for bioethanol production. Estimations of the availability of wheat straw for bioethanol production varies greatly depending on the assumptions. Wheat straw is plowed back into the soil to prevent loss of organic matter and nutrients which would lead to soil deterioration. For this reason, a percentage of the wheat straw needs to remain in the field, but this amount depends on several factors such as soil type, amount of standing stubble (Stumborg et al., 1996). In addition, weather, crop rotation, the slope of the land, wind and rainfall patterns as well as tillage practices are also factors that determine the amount of straw that can be removed from the field (Kerstetter & Lyons, 2001). A conservative estimate of 60% ground cover (Kim & Dale, 2004), which is the equivalent of 1.7 Mg of wheat straw residue per hectare, would result in 354 Tg of wheat straw available for ethanol production worldwide. It is also estimated that the lignin from this amount of straw could produce 122 TWh of electricity and 698 PJ steam. Others have estimated that 30% ground cover is sufficient (Volynets & Dahman, 2010), which is in accordance to the USDA standards. This would increase wheat straw availability to 481 Tg which could potentially result in 141 GL of ethanol and 141 TWh of electricity (Volynets & Dahman, 2010).

The most recent study (Li et al., 2012) on crop residue supply in Canada report that Saskatchewan, Alberta, Ontario, Manitoba and Quebec have the highest potential to produce ethanol from agricultural residues, which is estimated to be 6.6-13 billion litres. Most of the ethanol production will be from wheat straw because it is the dominant crop

species cultivated. Between 2001 and 2010, Saskatchewan, Alberta and Manitoba produced 92% of all wheat, while Ontario produced 7% for an annual total of 23.8 million dry Mg (Li et al., 2012).

1.3.Amenability of wheat straw for bioconversion processes

The relative ease of conversion of wheat straw when compared to softwoods or hardwoods is the main advantage of using this feedstock for bioconversion. Agricultural residues are relatively less recalcitrant and can be processed at lower temperatures, with less consumption of chemicals and without the need of a post-treatment to enhance the digestibility (Bura et al., 2009; Kumar & Wyman, 2009b). Furthermore, some pretreatment technologies have been developed for agricultural residues that do not require chemicals. The lower recalcitrance of wheat straw could be mainly attributed to the anatomical and chemical differences of the plant tissues. Straw materials have narrow, short cells in the straw tissue and do not exhibit secondary growth like softwoods and hardwoods so the lignin content is lower (Judt, 1993). The chemical composition of agricultural residues such as straw differs from that of softwoods and hardwoods, which brings about specific challenges for its processing. All lignocellulosic materials are made of cellulose, hemicellulose and lignin. Cellulose is a semi-crystalline homopolymer of β-(1→4)-linked D-glucopyranose units (Wyman et al., 2005). About 30% of the dry weight of agricultural crops is composed of hemicellulose, a heteropolymer that consists of a xylan backbone substituted by acetyl and arabinose groups. Removal of hemicelluloses increases the conversion of cellulose to glucose (Bura et al., 2009; García-Aparicio et al., 2007; Kumar & Wyman, 2009a; Öhgren et al., 2007).

The mechanism of hemicellulose inhibition on enzymatic hydrolysis is not fully understood, but it is likely that hemicellulose restricts cellulose accessibility because it coats the cellulose microfibrils (Chandra et al., 2011; Hu et al., 2011; Yoshida et al., 2008). Due to its abundance, its recovery must also be maximized in order to produce value-added co-products or to ferment into ethanol. Maximizing the recovery of the cellulose and hemicellulose into its respective glucose and xylose components is a challenge because hemicellulose is amorphous and has a lower solubilization temperature than cellulose (Talebnia et al., 2010). The temperatures applied to increase the ease of hydrolysis of the cellulose component of lignocellulosic biomass are high enough to cause degradation of the hemicellulose into products that are toxic to the yeast in the fermentation process. On the other hand, retaining the hemicellulose in the solid fraction also reduces the breakdown of cellulose into glucose, so the challenge is to optimize the fractionation process to ensure recovery of the glucose and xylose without compromising downstream processes.

Lignin is a polymer of phenylpropanoid units known as *p*-hydroxymethyl (H), syringyl (S) and guaiacyl (G). Unlike softwoods and hardwoods, lignin from herbaceous crops contains significant amounts of all three phenylpropanoid units (Sjöström, 1993; Vogel, 2008) and it constitutes about 10-20% of the straw tissue (Fan et al., 1982). Lignin acts as a physical barrier to cellulases due to its association with the cellulose and hemicellulose in the cell wall and by nonproductive binding to cellulases (Berlin et al., 2006; Fan et al., 1982; Mansfield et al., 1999). Removal of lignin results in an increase in enzymatic hydrolysis yields but the extent of delignification does not always correlate to the increase in sugar yields. A comparison of isolated lignins from corn stover, poplar

and lodgepole pine demonstrated that corn stover lignin did not affect the hydrolysis of Avicel, regardless of pretreatment or isolation method (Nakagame et al., 2010). Therefore, it is likely that the bioconversion of agricultural residues into ethanol will not require a treatment of the substrate to remove or modify the lignin which has been shown to be necessary in the case of softwoods (Kumar et al., 2011).

1.4. Pretreatment technologies commonly used for agricultural residues

1.4.1. The role of pretreatment in the bioconversion process

The goal of the bioconversion process is to break down the cellulose and hemicellulose into simple sugars that the yeast can utilize for fermentation (Alvira et al., 2010). The conversion of lignocellulosic biomass into ethanol consists of several steps: particle size reduction, pretreatment, hydrolysis, fermentation and distillation. The sugars are extracted and broken down into monosaccharides during pretreatment and hydrolysis. The pretreatment step breaks down the lignocellulosic matrix so that the enzymes can access the cellulose and hydrolyze it into glucose in the hydrolysis step (Mosier et al., 2005a). Hydrolysis of lignocellulosic substrates is achieved by a complex mixture of cellulases that are naturally found in fungi or bacteria. The most commonly studied and used cellulase mixture is from the fungus *Trichoderma reesei*. It produces two cellobiohydrolases (CBHI, CBHII), five endoglucanases (EGI, EGII, EGIII, EGIV and EGV) and two β−glucosidases (BGLI, BGLII) (Lynd et al., 2002).

Pretreatment is one of the most important steps in the bioconversion process as it determines the efficiency of all downstream processes. Its efficiency is in turn determined

by the type of biomass. In the case of agricultural residues, AFEX (Ammonia Fiber Expansion) and steam pretreatment are two of the most commonly used technologies. Pretreatment alters the chemical properties of cellulose, hemicellulose and lignin as well as the physical properties of the substrate. Pretreatment has effects on the chemical properties of cellulose such as degree of polymerization and crystallinity, and on the chemical properties of lignin such as hydrophilicity and degree of condensation (Li et al., 2007). The hemicellulose and lignin that remain in the water insoluble fraction after pretreatment have the greatest impact during enzymatic hydrolysis because these polymers coat the cellulose microfibrils and thus limit the accessibility of the cellulases to the cellulose (Mansfield et al., 1999). In addition, the phenolic compounds released from the lignin during pretreatment have been shown to inhibit and deactivate enzymes during hydrolysis (Ximenes et al., 2010; Ximenes et al., 2011) and they can also be toxic to the yeast during fermentation (Hahn-HäGerdal et al., 1991). Physical properties altered during pretreatment that affect enzymatic hydrolysis include porosity (or accessible surface area), swelling and particle size (Table 1).

Table 1 Substrate factors and enzyme factors that inhibit hydrolysis (Mansfield et al., 1999).

Substrate Factors	Effects
Degree of polymerization	Solubilization of cellulose
Cellulose crystallinity	Initial rate of enzymatic hydrolysis
Available surface area	Access to cellulose microfibrils
Lignin content	Barrier to cellulases, non-productive binding
Hemicellulose content	Barrier to cellulases, steric hindrance due to substituents
Particle size	Might have an effect on the initial rate of hydrolysis

Pretreatment also influences the sugar recovery from the raw material and the efficiency of hydrolysis and fermentation (Chandra et al., 2007). In order to achieve a cost-efficient process for ethanol production, we need to achieve a high recovery at every step of the process while minimizing the input of chemicals and enzymes (Merino & Cherry, 2007). The chemical differences between cellulose and hemicellulose make it difficult to maximize recovery of both components during pretreatment. Hemicellulose is an amorphous polymer and has a lower solubilization temperature than cellulose at 150°C (Talebnia et al., 2010). At this temperature it would be difficult to obtain a cellulose substrate that can be easily hydrolyzed. The higher temperatures of 180-210°C that are normally used to pretreat agricultural residues results in hemicellulose degradation.

The degradation products produced from hemicellulose and lignin during pretreatment are inhibitory or toxic to the fermenting yeast. The toxicity will depend on the pretreatment technology and also the pretreatment conditions used. Several biological and chemical strategies have been studied to overcome the toxicity of the water soluble fraction. Biological treatments include the use of peroxidases and lacasses to remove low molecular weight phenolic compounds and treatment with *T.reesei* to remove acetic acid, furfural and benzoic acid. Chemical treatments involve adjusting the pH of the slurry to 9-10 with $Ca(OH)_2$, also known as overliming (Palmqvist & Hahn-Hägerdal, 2000). Unfortunately, detoxification techniques add to the cost of production and may result in sugar loss. Furthermore, such strategies are unlikely to be used at large scale facilities (Hahn-Hägerdal et al., 2006). It is necessary to reduce the degradation of sugars during pretreatment to minimize sugar loss and also the cost of detoxification of the liquor.

An ideal pretreatment would be able to handle different types of biomass and recover the sugars in high yields in a form that is also highly digestible by the enzymes at a minimal enzyme loading, while minimizing the formation of degradation products (Alvira et al., 2010). Pretreatment technologies should also have low capital and operations costs and use limited chemical addition, especially in cases where chemicals such as H_2SO4 and SO_2 are used because they require special metal alloys to limit corrosion. Ideally, pretreatment chemicals should be recoverable so that they can be either re-used or can be converted to a useful by-product. Pretreatment technologies are energy intensive processes, but the energy use can be decreased greatly if the material can be utilized at low moisture contents and without significant particle size reduction. Other key requirements that can reduce the production cost are the recovery of sugars at high concentrations to minimize the energy spent during distillation and the recovery of lignin for value added co-products or heat and power production.

Achieving all the characteristics of an ideal pretreatment is difficult due to the inherent recalcitrance of lignocellulosics. There is a trade-off between achieving high sugar recovery and high digestibility at low enzyme loadings as mild pretreatment conditions that favour sugar recovery usually come at the expense of requiring higher enzyme loadings for complete conversion of the cellulose (Chandra et al., 2007; Gírio et al., 2010) (Figure 1).

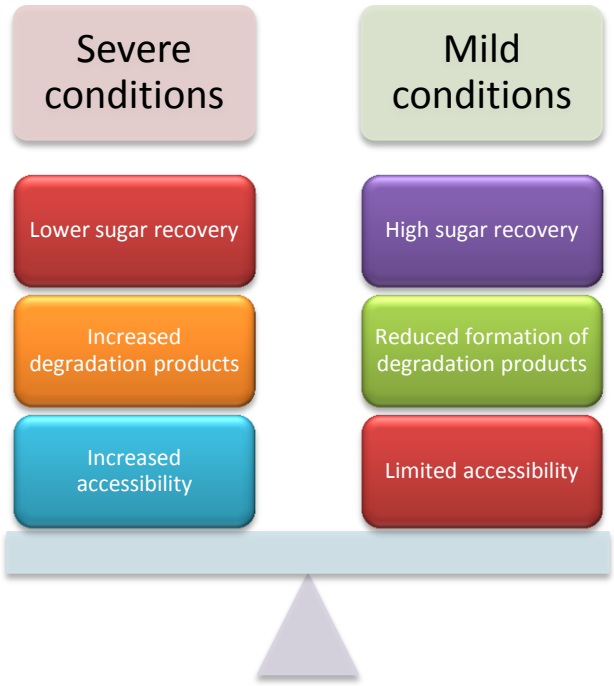

Figure 1 The trade-off between increasing accessibility to cellulases and increasing sugar recovery from the raw material.

1.4.2. Common pretreatment technologies for agricultural residues

There are various pretreatment technologies available and each has a different mode of action on the lignocellulosic substrate. The most common pretreatment technologies for agricultural residues are alkaline pretreatments, liquid hot water, dilute acid and steam pretreatment. Alkaline pretreatments include lime, ammonia recycle percolation (ARP) and ammonia fiber expansion (AFEX). Unlike acidic pretreatments, alkaline pretreatments do not degrade and only solubilize a small amount of the hemicellulose component. Although this may represent an advantage, high enzyme dosages or supplementation with xylanases is required to hydrolyze the hemicellulose and cellulose in the water insoluble fraction after pretreatment.

Lime pretreatment is one of the cheapest alkali pretreatments due to the low cost of lime and the ease of recovery. Its use has been reported for switchgrass, bagasse,

wheat straw and poplar (Chang & Holtzapple, 2000). The pretreatment can be performed at low temperatures but the reactions can take hours or days to complete and it also requires a reduction in particle size to 10 mm (Mosier et al., 2005a). Ammonia recycle percolation (ARP) removes up to 70% of the lignin and solubilizes 50% of the hemicellulose to produce a highly digestible substrate (Volynets & Dahman, 2010). Although the degree of delignification is quite high and selective, enzyme loadings of 40 FPU/g cellulose were required to achieve 90% conversion in wheat straw (Han et al., 2009), but better results have been reported for corn stover, where 15 FPU/ g cellulose resulted in a release of 85-90% of glucose in 72 hr. As with lime pretreatment, particle size reduction is also required (Han et al., 2009).

Ammonia fiber expansion is similar to steam pretreatment in that it uses a rapid decompression to break apart the material, but instead of an acid catalyst, it uses liquid ammonia. This process has been demonstrated to work well on several types of agricultural residues such as wheat straw (Mes-Hartree et al., 1988), corn stover, barley straw (Sun & Cheng, 2002), but it is not as effective for hardwoods or softwoods. Typical conditions are 1:1 ratio of liquid ammonia to biomass, 90°C and 30 minutes. The high dosage of ammonia makes it necessary to recover and recycle the ammonia in order to reduce costs (Sun & Cheng, 2002). One advantage of AFEX pretreatment is that no washing is required because no inhibitors are produced (Mes-Hartree et al., 1988). Particle size reduction is not a requirement and any residual ammonia can be a source of nitrogen for the yeast during fermentation (Mes-Hartree et al., 1988; Sun & Cheng, 2002). Since hemicellulose is not solubilized after pretreatment, an enzyme preparation

with hemicellulase activity is required to obtain high hydrolysis yields on agricultural residues (Elander et al., 2009).

Liquid hot water (LHW), also known as autohydrolysis, is a hydrothermal pretreatment that uses compressed hot water above saturation point to selectively solubilize the hemicellulose. This is accomplished by the autoionization of water and the acetic acid content of hemicellulose, which produces hydronium ions and results in an autohydrolysis reaction of the hemicellulose (Gírio et al., 2010). The remaining water insoluble stream consists mostly of cellulose and lignin and there is little sugar degradation products formed due to the mild pH conditions (Carvalheiro et al., 2009). Hydrothermal pretreatments can use a combination of long residence times and low temperatures (150°C) or short residence times and high temperatures (230°C) depending on the biomass type (Gírio et al., 2010). The liquid to solid ratio can also vary between 2 and 10 (w/w), but most reports in the literature use a ratio of 10:1 (Gírio et al., 2010).

Hydrothermal pretreatment such as liquid hot water (LHW) has the advantage that it does not require chemicals, which reduces the capital cost since there are no chemical recovery issues or problems with corrosion. Removal of precipitates is not required since there are no neutralization reactions (Mosier et al., 2005b). However, this process uses a high liquid to solid ratio, which requires a high energy usage for heating (Petersen et al., 2009; Volynets & Dahman, 2010) and also results in dilute sugar streams. The percent of solids that can be used in this process depends on the reactor, but it has been observed that as solid concentration increases the recovery efficiency of xylan decreases (Laser et al., 2002). This could be because high solid concentrations increase the organic acid concentration in the reaction, thus increasing the autohydrolysis reactions (Laser et al.,

2002). Since the pretreatment conditions are mild, the hemicellulose that is recovered in the soluble stream is mostly in oligomeric form (Petersen et al., 2009). This is beneficial because formation of degradation products is decreased, but it is also a challenge because the oligomers will require further processing to break down into monomers to be used for fermentation.

Dilute acid uses H_2SO_4 as an acid catalyst at low concentrations (0.5-1% w/w) to produce a substrate that is highly susceptible to enzymatic hydrolysis due to its ability to solubilize the hemicellulose component. Reaction temperatures can range between 140-200°C, which will depend on the reaction time which ranges between 2-30 minutes. High xylose recoveries of 77% and 74% have been reported for corn stover (Tucker et al., 2003) and wheat straw (Saha et al., 2005), respectively. Dilute acid can be costly since the particle size of the biomass needs to be reduced in an energy-intensive process prior to pretreatment. The dilute acid pretreated slurry must also be neutralized prior to fermentation due to its acidity (Mosier et al., 2005a; Talebnia et al., 2010).

Dilute acid has the advantage that it produces a highly digestible substrate while recovering most of the hemicellulose in the water soluble fraction. However, the optimum pretreatment conditions for hemicellulose and cellulose recovery are different which makes the recovery of both components, without producing degradation compounds, a challenge (Talebnia et al., 2010). Some studies have tried a two-step dilute acid pretreatment to increase the recovery of cellulose and hemicellulose while minimizing the formation of degradation products (Bosch et al., 2010; Saha et al., 2005), but this increases the capital costs and energy use since additional equipment is required and the biomass might need to be washed or impregnated again between steps (Bosch et

al., 2010; Wingren et al., 2004). Lignin removal during dilute acid pretreatment is ineffective. It has been observed that dilute acid pretreatment of corn stover stem rinds caused the formation of lignin droplets that could redeposit on the surface of the cell walls, thus decreasing the enzymatic hydrolysis yields (Selig et al., 2007). Unlike alkaline pretreatments that do not degrade cellulose, dilute acid can result in cellulose degradation.

The Biomass Refining Consortium for Applied Fundamentals and Innovation (CAFI) compared the performance of several pretreatment technologies including AFEX, ammonia recycle percolation, lime, dilute acid and steam pretreatment on the same source of corn stover. AFEX and SO_2-catalyzed steam pretreatment were identified as the most favorable pretreatment methods due to their high sugar recovery after pretreatment and enzymatic hydrolysis (Elander et al., 2009). However, steam pretreatment has additional advantages since it has a low consumption of chemicals and uses short residence times to produce a digestible substrate. In addition, steam pretreatment is one of the few processes used in pilot and commercial scale plants that is applicable to softwoods, hardwoods and agricultural residues (Chandra et al., 2007). Unlike AFEX, SO_2-catalyzed steam pretreatment solubilizes the hemicellulose, which reduces the need for additional enzymes to remove the hemicellulose from the solid fraction (Eggeman & Elander, 2005). Particle size reduction is also not necessary, which reduces the energy requirements of the process (Sun & Cheng, 2002).

Among the acid pretreatments, steam pretreatment can produce the highest solid concentrations after pretreatment (Elander et al., 2009; Laser et al., 2002). This process was developed in the 1930s and it was later proposed as an alternative to

chemithermomechanical (CTMP) and chemimechanical (CMP) pulping process due to the lower energy requirements in refining and the high solid yields of up to 75% (Kokta & Ahmed, 1998).

During steam pretreatment the biomass is subjected to high pressurized steam for a short period of time and the pressure is released quickly (Kokta & Ahmed, 1998). Steam pretreatment efficiency is influenced by the time, temperature, particle size, moisture content and type of the biomass. The fractionation of lignocellulosic substrates can be defined mathematically using the severity factor or combined severity factor. The severity factor takes into account the time and temperature whereas the combined severity factor also takes into account the concentration of acid catalyst used. The severity factor was introduced to predict the solubilisation of xylan during hydrothermal pretreatment such as steam pretreatment without the addition of an acid catalyst (Overend & Chornet, 1987). The severity factor is defined as R_o=t*e^ [(T-100)/14.75], where t represents time in minutes and T represents temperature in degrees Celsius.

Steam pretreatment of agricultural residues has been performed without a catalyst or with catalysts such as H_2SO_4 or SO_2. Autohydrolysis is essentially steam pretreatment with no external application of chemicals. Similar to liquid hot water pretreatment, this process relies on the acetyl content of the biomass to cleave the hemicellulose and lignin linkages and thus increase the available surface area of the substrate. The advantage of autohydrolysis is that it does not use chemicals so there are no issues with equipment corrosion or chemical recycling. However, the recovery of hemicellulose is reduced when compared to steam explosion treatment with an acid catalyst (Ballesteros et al., 2006; Chandra et al., 2007; Horn et al., 2011; Sassner et al., 2005).

The use of acid catalysts like H_2SO_4 or SO_2 increases hemicellulose recovery because it solubilizes the hemicellulose faster and it also reduces the temperature and reaction times required to produce a hydrolysable substrate (Chandra et al., 2007; Sassner et al., 2005). Depending on the conditions and acid concentration used, the acid catalyst can also increase the concentration of monomers in the liquid stream, whereas with autohydrolysis reactions there may be a higher concentration of oligomeric sugars. Recovering the sugars in monomeric form is desirable because they can be used directly for fermentation. Since steam pretreatment of agricultural residues is done for short periods of time (5-10 minutes), there is a lower amount of water usage when compared to dilute acid or hydrothermal pretreatments. This could possibly result in an increase in the sugar concentration, particularly of the solubilized hemicellulose fraction.

Steam pretreatment imparts changes to the substrate similar to dilute acid and liquid hot water. For example, the lignin usually remains in the water insoluble fraction and several studies have reported the presence of lignin droplets on the surface of the cell walls (Donaldson et al., 1988; Hemmingson, 1986; Toussaint et al., 1991). It also seems that the formation of lignin droplets might be greater in steam pretreated substrates (Kristensen et al., 2008). It is hypothesized that during steam pretreatment, the cleavage of β-O-4 bonds lead to the depolymerization of lignin. As the severity increases, repolymerization reactions become predominant and lead to an increase in the molecular size and heterogeneity of lignin (Li et al., 2007). Pseudo-lignin, which is the repolymerization of degradation products and polysaccharides, also increase with pretreatment severity. Other substrate changes include cleavage of the lignin-carbohydrate complex (LCC) and a reduction in the cellulose degree of polymerization.

An increase in the carboxylic acid groups has also been reported (Li et al., 2009; Li et al., 2007). All these changes contribute to an increase in accessible surface area, which enhances the susceptibility of the substrate to enzymatic hydrolysis.

Although steam pretreatment is an effective technology for the bioconversion of agricultural residues, some challenges remain to be addressed.

1.5.Challenges of steam pretreatment of agricultural residues

1.5.1. Biomass utilization and sugar recovery

Technoeconomic studies have identified several bottlenecks in the bioconversion process that have guided the research efforts to reduce the cost of ethanol production (Balat et al., 2008; Galbe et al., 2007; Huang et al., 2009; Piccolo & Bezzo, 2009). There are several possibilities in the configuration of the process to reduce the cost of lignocellulosic ethanol such as coupling lignocellulosic ethanol with existing first-generation ethanol plants, integration to pulp and paper mills or co-location with a combined heat and power plant. These possibilities may result in a reduction of the ethanol cost, but it is undeniable that the process itself is capital and energy intensive and further improvement is necessary.

Ethanol cost is sensitive to feedstock cost, enzyme cost and conversion yields. Feedstock cost can make up to 40% of the total production cost. In the future, this may be reduced by switching to lower value residues that have little competition in the market (Balat et al., 2008). Regardless of the source of biomass, efficient utilization of all the lignocellulosic components will be necessary to lower the cost of ethanol. As for the enzyme cost, we have seen great improvements over the last 20 years with respect to

enzyme efficiency and a reduction in the cost of enzyme production (Merino & Cherry, 2007). In most technoeconomic analyses the enzyme cost is quite variable, but it is estimated that it can make up to 11% of the cost (Aden & Foust, 2009).

Even if further technological improvements result in better and cheaper enzymes, ethanol costs cannot be lowered if the bioconversion process cannot generate high conversion yields in a cost effective manner. Crucial issues that must be addressed to decrease the cost of production are: increasing the recovery of all sugars that can be easily hydrolyzed at low enzyme loadings and increasing the sugar concentration of the liquid streams for fermentation, which can result in a more concentrated ethanol stream after fermentation (Eggeman & Elander, 2005; Galbe et al., 2007; Gnansounou, 2010; Sassner et al., 2008). In the case of agricultural residues like wheat straw, the recovery of the hemicellulose component without sacrificing the efficiency of enzymatic hydrolysis is the most crucial parameter that will determine the efficiency of the pretreatment process.

1.5.2. Pretreatment configuration and gross handling of biomass

The preparation of the biomass prior to steam pretreatment and the method used to load the biomass into the steam pretreatment digester can have a significant effect on sugar recovery. Steam pretreatment uses steam and high temperatures (170-210°C) to fractionate the biomass followed by a quick pressure release. The quick pressure release during steam pretreatment results in an explosion of the biomass, and it has been a matter of debate as to whether or not this explosive decompression aids in the ease of hydrolysis of the substrate by increasing accessibility of the substrate to the enzymes (Brownell & Saddler, 1987). Another study investigated the effects of chip size, acid, moisture content and pressure drop on the pretreatment and enzymatic hydrolysis on aspenwood chips. It

was possible to manipulate the pressure release in order to avoid the explosive decompression and concluded that the explosion effect was not required as the enzymatic hydrolysis yields between the two treatments were comparable. However, this work only analyzed the sugar recovery of the pentose and hexose fraction of the pretreatment done with the explosive decompression, with and without SO_2 as an acid catalyst (Brownell et al., 1986).

This earlier work by Brownell and Saddler (1987) also confirmed that the explosion treatment had no significant effect on the ease of hydrolysis of the substrates. In this case, there seemed to be a greater destruction of the pentosan fraction in the treatment with no explosion. However, it remains unclear whether the explosion pretreatment affected sugar recovery because the unexploded substrates were pretreated at 190°C for longer residence times (12-100 min) while the exploded substrates were pretreated at 210°C for shorter residence times (0.33-4 minutes) (Table 2). It should be noted that the treatments at 240°C correspond to a pressure in the steam gun of 514 psi compared to only 194 psi at 190°C which could possibly affect the results when comparing the effects of explosion vs. "no-explosion" treatments. Thus, it is likely that the pentosan recovery was affected by the higher severity conditions but it is not possible to determine if the explosion vs. no explosion treatment had an additional effect on the sugar recovery.

Table 2 Severity factor (log R_o) of the conditions applied by Brownell and Saddler (1987) on steam pretreatment of aspenwood chips with and without explosion treatment

Treatment	Temperature (°C)	Time (min)	log (R_o)
No explosion	190	12	3.73
	190	25	4.05
	190	50	4.35
	190	100	4.65
Explosion	240	0.33	3.64
	240	0.67	3.95
	240	1.33	4.25
	240	2	4.42
	240	3	4.60
	240	4	4.72

The moisture content of the biomass prior to pretreatment has also been demonstrated to have an effect on the sugar recovery as well as the ease of hydrolysis of the substrates. It seems that the higher moisture content can increase the solids recovery and hemicellulose recovery by decreasing the relative severity of the pretreatment (Cullis et al., 2004). Others have shown that pre-soaking switchgrass and sugarcane bagasse prior to pretreatment increased the sugar recovery after pretreatment and the ease of enzymatic hydrolysis of the resulting substrates. In this case it was hypothesized that the addition of water increased the permeability and reactivity of the SO_2, which was the acid catalyst used in this study (Ewanick & Bura, 2011).

1.5.3. Choice of acid catalyst for pretreatment

The choice of catalyst has also been shown to play a role in affecting the ease of biomass pretreatment. In most cases, steam pretreatment of agricultural residues has been carried out employing either H_2SO_4 or SO_2 as an acid catalyst. There are few studies that have compared the hydrolysis and fermentation of steam pretreated substrates using SO_2 or H_2SO_4 as an acid catalyst (Eklund et al., 1995; Martín et al., 2002; Tengborg et al.,

1998). It seems that evaluating the efficiency of the impregnation agents varies with the substrate, the pretreatment conditions and the amount of catalyst used. For example, Martín et al. (2002) concluded that SO_2 was a better catalyst for sugarcane bagasse because it resulted in higher glucose and xylose yields (after pretreatment and enzymatic hydrolysis) and increased fermentability compared to H_2SO_4 pretreated substrates. However, this study compared SO_2 and H_2SO_4 at 1.1% and 1.0% (w/w) loadings, respectively, and this may have been an excessive amount of H_2SO_4 considering that the pretreatment temperature and time were 205°C and 10 min (Martín et al., 2002). The molar mass of H_2SO_4 (98.08 g/mol) is higher than that of SO_2 (64.06 g/mol), while H_2SO_3 has pKa values of 1.857 and 7.172 and H_2SO_4 has pKa values of -3 and 1.99. Therefore, comparing these catalysts at the same loading will inevitably result in a higher severity and higher degradation of sugars for the H_2SO_4-pretreated substrates.

H_2SO_4 is added directly to the biomass as an acid, while SO_2 must undergo further chemical reactions to convert to acid when loaded on the biomass. The amount of SO_2 that converts to H_2SO_4 during steam pretreatment remains unclear since industrial production of H_2SO_4 from SO_2 requires extremely high temperature (400-450°C) and a vanadium oxide catalyst (Dunn et al., 1999). Eklund et al. (1995) found that the use of H_2SO_4 as a catalyst resulted in the highest xylose yields for steam pretreated willow, but SO_2 gave the highest glucose yields after pretreatment and enzymatic hydrolysis. Tengborg et al. (1998) also compared both catalysts on a mass basis and concluded that SO_2 generally gave similar sugar recovery after pretreatment and enzymatic hydrolysis of spruce when compared to H_2SO_4-pretreated substrates, but the fermentability was higher in SO_2-pretreated substrates because there were less inhibitors formed during the process.

It was also noted that the pattern of sugar release differs for both catalysts. SO_2-pretreated substrates had a higher glucose release during enzymatic hydrolysis whereas H_2SO_4-pretreated substrates had a higher sugar release after pretreatment likely due to the hydrolytic power of the two protons in the H_2SO_4 added directly to the pretreatment reaction. The study by Tengborg et al. (1998) compared the steam pretreatment, enzymatic hydrolysis and fermentation of softwood substrates preatreated with SO_2 or H_2SO_4. The comparison was based on two different studies (Stenberg et al., 1998; Tengborg et al., 1998), and it was assumed that the results were comparable since the substrate, equipment and pretreatment method were the same. However, the pretreatment conditions differed for each acid catalyst. The pretreatment with SO_2 was done using a concentration of 1-6% (w/w), 190-230°C and 2-15 min whereas the pretreatment with H_2SO_4 was done using a concentration of 0.5-4.4% (w/w), 180-190°C and 1-20 min. The enzymatic hydrolysis was also performed for 96 hours with 0.2 g Celluclast plus 0.05 g beta-glucosidase per g of dry substrate. At such enzyme loadings, the SO_2-substrates had higher yields than the H_2SO_4 substrates, but this amount of enzyme used could be considered to be high.

Sulfuric acid could be a more effective catalyst than SO_2 at equal doses because H_2SO_4 is normally added at lower mass and molar loadings during pretreatment compared to SO_2 (Linde et al., 2006; Linde et al., 2006; Sassner et al., 2005; Sassner, Mårtensson et al., 2008). This could potentially lead to cost savings since less catalyst is needed to achieve a similar level of sugar release during pretreatment and enzymatic hydrolysis. The handling and control of the dosage of H_2SO_4 is also easier when compared to SO_2 as this gas is adsorbed to various degrees depending on the biomass

substrate. For example, in our laboratory we have shown that moist biomass (50% solids) can adsorb SO_2 directly and the SO_2 loadings can be controlled more effectively than loading the SO_2 on dry biomass and leaving overnight as has also been reported in the literature (De Bari et al., 2007). As well as better dosage control with H_2SO_4, if more sugars are released in the pretreatment step with H_2SO_4, it could be an advantage since we could potentially reduce the amount of cellulolytic enzymes and accessory enzymes such as xylanase required during hydrolysis (Hu et al., 2011). One potential advantage of SO_2 over H_2SO_4 may be that the gas phase addition allows for an even distribution of the catalysts. However, pre-wetting the biomass prior to pretreatment with a solution containing the H_2SO_4 may aid in the distribution of the acid catalyst. Pre-wetting is usually performed during dilute acid and steam pretreatment with H_2SO_4, but the material is usually soaked with exorbitant amounts of water, as high as 1:10 solids to liquids ratio (Ballesteros et al., 2006). In our study, we will minimize the pre-wetting of the biomass to decrease the dilution of the liquid fraction from steam pretreatment.

1.5.4. Current research on steam pretreatment of agricultural residues

Although the type of catalyst and biomass handling play a crucial role in the ability to recover sugars after pretreatment and hydrolysis, the combination of time, temperature and acid catalyst loading have been the typical pretreatment parameters that have been used to maximize sugar recovery and to obtain a substrate susceptible to enzymatic hydrolysis. In order to increase the recovery of sugars from agricultural residues, hemicellulose recovery must be maximized since it is the most sensitive component to degradation and it can compose between 25-35% of the dry weight of the straw (Kabel et al., 2007). The challenge is to increase sugar recovery without reducing

the efficiency of downstream processes of hydrolysis and fermentation. During steam pretreatment, the hemicellulose is solubilized while there is minimal solubilisation of the cellulose and lignin (Ramos, 2003). If the severity is too low (low temperature or short residence time), sugar recovery can be maximized, but breaking down this substrate into monomeric sugars will be expensive since it will require excessive amounts of enzymes. If the severity is too high (high temperature or long residence time), most of the acid-labile hemicellulose fraction will dissolve and partially degrade into toxic compounds that inhibit the yeast used during fermentation and result in sugar loss (Ramos, 2003).

There are several reports that have evaluated and determined optimal steam pretreatment conditions of agricultural residues with or without a catalyst. However, it is difficult to determine if the conditions reported could be applied in large scale operations for the following reasons:

- In most cases when the pretreatment is done with sulfuric acid, impregnation is done at a 10:1 (Ballesteros et al., 2006) up to a 20:1 liquid to solids ratio. Impregnation time can be anywhere between 3 hours (Tucker et al., 2003) up to 12 hours (Ballesteros et al., 2006) and it is usually done at 45-80°C (Ballesteros et al., 2006; Tucker et al., 2003). Studies that have evaluated the optimal conditions for steam pretreatment of wheat straw do not take into account that the efficiency of the pretreatment has been increased at the expense of increasing energy and water usage by impregnating for several hours at moderate temperatures.
- Extensive washing of the substrate is usually done after pretreatment (Ballesteros et al., 2006) and in some cases the material is washed before and after pretreatment (Tucker et al., 2003). It is common to find reports that use a ratio of 20:1 water to

solids to wash the substrates before pretreatment. This improves the efficiency of the enzymatic hydrolysis because toxic products and oligomers are removed during washing, but it is done at the expense of increasing water use and diluting the sugar streams.

- Studies that assess the susceptibility of a substrate to pretreatment and enzymatic hydrolysis often report enzymatic hydrolysis results done at unrealistic conditions. For example, hydrolysis is performed at very high enzyme loadings or at a low solids loading. In other cases the hydrolysis is performed for 96 hours. Thus, it can be hypothesized that the optimal condition would differ greatly if the enzymatic hydrolysis experiments were done at high solids loading, in shorter time periods and with moderately low enzyme loadings.

In summary, if a pretreatment technology is to meet the conditions required for commercialization of ethanol we must report yields based on raw material, impregnate for short periods of time at room temperature while reducing the water usage and also minimize the washing of the substrate after pretreatment. In addition, enzymatic hydrolysis should be performed in unwashed substrates at high consistency to assess the real potential of the pretreatment technology and the biomass as feedstock for ethanol production (Eggeman & Elander, 2005).

The second main challenge in the bioconversion of agricultural residues is to achieve high sugar concentrations in the water soluble streams after steam pretreatment and hydrolysis. This is a major component of the process cost since the sugar streams produced are too dilute and result in an ethanol stream that is 4% (w/v). As a result, energy intensive processes such as distillation and evaporation must be employed to

increase the ethanol concentration after fermentation (Galbe et al., 2007). Achieving an ethanol concentration like that of the starch ethanol industry of 8% would already be a great improvement in the process economics. The concentration of the liquid stream after enzymatic hydrolysis can be increased by avoiding washing the substrate after pretreatment, hydrolyzing the solid fraction at high-solids concentration or by combining the sugars from the solid and liquid fraction to perform a whole slurry hydrolysis.

During pretreatment, most studies impregnate the biomass at a high liquid to solids ratio to improve the recovery of sugars and the digestibility of the substrate. The water use is so high that it may require an extra step in which the slurry is filtered and pressed to increase the concentration of solids prior to enzymatic hydrolysis. Otherwise, the sugar streams would become too dilute by the end of pretreatment and hydrolysis. Another way of decreasing the liquid to solids ratio is to increase the amount of solids processed during pretreatment. Increasing the solid loading has been shown to decrease the recovery of xylose and it also results in a less efficient pretreatment, as demonstrated by the lower hydrolysis yields (Tucker et al., 2003). Most laboratory scale studies also wash the substrates with water prior to enzymatic hydrolysis. Several studies have shown that enzymatic hydrolysis yields increase after washing the substrate (Alfani et al., 2000; Carrasco et al., 2010; Merino & Cherry, 2007; Vlasenko et al., 1997). Despite the fact that washing is beneficial for enzymatic hydrolysis, this is not a feasible strategy for a large scale facility due to the energy required for heating (Petersen et al., 2009).

Hydrolysis at high solids concentration is defined by the little amount of free water available (Kristensen, Felby, & Jørgensen, 2009). The advantages of high solids hydrolysis is an increase in the system capacity, less energy is required for heating and

cooling of the slurry, and less waste water production (Kristensen, Felby, & Jorgensen, 2009). However, hydrolysis at high solids concentrations is problematic due to the high viscosity of the material that decreases the efficiency of mixing (Jørgensen et al., 2007). Enzymatic hydrolysis yields decrease with an increase in solids concentration and this is likely due to several factors such as the stirring mechanism, which may result in enzyme deactivation and denaturing (Mohagheghi et al., 1992) and end-product inhibition due to an increase in the concentration of sugars (Hodge et al., 2008).

Table 3 Summary of literature on steam pretreatment of agricultural residues

Reference	Feedstock	Catalyst	Best Conditions	Yields	Biomass handling before pretreatment	Washing	Enzymatic hydrolysis conditions
(Ballesteros et al., 2006)	Wheat Straw	H_2SO_4	180°C, 10 min, 0.9% H_2SO_4	70% theoretical (ethanol)	Presoaked 18h, 45°C, 1:10 solid to liquid ratio	Yes	10% solids, 72 h, 50°C, 15 FPU/g dry WIS Celluclast, 12.6 IU.g dry WIS b-glucosidase
(Tucker et al., 2003)	Corn Stover	H_2SO_4	190°C, 90-110 s, 1% H_2SO_4	>90% theoretical (soluble xylose), 85% (ethanol) based on cellulose from WIF	Washed 20:1 liquid to solid ratio for 45 min followed by impregnation for 3-4 hr at 50°C	Washed for 30 minutes with hot tap water and filtered 5 times with 40°C tap water and once with 60°C deionized water	Simultaneous Saccharification and Fermentation (SSF) (15 FPU/g glucan)
(Linde et al., 2008)	Wheat Straw	H_2SO_4	190°C, 10 min, 0.2% H_2SO_4	102% glucose, 96% xylose (theoretical), 67% ethanol (theoretical)	Impregnation 20:1 liquid to solid ratio	No washing prior to SSF	96 h, 2% solids, 40°C, 15FPU/g solid (approx. 46 FPU/g glucan) SSF conditions 14 FPU/g WIS
(Carrasco et al., 2010)	Sugarcane bagasse	SO_2	190°C, 5 min, 2% SO_2	87.3% (theoretical for Glu, Xyl and Ara)	30 min, room temperature (no soaking but biomass water content 75-77%)	Yes	2% solids, 40°C, 72h, 2.32 g Celluclast and 0.52 g b-glucosidase per 10 g of solids
(Horn et al., 2011)	Wheat Straw	No catalyst	210°C, 10 min	90% glucose (*at the expense of xylose degradation)	Not specified	Not specified	5% solids, 20 FPU/g solids (approx. 51 FPU/g glucan)

Reference	Feedstock	Catalyst	Best Conditions	Yields	Biomass handling before pretreatment	Washing	Enzymatic hydrolysis conditions
(Palmarola-Adrados et al., 2004)	Starch free wheat fibers	No catalyst	190°C, 10 min	74% theoretical (Ara. Glu, Xyl)	Not specified	Not specified	5% solids, 72 h, 50°C, Celluclast and Ultraflow (hemicellulose-degrading enzymes) at a 1:1 ratio, 2g enzyme/100 g slurry
(Petersen et al., 2009)	Wheat Straw	No catalyst (hydrothermal)	195°C, 6-12 min	70% hemicellulose, 94% cellulose (89% hydrolysis yield without xylanases) Not based on theoretical	Soaked at 80°C for 5-10 min	Yes	Prehydrolysis and SSF Prehydrolysis: 10% solids, 50°C, 24 hr, 15 FPU/g DM Celluclast, Novozym 188 (5:1 w/w), Multifect Xylanase (0.1 g/ g DM) SSF: 15 FPU/g DM
(Nidetzky et al., 1993)	Wheat Straw	No catalyst	210°C, 10 min	93% glucose (theoretical), no mention of xylose recovery	Not specified	Yes (hot water)	2% solids, 100 hr, 100 FPU/g substrate

1.6.Research approach and objectives

The main objective of this thesis was to assess pretreatment strategies to maximize the xylose solubilization and recovery while increasing accessibility to the water insoluble cellulosic component such that much lower enzyme loadings could be used at high substrate concentrations over a short period of time to maximize overall sugar recovery from wheat straw. Although many studies have shown enzymatic hydrolysis yields and sugar recoveries after pretreatment and enzymatic hydrolysis, there are only a few that have assessed the total amount of sugars solubilized during the pretreatment and enzymatic hydrolysis processes to try to quantify the effectiveness of the pretreatment conditions. Many studies equate a high sugar recovery in the combined water insoluble/soluble fractions as a so-called effective pretreatment, but high enzyme loadings are typically used to hydrolyze the resulting substrates and the actual amount of total soluble sugars is not clearly quantified.

One of the goals in the work described in this thesis was to increase the overall sugar recovery by assessing different modes of biomass handling while comparing two acid catalysts (SO_2 and H_2SO_4) commonly used for steam pretreatment of agricultural residues. The pretreatment conditions were subsequently altered using a statistical design approach to try to determine the "optimum compromise" between good, water soluble, hemicellulose sugar recovery while enhancing the ease of enzymatic hydrolysis of the water insoluble cellulosic fraction. It was hoped that this work would demonstrate some mechanistic differences between SO_2 and H_2SO_4. We hoped that this work would provide some insights with regard to the compromise between pretreatment and enzymatic hydrolysis. We anticipated that some sugar loss in the hemicellulose fraction would be

offset by the better accessibility to the cellulosic fraction such that lower protein/enzyme loadings could be used to hydrolyze the cellulosic fraction at high substrate concentrations over a short period of time. Some of the issues that will be addressed in this thesis in order to obtain good sugar recovery at high substrate concentrations after steam pretreatment of wheat straw are:

- Determine if differences in initial, gross biomass characteristics and handling will influence the effectiveness of steam pretreatment. Although there has been some work on how the handling of woody biomass affects the efficiency of pretreatment, there have only been limited studies on potential improvements to biomass handling that could increase the efficiency of pretreatment in agricultural residues. The work described in the thesis looked at the influence of biomass moisture content while determining if there is a need for explosive decompression during steam pretreatment. The effect on enzymatic hydrolysis and overall sugar recovery of these two parameters was studied simultaneously. It was expected that increasing the moisture content would decrease the relative severity of the pretreatment and thus result in a higher xylose recovery. It was thought that the explosion treatment might result in higher yields since the method in which the biomass is loaded into the steam gun allows for a more homogeneous treatment. Conversely loading the substrates in steel baskets that restrain the explosive decompression could result in a higher degradation of sugars and a more heterogeneous pretreatment since the straw biomass in the outermost part of the steel basket might be exposed to a greater amount of steam than the inner part of the basket. The explosive decompression treatment might also result in a larger amount of the water soluble stream at the conclusion of steam pretreatment

since the substrate requires washing with additional water to quantitatively transfer it from the vessel for subsequent processing. We also wanted to determine whether it was possible to recover the sugars at a higher concentration in the water soluble fraction after pretreating in the steel basket since this method did not require washing to transfer the biomass from the reaction vessel and thus only results in a single water soluble stream.

- The SO_2 and H_2SO_4 catalysts were also compared for their ability to increase sugar recovery and their effect on the ease of enzymatic hydrolysis of the cellulosic fraction. Previous studies have compared these catalysts based on equal loadings or at different pretreatment conditions. Since the molecular weight of H_2SO_4 is higher than that of SO_2 this type of comparison may be not be equitable. It may be possible to use much lower loadings of H_2SO_4 to have a comparable effect to SO_2 during steam pretreatment. Therefore, in this study, we applied different loadings of SO_2 and H_2SO_4 in order to test the hypothesis that H_2SO_4 could result in comparable yields at lower loadings at the same pretreatment conditions.
- After determining the best combination of biomass handling and acid catalyst, we used a Box-Behnken statistical design to evaluate the different combination of process conditions. The overall experiment was conducted with the working hypothesis that steam pretreatment is a compromise whereby increasing the severity will improve the ease of enzymatic hydrolysis of the water insoluble cellulosic fraction at the expense hemicellulose sugar degradation in the water soluble fraction. We anticipate that a substrate that has been pretreated at a sufficient severity to allow for good cellulose hydrolysis at low enzyme loadings after hydrolysis will likely

undergo some hemicellulose degradation but result in overall higher yields of recoverable glucose and xylose. Our approach also evaluated the efficiency of pretreatment and hydrolysis in more realistic conditions by conducting the enzymatic hydrolysis on unwashed substrates at relatively low enzyme loadings and for a shorter reaction time of 48 hours. The pretreatment was performed on pre-wetted substrates with an acid catalyst, but the volume of water was reduced so that the overall water usage during the process was minimized. The total soluble sugar yield (TSSY) was assessed, as this reflects the total amount of sugars that can be provided to the downstream fermentative process.

- The final work in this thesis was performed to gain insights into the alteration of biomass properties when sulfur dioxide is used as a catalyst during steam pretreatment. Although H_2SO_4 and SO_2 were compared during the earlier work reported in the thesis, SO_2 has tended to be the acid catalyst of choice for many groups working on pretreatment. Unlike H_2SO_4, SO_2 has the potential to sulfonate the lignin component of biomass which is a property exploited during sulfite pulping of biomass for pulp production (Sjöström, 1993). It has been suggested that the sulfonation of lignin increases hydrophilicity and swelling of the substrate thereby reducing hydrophobic interactions between cellulase enzymes and lignin and improving cellulose accessibility (Del Rio et al., 2011). It is possible that the abundant ash component (5-10% ash content) of agricultural residues can provide adequate pH buffering capacity to facilitate sulfonation of the lignin component similar to a sulfite pulping reaction. Previous studies had only studied the acetone soluble "subset" of the lignin component or only the liquid stream of stream after SO_2

catalyzed steam pretreatment to determine if sulfonation occurred during steam pretreatment. Thus the last chapter of the thesis looked at the mechanisms behind why SO_2 catalyzed steam pretreatment appeared to be a more effective pretreatment.

2. Materials and Methods

2.1. Chemical composition of raw material

The wheat straw was cultivated in Indian Head, Saskatoon (2010 harvest) and it was kindly provided by Agriculture Canada. The corn stover was kindly provided by NREL (National Renewable Energy Laboratory, USA). Prior to the chemical analysis of the raw material, wheat straw and corn stover were subjected to a two-step extraction procedure using water and ethanol (Sluiter et al., 2005). The carbohydrates extracted in the water and ethanol extraction steps were measured in a Dionex (Sunnyvale, CA) HPLC (ICS-3000) equipped with an AS 50 auto sampler, ED50 electrochemical detector, GP 50 gradient pump and anion exchange column (Dionex CarboPac PA1).

2.2. Chemical composition of water insoluble fraction (WIF) and water soluble fraction (WSF) after steam pretreatment

The chemical composition of the substrates' water insoluble fraction (WIF) was done following the procedure in the NREL Technical Report TP-510-42618 (Sluiter et al., 2008). The water soluble fraction (WSF) was measured by hydrolyzing the oligomeric sugars into monomeric sugars with 72% H_2SO_4 followed by HPLC analysis. Sugars from the WIF and WSF fractions were measured in a Dionex (Sunnyvale, CA) HPLC (ICS-3000) system, equipped with an ion exchange Carbopac PA-1 column (4 × 250 mm) equilibrated with 0.2 M NaOH and eluted with nanopure water at a flow rate of 1 mL min-1 (Dionex Corp.), an ED 50 electrochemical detector (gold electrode), AD 50 absorbance detector and autosampler (Dionex Corp., Sunnyvale, CA, U.S.). Sodium hydroxide (0.2 M) was added post-column (for detection) at a flow rate of 0.5 mL min^{-1}.

Prior to injection, samples were filtered through 0.2 μm HV filters (Millipore, MA, U.S.) and a volume of 20 μL was loaded. Analytical-grade standards: D-arabinose, D-galactose, D-glucose, D-xylose and D-mannose (Sigma) were used to quantify the concentration of sugars. In addition, L-fucose (Sigma) was used as an internal standard. The ash content of corn fibre was determined by ignition at 575°C, according to TAPPI standard T-211.

2.3.Pretreatment and enzymatic hydrolysis of corn stover

Steam pretreatment was conducted in a 2L StakeTech steam gun at 190°C, 3% SO_2, 5 min. Corn stover screened through a 2 mm mesh was used in this study as the pretreatment conditions for this substrate had been optimized previously (Bura et al., 2009). A schematic of the pretreatment and separation of the water soluble fraction (WSF) and water insoluble fraction (WIF) is shown in Figure 2. Two batches of 50 g (O.D.W.) were loaded directly in the steam gun to produce the explosion treatment. The "no explosion" treatment was achieved by loading the biomass inside a stainless steel wire mesh basket and they were later disintegrated for 5 minutes. The exploded substrates were washed with 1L of water to collect the substrate from the vessel. The biomass was also pretreated at different moisture contents. The initial moisture content of corn stover was 7.8% and prior to impregnation with SO_2 the substrates were wetted with 50 mL of distilled water to increase the moisture content to 50%. After the pretreatment, the water soluble (WSF) and insoluble (WIF) fractions were separated by vacuum filtration. The WIF of the unexploded substrates were subjected to further particle size reduction in a disintegrator for 5 minutes.

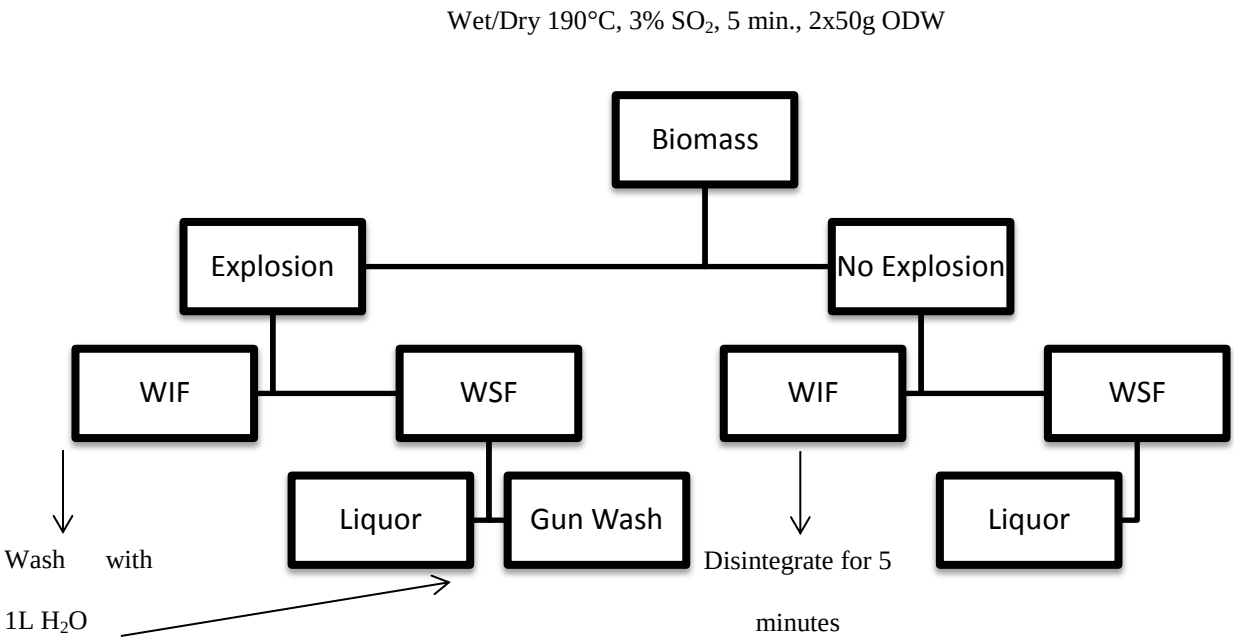

Figure 2 Procedure for steam pretreatment of corn stover to study the effects of explosion and moisture content on the sugar recovery and hydrolysis of the substrates.
WSF=water soluble fraction, WIF=water insoluble fraction

The second set of pretreatments involved a comparison of acid catalysts. Steam pretreatment was conducted in a 2L StakeTech steam gun at 190°C and 5 min. The following acid catalyst loadings were used: 3% SO_2, 0.75% H_2SO_4, 1.5% H_2SO_4. The acid catalyst loadings were based on values commonly used in the literature (Ballesteros et al., 2006; Bura et al., 2009). After pretreatment and characterization of the chemical composition of the substrates, batch hydrolysis was then conducted at 2% solids in 0.05 M sodium acetate buffer (pH=4.8), in a FINE PCR hybridization incubator Combi D-24, 50°C for 72 hours inside 2.5 mL microcentrifuge tubes. The final volume of the reaction was 1 mL. Cellic Ctec2 (Novozymes) was used in the enzymatic hydrolysis experiment at a loading of 10 mg protein/g glucan, which was equivalent to 4.8 FPU/g glucan. Protein content of the enzyme cocktail was determined using the ninhydrin method (209.8 mg

protein/mL) and enzyme activity was determined using the filter paper assay (102.4 FPU/mL) (Ghose, 1987).

2.4.Pretreatment and enzymatic hydrolysis of wheat straw

In order to determine the pretreatment conditions that would allow a high sugar recovery of sugars, a second set of wheat straw substrates were pretreated using conditions based on a Box Behnken design. The pretreatment conditions are described in detail in Table 9. All pretreatment conditions were done using 75 g (ODW) of wheat straw. Prior to pretreatment, the substrates were prewetted with 75 mL of distilled water in which the acid catalyst was added. The combined severity factor (CS) was calculated using the equation: $R'_o = [H+]*R_o$. R_o is the severity factor defined as $R_o=t*e^{[(T-100)/14.75]}$ where t represents time (minutes) and T represents temperature in Celsius (Overend & Chornet, 1987). The concentration of hydrogen ions was obtained by measuring the pH of the WSF and then calculating the antilog of the pH values. Statistical analysis was conducted using SAS 9.2.

Hydrolysis experiments were conducted at 2% and 10% solids. Cellic Ctec2 (Novozymes) was used in all the enzymatic hydrolysis experiments at a loading of 10 mg protein/g glucan, which was equivalent to 4.8 FPU/g glucan. Batch hydrolysis was conducted in triplicate at 2% solids in 0.05 M sodium acetate buffer (pH=4.8), in a FINE PCR hybridization incubator Combi D-24, 50°C for 48 hours inside 2.5 mL microcentrifuge tubes. The final volume of the reaction was 1 mL. Batch hydrolysis was conducted in duplicates at 10% solids in 0.05 M sodium acetate buffer (pH=4.8), 50°C for 48 hours at 150 rpm. The final volume of the reaction was 10 mL. An additional enzymatic hydrolysis experiment was conducted at 20 mg protein/g glucan (9.7 FPU/g

glucan). This enzyme loading was applied to all substrates at 10% solids in order to increase the total soluble sugar yield.

A hydrolysis experiment was conducted to compare the effects of washing on conditions 9, 15 and 12 as they correspond to the low, medium and high severity pretreatments of the Box Behnken design. Approximately 5 g wet weight of the substrates was washed in 500 mL of distilled water and filtered through a Buchner funnel. Moisture content was recalculated for the washed substrates and the batch hydrolysis was conducted in triplicate at 2% solids in 0.05 M sodium acetate buffer (pH=4.8), in a FINE PCR hybridization incubator Combi D-24, 50°C for 48 hours inside 2.5 mL microcentrifuge tubes. The final volume of the reaction was 1 mL.

The effect of xylanase supplementation at high solids hydrolysis was determined by hydrolyzing the substrate of condition 14, which corresponds to the medium severity substrate, at different cellulase to xylanase ratios. Three different combinations of cellulase and xylanase loadings were used: 4mg:16mg, 10mg:10mg and 16mg:4mg. Hydrolysis experiments were conducted in duplicate using a total protein loading of 20 mg/g glucan. Batch hydrolysis was conducted at 10% solids in 0.05 M sodium acetate buffer (pH=4.8), 50°C for 48 hours at 150 rpm. The final volume of the reaction was 10 mL. Cellic Ctec2 and Htec (Novozymes) were used for this experiment.

In the study of the effects of SO_2 on the substrate properties of wheat straw, steam pretreatment was conducted in a 2L StakeTech steam gun at 190°C and 10 minutes. The substrates were loaded in a stainless steel wire mesh basket to facilitate loading and unloading. Sulfur dioxide loadings ranged from 0, 1, 3, 5 and 10% (w/w) and were

applied to 50 g of substrate in sealable plastic bags. Prior to impregnation with SO_2, the samples were wetted with 200 mL of water. After the pretreatment, the entire slurry was disintegrated for 5 min and the water soluble (WSF) and insoluble (WIF) fractions were separated by vacuum filtration. The WIF fraction was washed with approximately 3L of water and vacuum filtered to a final moisture content of 76–82%. Only the solid fractions of pretreated materials were used for this study.

Batch hydrolysis was then conducted at 2% solids in 0.05 M sodium acetate buffer (pH=4.8), in a FINE PCR hybridization incubator Combi D-24, 50°C for 24 hours inside 2.5 mL microcentrifuge tubes. The final volume of the reaction was 1 mL. Two commercial preparations (both Novozymes, Franklinton, NC, USA) - a cellulase cocktail (Celluclast 1.5 L; protein content 130 mg/mL) derived from *Trichoderma reesei* and a β-glucosidase preparation (Novozym 188; protein content 233 mg/mL) derived from *Aspergillus niger* were used in the enzymatic hydrolysis experiments at a 1:2 cellulase to β-glucosidase ratio. Celluclast enzyme loading was based on 20 mg protein/g glucan, which was equivalent to 7.5 FPU/g glucan.

2.5.Analysis of hydrolysates

Glucose and xylose concentrations of all hydrolysis samples were analyzed in a Dionex (Sunnyvale, CA) HPLC (ICS-3000) equipped with an AS 50 auto sampler, ED 50 electrochemical detector, GP 50 gradient pump and anion exchange column (Dionex CarboPac PA1) equilibrated with 0.2 M NaOH and eluted with nanopure water at a flow rate of 1 mL min^{-1} (Dionex Corp.) Prior to injection, samples were filtered through 0.20 mm filters (Millipore, MA, U.S.) and a volume of 20 µL was loaded. Analytical-grade standards: D-glucose and D-xylose (Sigma) were used to quantify the concentration of sugars. In addition, L-fucose (Sigma) was used as an internal standard.

2.6.Substrate characterization of wheat straw substrates

Sulfonic and carboxylic group content, fiber length, cellulose crystallinity, cellulose degree of polymerization, available surface area and swelling were measured in the substrates pretreated with 0-10% SO_2. Sulfonic and carboxylic acid group content was measured by the conductometric titration method (Katz et al., 1984). In brief, each sample was measured in duplicate. About 0.5 g (ODW) were weighed and soaked in 0.1 M HCl overnight. The samples were then washed and filtered with nanopure water three times and then resuspended in 200 mL of 0.001 M NaCl. Then, 0.4 mL of 0.1 M HCl were added to the solution and the conductivity was recorded. The samples were titrated with 0.1 mL aliquots of 0.05 M NaOH and the conductivity was recorded each time.

An Optest Hi-Resolution benchtop fiber quality analyzer (FQA) was used to measure the fiber length of the samples. The length-weighted average was reported in this study as it reduces the effect of smaller particles on the fiber length calculations. Cellulose crystallinity was measured using a Perkin Elmer Spectrum One FT-IR

Spectrometer with a PIKE MIRacle™ single bounce attenuated total reflectance (ATR) accessory (Perkin Elmer, Wellesley, MA). Briefly, approximately 0.1 g ODW of never dried substrate was re-suspended in 50 ml of deionized water and shaken vigorously to disperse the material. The re-suspended sample was filtered in a Buchner funnel fitted with a Whatman no. 1 filter paper to create a pulp pad and subsequently air-dried overnight. The samples were pressed uniformly against the diamond surface, and mid-IR spectra were obtained by averaging 64 scans from 4000 to 600 cm^{-1} at 4 cm^{-1} resolution. The spectra were normalized at 1900 cm^{-1} using the Spectrum One software supplied with the equipment.

The viscosity of the cellulose component of the substrates was determined according to the TAPPI method T 230 os-76. Prior to the viscosity measurements, the samples were delignified at room temperature according to PAPTAC useful methods G10. The samples that were pretreated with 0, 1 and 3% SO_2 were subjected to an additional treatment with 17.5% NaOH to remove the hemicellulose so that the viscosity measurements were representative of the cellulose component only (TAPPI method T 203 om-93). Approximately 0.75 g (wet weight) were weighed into 50 mL falcon tubes. Then, 25 mL of 17.5% NaOH were added and the samples were shaken for 30 minutes. An additional 25 mL of distilled water were added to the tube and the samples were shaken for an additional 30 minutes. The tubes were then centrifuged and the supernatant was discarded and the samples were washed thoroughly with water and then filtered.

Water retention value (WRV) was performed and calculated according to TAPPI Useful Method-256 with the exception that all substrate samples were filtered and the filtrate was recycled twice to improve the retention of the smaller particles. Simon's stain

was performed according to the method developed by Chandra et. al (2008), however the method was scaled down to a total volume of 1mL and only the Direct Orange dye (DO) (Pontamine Fast Orange 6RN, Pylam Products Co. Inc., Garden City, NY) was used.

3. Results and Discussion

3.1. Influence of biomass characteristics and handling on the sugar recovery and enzymatic hydrolysis of steam pretreated corn stover

3.1.1. To assess the effects of gross handling of biomass and pretreatment configuration

Although the thesis work was primarily focused on the utilization of wheat straw, because so much past work had been carried out on corn stover we first wanted to try and establish some base conditions with this more intensively studied feedstock. The corn stover feedstock was well characterized as it had been the subject of numerous studies investigating the effects of leading pretreatment technologies in our laboratory and laboratories across the United States as part of the Consortium for Applied Fundamentals and Innovation (CAFI) (Bura et al., 2009; Elander et al., 2009). The CAFI consortium was originally established to compare the performance of the most prevalent pretreatment technologies in the United States and the University of British Columbia in Canada, on a common biomass substrate. The main performance metric in the CAFI consortium was the ability of a given pretreatment to maximize the recovery of glucose and xylose from the biomass. Although the CAFI approach was useful, the enzyme loadings that were utilized during the project were high, at 15 FPU/g glucan, which were close to 30 mg protein/g gram of cellulose. As a result, this tended to improve the performance of all of the pretreatments tested. Regardless, SO_2 catalyzed steam pretreatment was among the top performing technologies tested on the corn stover. Thus, we first wanted to assess if

this well characterized corn stover would perform in the same way as wheat straw while using lower enzyme loading than those previously employed in the previously reported CAFI studies. To ensure comparability we pretreated the corn stover in parallel with the wheat straw.

Both the corn stover and wheat biomass samples were pretreated at 190°C, 5 min and 3% SO_2. These conditions had previously been shown to provide the best overall compromise between xylose recovery and the ease of hydrolysis of the cellulose component of corn stover (Bura et al., 2009). Both feedstocks resulted in similar glucose and xylose recovery after pretreatment and a similar chemical composition of the water insoluble fraction (Table 4). The enzymatic hydrolysis of the corn stover and wheat straw substrates at 10 mg/g glucan (4.8 FPU/g glucan) resulted in similar glucose yields of 72% and 67% respectively. Since both the corn stover and wheat straw substrates behaved similarly during the steam pretreatment experiments, the subsequent experiments assessing the effects of pre-moistening and subjecting the biomass to explosive decompression were performed on the corn stover biomass to conserve the wheat straw biomass for subsequent pretreatment testing.

Table 4 Chemical composition, glucose and xylose recovery and enzymatic hydrolysis yields of wheat straw and corn stover pretreated at 190°C, 3% SO_2, 5 minutes.
Enzymatic hydrolysis was conducted at 50°C, 72 hours and 10 mg protein/g glucan (4.8 FPU/g glucan). Recovery of arabinan, galactan and mannan was was beyond detectable limit (bdl) where noted.

Substrate	Glucose Recovery	Xylose Recovery	Glucose Hydrolysis	Klason Chemical composition (%)							
				Ara	Gal	Glu	Xyl	Man	AIL	ASL	Ash
Wheat Straw	84%	74%	67%	bdl	bdl	65.9	8.0	bdl	26.1	1.1	4.2
Corn Stover	85%	74%	72%	0.5	bdl	62.9	10.5	bdl	19.4	0.9	6.1

3.1.2. Effect of moisture content and explosion treatment on the sugar recovery and enzymatic hydrolysis of corn stover substrates

3.1.2.1. Effect of moisture content and explosion treatment on sugar recovery

The main parameters investigated in this section were the biomass moisture content and the use of an explosive decompression step during steam pretreatment and their effects on sugar recovery and ease of enzymatic hydrolysis. All pretreatments were performed at the optimal condition that was determined in the CAFI study (Bura et al., 2009). The effects of moisture content and the explosive decompression were compared at 190°C, 5 min and 3% SO_2. It was hypothesized that moistening of the corn stover prior to pretreatment would result in higher sugar yields as it has been shown on switchgrass, sugar cane bagasse and wood chips (Cullis et al., 2004; Ewanick & Bura, 2011).

In addition to assessing the effect of moisture content, the effects of explosive decompression during steam pretreatment were evaluated. Normally, steam pretreatment has been performed where the biomass is depressurized or "exploded" into a holding tank after the biomass is pressurized in the steam digester. However, the explosive decompression step requires the use of additional wash water for removing the steam pretreated biomass from the collection vessel, which results in a dilution of the water soluble fraction after pretreatment. Previous work has shown that the explosive decompression did not result in improvements in the subsequent enzymatic hydrolysis of the substrate (Brownell & Saddler, 1987; Brownell et al., 1986). Therefore, a basket was used to hold the biomass during the steaming in the steam gun to eliminate the need for additional wash water and to try to obtain higher sugar concentrations after steam

pretreatment. Although the use of the basket may allow for higher sugar concentration, it was also anticipated that the explosion treatment might also result in higher sugar yields because the biomass would be evenly pretreated, as opposed to the pretreatment in the basket (Figure 3), where presumably the biomass located at the outermost parts of the basket would receive a more severe pretreatment than the biomass located in the middle of the basket. The moisture content was increased by presoaking 50g ODW of corn stover with 50 mL of distilled water. The original moisture content of the corn stover was 7%.

Figure 3 Steel basket used to conduct pretreatment experiments without explosive decompression

When the chemical composition of the water insoluble fraction (WIF) was assessed for both the wet and dry substrates, the explosion treatment was shown to result in an increase in the xylan content of both the WIF fractions (Table 5). It was apparent

that the explosion treatment may have been less severe than the treatment with no explosion. The decrease in xylan content in the WIF from the pretreatment performed with no explosion was probably due to the severe treatment undergone by the biomass on the outer portions, which likely resulted in an increase in xylan degradation.

Table 5 Chemical composition of the raw and the WIF (water insoluble fraction) of steam pretreated corn stover substrates.
Pretreatment conditions were 190°C, 3% SO_2 and 5 min. Each pretreatment condition was performed in two batches of 50 g ODW. Recovery of galactan and mannan were beyond detectable limits (bdl). Ext = extractives

Substrate	ID	Ara (%)	Gal (%)	Gluc (%)	Xyl (%)	Man (%)	AIL (%)	ASL (%)	Ash (%)	Ext (%)
Raw Material	CS0	3.5	1.1	47.4	26.5	bdl	14.3	2.0	4.3	14
Wet + no explosion	CS1	0.5	bdl	59.9	6.9	bdl	21.3	1.8	6.8	-
Wet + explosion	CS2	0.6	bdl	67.2	10.9	bdl	17.6	1.9	5.5	-
Dry + no explosion	CS3	0.6	bdl	60.6	6.8	bdl	22.3	2.1	6.7	-
Dry + explosion	CS4	0.5	bdl	62.9	10.4	bdl	19.4	0.9	6.1	-

Noticeable differences were found in the xylose recovery of the respective water soluble fractions (Table 6). The lowest total xylose recoveries regardless of the moisture content were found in the substrates CS1 and CS3, which were pretreated in the basket. The low xylose recovery was likely due to the heterogeneous treatment received by the biomass in the basket. Of note, although the highest total xylan recovery was observed with the samples CS2 and CS4, which were pretreated with the explosion treatment, the same samples also had slightly lower glucose recovery. It was apparent that moistening the biomass prior to pretreatment and subjecting the biomass to the explosion treatment increased both hemicellulose and glucose recovery (Table 6).

Table 6 Effect of explosion vs. no explosion treatment on the glucose and xylose recovery of corn stover substrates pretreated at 190°C, 5min, 3% SO_2.
WIF=water insoluble fraction, WSF=water soluble fraction

Substrate	ID	WIF		WSF		Total Sugar recovery	
		Glucose	Xylose	Glucose	Xylose	Glucose	Xylose
wet + no explosion	CS1	85%	18%	6%	35%	91%	53%
wet + explosion	CS2	82%	24%	9%	62%	91%	86%
dry + no explosion	CS3	91%	18%	5%	38%	96%	56%
dry + explosion	CS4	79%	24%	6%	51%	85%	74%

The mechanism by which moisture content increases the sugar recovery of steam pretreated substrates is not well understood. It is possible that the moisture content simply reduces the apparent severity of the pretreatment, thus resulting in less sugar degradation (Cullis et al., 2004). Others have suggested that increasing the moisture content of the biomass allows for a fast and homogeneous permeation of the catalyst (Ewanick & Bura, 2011). The effect of moisture content on the sugar recovery, particularly of xylose, is in accordance with the trend previously reported for switchgrass, where higher moisture contents resulted in an increase in xylose recovery (Ewanick & Bura, 2011). It may also be possible that increased moisture facilitates the conversion of the SO_2 catalyst to sulfurous acid thus providing a greater number of protons for the hydrolysis of the hemicellulose component at lower pretreatment severities (Biermann, 1996). It seems that high moisture content coupled with the explosive decompression increases xylose recovery, but when the substrates did not undergo explosive decompression the xylose recovery was only 53-56%, regardless of the moisture content. Therefore, the use of the steel basket to prevent explosive decompression would not result in higher sugar recovery in the liquid and it is not a suitable method for pretreatment of agricultural residues.

3.1.2.2. Effect of moisture content and explosion treatment on the ease of enzymatic hydrolysis

After determining the total glucose and xylose recovery of the substrates after steam pretreatment, the water insoluble fractions were hydrolyzed at a cellulase loading of 10 mg protein/g glucan, which corresponds to 4.8 FPU/g glucan, in order to evaluate the effects of the explosion treatment on the ease of enzymatic hydrolysis of the substrates. Our results show that, when the substrates undergo explosive decompression, the enzymatic hydrolysis yields of the prewetted and dry biomass were similar (Figure 4). This is in accordance with the results reported by Brownell et al. (1986) on small wood chips, where the moisture content had no effect on the enzymatic hydrolysis yields. Ewanick and Bura (2011), however, found that the increase in moisture content coupled with a catalyst resulted in an increase in enzymatic hydrolysis yields.

It is likely that the method employed to increase the moisture content of the biomass in the work of Ewanick et al. (2011) contributed to the enhanced hydrolysis yields as the substrates were submerged in water for 48 hours prior to pretreatment and subsequently filtered to a moisture content of 80%. In addition to moistening the biomass and changing the heating rate during steam pretreatment, it is possible that water can remove a portion of the ash from the straw. The ash content of agricultural residues has been shown to inhibit cellulolytic enzymes and also contribute to the buffering capacity of the substrate. Therefore, the mineral cations that comprise the ash in straw can "consume" the acid catalyst, thereby reducing the efficiency of the pretreatment and the apparent severity (Öhgren et al., 2007). In the work reported here, rather than saturating the biomass with water, water was applied to the biomass to moisten the corn stover to a

50% moisture level on the same day as the steam pretreatment was carried out. The material was not filtered prior to loading the material in the steam gun and the substrates were not washed with additional water as described in the work of Ewanick and Bura (2011). Therefore, it can be speculated that the method of increasing the moisture of the biomass used in the work reported here did not remove a significant amount of ash from the corn stover. It is likely that the retention of ash in the biomass may have influenced the enzymatic hydrolysis yields since the ash content has been shown to inhibit the enzymatic hydrolysis on steam exploded rice straw in addition to neutralizing the added SO_2 catalyst during pretreatment (Bin & Hongzhang, 2010; Öhgren et al., 2007).

Comparing the samples that were pretreated in the basket to those that were subjected to the explosive decompression it was apparent that there was little difference in the ease of enzymatic hydrolysis of the substrates that were pre-moistened (Figure 4). This seems to confirm the results previously reported by Brownell and Saddler (1987) who pretreated "green" aspen wood chips (~50% moisture content). However, when the biomass was pretreated at a low moisture content of 7%, the explosion treatment had a significant impact on the enzymatic hydrolysis results. The substrate that was pretreated with no explosion (CS3) had a glucose yield of 63%, compared to 72% for sample CS4, which was also dry but was subjected to explosive decompression. It was evident that sample CS3 had undergone the most severe pretreatment as both glucose and xylose recoveries were compromised when dry biomass was treated in the basket. This is further confirmed by the combined severity factor of sample CS3, which was 0.38, whereas the combined severity factor of sample CS4 was 0.09. As the use of the basket appeared to increase the pretreatment severity, it was likely that this led to a decrease in

enzymatic hydrolysis yields and degradation of sugars to furans. Recent work has shown that, during the steam pretreatment of Douglas-fir, an increase in pretreatment severity resulted in the condensation of lignin, which increased its hydrophobicity and thereby increased its tendency to bind non-productively to cellulases (Nakagame et al., 2011b). Similar results on the effect of pretreatment severity on lignin condensation have been found for steam pretreated wheat straw (Sun et al., 2004). Therefore, it is possible that the lignin component in sample CS3 was more condensed and inhibitory toward enzymatic hydrolysis, thus decreasing the hydrolysis yields. Overall it was evident that the best combination of sugar recovery and hydrolysis was obtained with moistened biomass that was subjected to the explosive decompression during steam pretreatment.

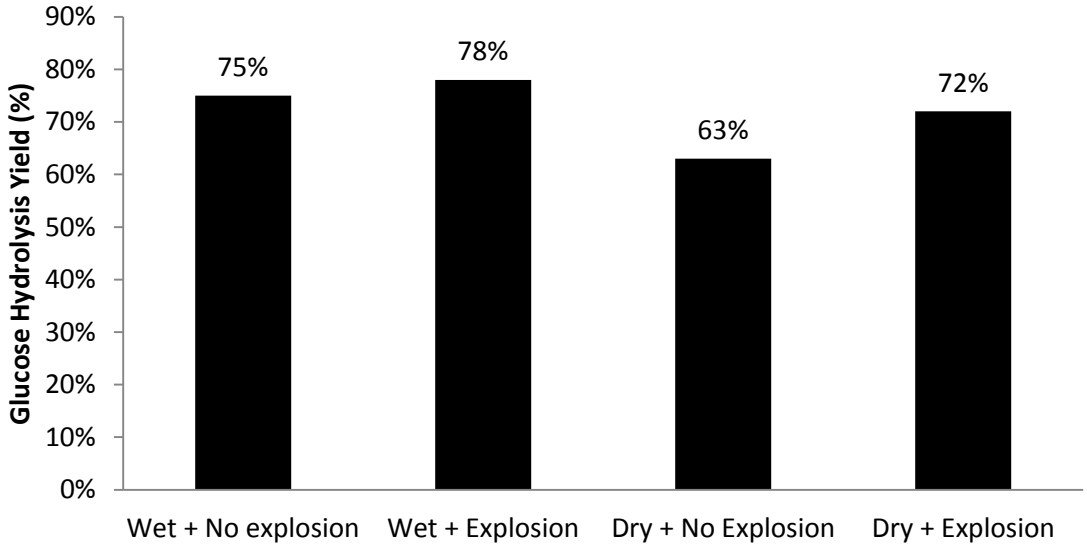

Figure 4 Enzymatic hydrolysis of corn stover substrates at 2% solids, 72 hours and 10 mg protein/g glucan (4.8 FPU/g glucan)

3.2.Choice of SO_2 vs H_2SO_4 as acid catalysts

3.2.1.1. Effect of acid catalysts on sugar recovery

Steam pretreatment of agricultural residues such as wheat straw can be performed without the addition of an acid catalyst due to the acetyl group moieties that constitute a portion of their hemicellulose component acting as the acid catalyst. During steam pretreatment, the acetyl groups are liberated to provide an acidic medium that facilitates the solubilization of the hemicellulose component (Chandra et al., 2007). However, several studies have shown that the addition of an acid catalyst such as SO_2 or H_2SO_4 can result in the solubilization of hemicellulose at lower pretreatment severities in addition to increases in enzymatic hydrolysis yields (Chandra et al., 2007). Even though H_2SO_4 has a much higher molecular weight than SO_2 (98 vs 64 g/mol), previous studies have compared SO_2 and H_2SO_4 as catalysts for steam pretreatment at equal mass loadings of each catalyst. Other studies comparing the two catalysts have either performed enzymatic hydrolysis experiments with relatively high enzyme loadings or have pretreated at different conditions for each catalyst rather than making the comparison at the same condition. Thus, it is unclear if there is an advantage to employing SO_2 over H_2SO_4 as a catalyst during steam pretreatment. In addition to its higher molecular weight, H_2SO_4 is also applied directly as an acid with two low pKa values (-3 and 1.99), while SO_2 must react with water to either form H_2SO_3 which can readily dissociate to HSO_3^- and H^+ (pKa=1.8) or undergo further oxidization to H_2SO_4. Therefore, it was thought that, if H_2SO_4 is used at a lower loading, it would be possible to obtain comparable sugar recovery and enzymatic hydrolysis yields to those obtained with SO_2 (Biermann, 1996).

Also, the use of a lower loading of acid catalyst may also be more economical and cause less corrosion of the steam gun.

In these experiments we compared the effects of SO_2 and H_2SO_4 on the sugar recovery and enzymatic hydrolysis of steam pretreated corn stover. H_2SO_4 was used at lower loadings of 0.75% and 1.5% compared to 3% SO_2. Based on the results from the previous section on moisture content and explosion treatment, all pretreatment conditions were done by pre-wetting the substrate and all substrates underwent explosive decompression. An additional substrate pretreated with 3% SO_2 at low moisture content was included as this represents the optimal condition from the CAFI study. Klason analysis of the water insoluble fraction obtained after steam pretreatment showed that the corn stover pretreated with 3% SO_2 had a similar xylan content regardless of the moisture content, whereas the water insoluble fraction of the corn stover pretreated with 0.75% and 1.5% H_2SO_4 had a xylan content of 12% and 7% respectively (Table 7). The uncatalyzed sample retained as much as 20% of the xylan in the water insoluble fraction (WIF). The glucan content remained fairly similar across all treatments. The acid insoluble lignin was 18-23%, the highest lignin content being that of the sample pretreated with 1.5% H_2SO_4.

Table 7 Chemical composition of the WIF of steam pretreated corn stover substrates using SO_2 or H_2SO_4 as an acid catalyst.
Except where noted, all substrates pretreated at 190°C, 5min, prewetted and with explosion treatment, 75 g ODW. Recovery of mannan was beyond detectable limit (bdl).

Substrates	Arabinan (%)	Galactan (%)	Glucan (%)	Xylan (%)	Mannan (%)	AIL (%)	ASL (%)	Ash (%)
No catalyst	1.37	0.42	58.86	20.30	bdl	17.58	1.97	4.24
3% SO_2	0.35	0.01	63.56	9.68	bdl	20.46	1.78	5.85
3% SO_2 (dry)	0.35	0.10	66.18	9.86	bdl	22.17	1.93	6.08
0.75% H_2SO_4	0.47	0.15	65.46	12.64	bdl	18.29	1.73	4.66
1.5% H_2SO_4	0.27	0.05	63.79	7.38	bdl	23.28	1.77	6.08

An approximately doubling of the loading of SO_2 was required to solubilize the xylan component from the biomass to a comparable level as to when 1.5% H_2SO_4 was used as the catalyst for steam pretreatment (Table 8). The xylose recovery in the water insoluble fraction was between 16-29% for all substrates pretreated with an acid catalyst (Table 8). Pretreatment without a catalyst resulted in about 50% of the original xylan remaining in association with the water insoluble cellulosic fraction. The xylose recovery in the water soluble fraction (WSF) was similar for the pre-wetted substrates pretreated with an acid catalyst (H_2SO_4 or SO_2). The lowest xylose recovery in the WSF was obtained during the pretreatment without an acidic catalyst thus it was apparent that the addition of the acid catalyst facilitates the solubilization and recovery of the xylan component. The total recovery for both glucose and xylose was quite comparable among the samples. Of the samples pretreated with an acid catalyst, the substrate pretreated with 0.75% H_2SO_4 had the highest total glucose and xylose recovery of 91% and 94%, respectively and also solubilized a similar amount of xylan to the sample pretreated using 3% SO_2 (Table 8).

Table 8 Glucose and xylose recovery of corn stover pretreated using different acid catalysts.
Except where noted, all substrates pretreated at 190°C, 5min, prewetted and with explosion treatment, 75 g ODW. WSF=water soluble fraction, WIF=water insoluble fraction

Substrate	WIF		WSF		Total Sugar recovery	
	Glucose	Xylose	Glucose	Xylose	Glucose	Xylose
No catalyst	82%	50%	7%	46%	89%	96%
3% SO_2	78%	21%	9%	67%	87%	88%
3% SO_2 (dry)	86%	23%	8%	58%	94%	81%
0.75% H_2SO_4	82%	29%	8%	66%	91%	94%
1.5% H_2SO_4	80%	16%	10%	68%	90%	84%

We further analyzed the proportion of oligomers present in the WSF. A greater sugar recovery in a monomeric form would be ideal in order to avoid post-treatments to facilitate sugar uptake for yeast fermentation. It was found that most of the xylose present in the WSF was in the form of xylo-oligomers. The substrate pretreated with 0.75% H_2SO_4 had the highest amount of xylo-oligomers, whereas the 3% SO_2 (prewet) and 1.5% H_2SO_4 substrates had comparable amounts of xylo-oligomers of 82% and 80%, respectively. The glucose oligomers ranged between 72-79%, and the substrates pretreated with 1.5% H_2SO_4 had the lowest amount of glucose oligomers.

3.2.1.2. Effect of acid catalysts on ease of enzymatic hydrolysis

The enzymatic hydrolysis of the corn stover substrates showed that the glucose yields after 72 hours were similar for the samples pretreated with an acid catalyst. The sample pretreated with 3% SO_2 had the highest glucose yield of 82%, but the substrate pretreated with 1.5% H_2SO_4 had a yield of 83%. From the results it was apparent that H_2SO_4 was just as efficient as SO_2 in producing substrates with a high cellulose recovery and degree of digestibility. However, H_2SO_4 could be applied at half the loading of SO_2 to produce similar results, thus indicating that the H_2SO_4 was a more potent catalyst than SO_2.

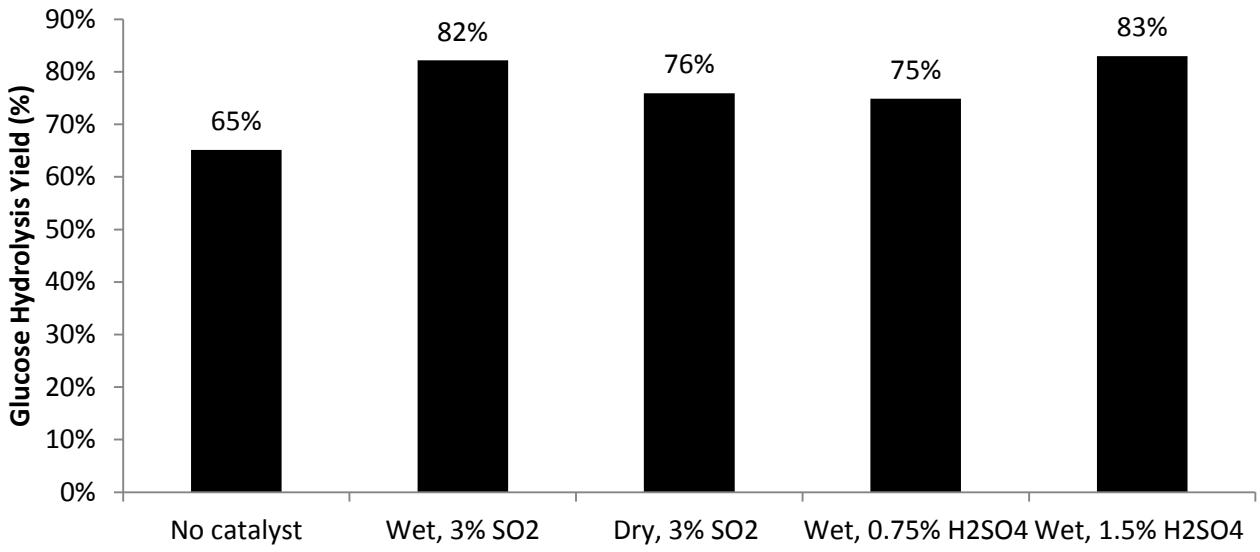

Figure 5 Hydrolysis of corn stover substrates pretreated with SO_2 or H_2SO_4.
Enzymatic hydrolysis was conducted at 50°C, 72 hours and 10 mg protein/g glucan (4.8 FPU/g glucan)

There are two studies in the literature that can serve as relevant comparisons to our results because they compared the catalysts at different concentrations. Tengborg et al. (1998) compared the results of their study using H_2SO_4 with the results reported by Stenberg et al. (1998) using SO_2 because both studies were done in the same equipment and with the same feedstock (Stenberg et al., 1998; Tengborg et al., 1998). However, the temperature and time used differed for SO_2 and H_2SO_4, the former being pretreated at higher temperatures and shorter time periods. One of the main conclusions of this comparison is that SO_2-pretreated substrates have a higher fermentability than the H_2SO_4-pretreated substrates, but this is probably due to the fact that the substrates pretreated with H_2SO_4 were generally pretreated for longer periods of time. Therefore, it is likely that the production of inhibitors was higher than that of the SO_2-pretreated substrates and this may have affected the fermentability of the substrates. The second study that compared H_2SO_4 and SO_2 at different concentrations was performed on willow substrates (Eklund et al., 1995) at the same temperature ranges (190-230°C) and residence time (10

min). Pretreatment with 1% SO_2 was compared to willow substrates pretreated with 0, 0.6, 1.5 or 3% H_2SO_4. In this case, it was concluded that SO_2 was a better catalyst because the glucose recovery and enzymatic hydrolysis yields were higher. The highest xylose recovery was achieved with one of the H_2SO_4-pretreated substrates, but at that given condition the glucose enzymatic hydrolysis yields were very low. It is possible that Eklund et al. (1995) obtained such results because the pretreatment conditions were not optimized for the pretreatment of willow with H_2SO_4. Our results demonstrate that when pretreatment conditions are close to optimal, compared to SO_2, it is possible to obtain comparable sugar recovery and enzymatic hydrolysis yields using lower loadings of H_2SO_4. Although the SO_2-pretreated substrate had slightly higher enzymatic hydrolysis yields, the H_2SO_4-pretreated substrates had a higher total sugar recovery. A potential drawback of using H_2SO_4 as an acid catalyst is that the xylose recovery in the WSF is mostly in oligomeric form and would require further processing if it is to be used for fermentation into ethanol.

The fact that less H_2SO_4 is needed to achieve comparable results to SO_2 also suggests that the mechanism of the acid catalysts during pretreatment may not be identical. The common assumption is that SO_2 converts to H_2SO_4 during the steam pretreatment process but it is not clear to what extent this occurs. It is also possible that SO_2 converts to sulfurous acid (H_2SO_3) which in the absence of a counterion to buffer the reaction, results in an acidic environment that solubilizes the hemicellulose similar to a so-called “black cook” in sulfite pulping which results in lignin condensation and a lack of lignin sulfonation (Sjöström, 1993). The condensation of lignin has also been shown to occur during SO_2 catalyzed steam pretreatment (Li et al., 2007). The SO_2 has also not

yet been shown to sulfonate the lignin component during SO_2 catalyzed steam pretreatment which will be a subject of a subsequent chapter.

3.2.2. Discussion

The effects of biomass handling, particle size and acid catalyst were studied for corn stover substrates. It was found that increasing moisture content and using the explosive decompression produced substrates with high glucose and xylose recoveries without compromising the enzymatic hydrolysis. A comparison of different moisture contents with explosive decompression was done on wheat straw and we observed similar trends as that of corn stover. It was apparent that the results on corn stover are comparable to those obtained with wheat straw. The comparison of SO_2 and H_2SO_4 as acid catalysts shows that it is possible to pretreat corn stover with less H_2SO_4 and obtain comparable results when SO_2 is used as a catalyst. As it appeared that the use of H_2SO_4 also resulted in a higher xylose recovery and that impregnation of the samples could be achieved more accurately, subsequent experiments with wheat straw were done using H_2SO_4 as an acid catalyst.

3.3.Increasing sugar recovery and sugar concentration of wheat straw after steam pretreatment and enzymatic hydrolysis

3.3.1. Pretreatment conditions, mass balance and sugar recovery

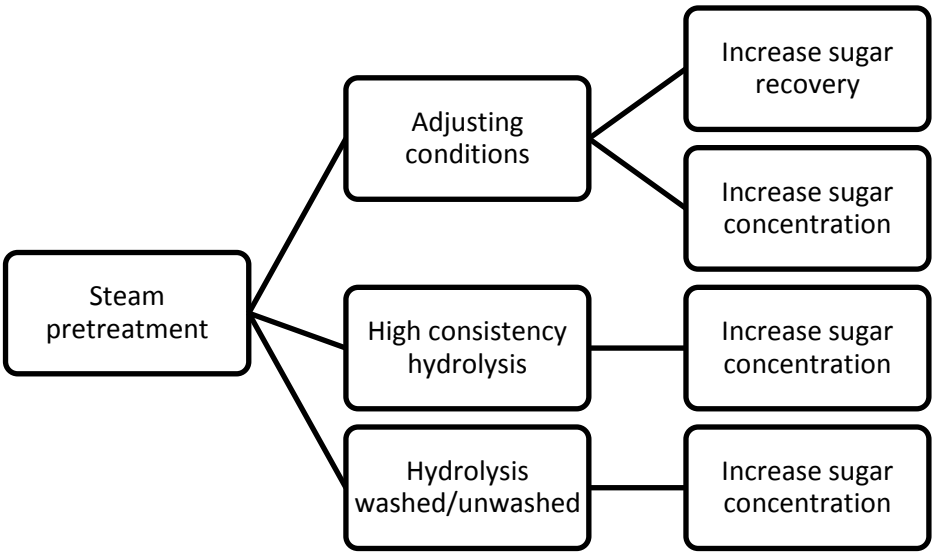

Figure 6 Schematic of experiments conducted to increase sugar recovery and concentration of steam pretreated substrates

Once the optimal biomass handling that would result in a high sugar recovery during the steam pretreatment of agricultural residues was determined, a range of pretreatment conditions were tested. The pretreatment conditions ranged from those of an intermediate severity to those of a relatively high severity. Using literature values as a guide, extremely mild or severe conditions were avoided (Ballesteros et al., 2006; Carvalheiro et al., 2009; Kabel et al., 2007). This would allow for a realistic range of conditions to test the hypothesis that maximizing the combined sugar recovery after both the pretreatment and hydrolysis step at low enzyme loadings may require sacrificing the

recovery of a portion of the hemicellulose. As shown in the previous chapter, H_2SO_4 was able to act as a catalyst to enhance the removal of hemicellulose during the steam pretreatment of corn stover sugars at a lower loading than SO_2. Therefore, the range of conditions selected for this study were based on pretreatment conditions found in the literature for dilute acid and steam pretreatment of agricultural residues using H_2SO_4 as the acid catalyst (Ballesteros et al., 2006; Kabel et al., 2007; Rosgaard et al., 2007; Zimbardi et al., 2007). Three factors, time, temperature and acid catalyst loading with three levels each were selected for the statistical design (Table 9). Due to the laborious nature of steam pretreatment and material balance, a Box-Behnken experimental design was used as it would allow the assessment of each variable while decreasing the number of experimental conditions to 15 rather than 27 as in the case of a full factorial design (Qi et al., 2009). Another advantage of the Box-Behnken design is that it does not include the extreme points in the statistical design. This eliminates the very low and very high severity pretreatment conditions, which for the purposes of this project would not result in reasonable sugar recovery and enzymatic hydrolysis yields. Based on the literature on the steam pretreatment of agricultural residues using H_2SO_4 as an acid catalyst, 1.5% was chosen as the highest concentration of H_2SO_4, while 210^{o}C and 8 min were chosen as the highest temperature and residence time. The centre point of the design was 190°C, 5 min and 0.75% H_2SO_4.

Table 9 Pretreatment conditions and combined severity factor (CS) of steam pretreated wheat straw substrates based on a Box-Behnken statistical design

Conditions	Temp (°C)	Time (min)	H_2SO_4 (% w/w)	CS
1	170	2	0.75	-1.99
2	170	8	0.75	-1.05
3	210	2	0.75	-0.26
4	210	8	0.75	0.74
5	190	2	0	-2.02
6	190	2	1.5	0.04
7	190	8	0	-0.69
8	190	8	1.5	0.57
9	170	5	0	-2.27
10	210	5	0	0.11
11	170	5	1.5	-0.39
12	210	5	1.5	1.00
13	190	5	0.75	-0.54
14	190	5	0.75	-0.48
15	190	5	0.75	-0.48

The chemical composition of the starting material was 2.3% arabinan, 0.5% galactan, 49.6% glucan, 24.7% xylan, 0.05% mannan, 18.8% acid insoluble lignin (AIL), 1.2% acid soluble lignin (ASL) and 6.5% ash content. The combined severity factor (R'_o= [H+]*R_o) was calculated using the severity factor R_o, where R_o=t*e^ [(T-100)/14.75], and multiplying by the concentration of H^+ ions found in the WSF. The combined severity factor is reported as the logarithm of R'_o. After the pretreatments, the substrates received limited washing to avoid dilution of the solubilized sugars. However, washing has been shown to play a significant role in influencing the ease of hydrolysis of the resulting substrate which will be discussed later. The pretreatment and enzymatic hydrolysis experiments that were conducted in this section are summarized in Figure 6.

Due to the labile nature of the hemicellulose component, it was anticipated that higher combined severity factors would result in a greater removal of the xylan component from the water insoluble substrates which has been observed previously in the case of corn stover (Bura et al., 2009). Indeed, the increase in combined severity showed a linear correlation with the content of the xylan component in the water insoluble fraction with an r^2 of 0.88 (Figure 7) which is in accordance with previous work on steam pretreated wheat straw (Kabel et al., 2007). The overall chemical compositions of the water insoluble fractions (WIF) show that the glucan content of the substrates ranged from 48-62%, while the xylan content ranged from 0.3-21% (Table 10).

In many cases, especially under acidic conditions, the severity of the pretreatment that is required to solubilize most of the xylan from the water insoluble fraction also results in a decrease in the overall recovery of the xylan fraction, as the xylan can undergo acidic dehydration to furfural. Therefore, the highest xylose recoveries were obtained with the substrates pretreated at lower severities such as condition 9 and 1 which reached yields of close to 80% (Table 11). Several pretreatment conditions resulted in intermediate xylose recoveries between 60-69%, but the partitioning of the sugars between the WIF and WSF varied according to the severity of the pretreatment. This range of recovery is well within the range reported previously for "compromise" conditions for SO_2-steam pretreated corn stover, which recovered 64% of the xylan fraction in the combined water soluble and insoluble fractions (Bura et al., 2009). Previous work by Ballesteros et al. (2006) on H_2SO_4-catalyzed steam pretreated wheat straw also reported high xylan recovery of 82%, but found that the conditions at which the sugar conversion yield was maximized gave a hemicellulose recovery of 70%. These

results were obtained at an acid loading of 0.9% (w/w), which is within the range of the conditions used in our experimental design. It should be noted that many of the previous studies have different criteria for determining the "compromise" conditions that increase sugar recovery. In some cases, the compromise conditions were defined as the sugars solubilized after pretreatment and enzymatic hydrolysis, but the enzyme loadings used were up to three-fold higher than the ones used in this study (Ballesteros et al., 2006; Linde et al., 2008; Palmarola-Adrados et al., 2004; Petersen et al., 2009). Others only examined the ease of enzymatic hydrolysis of the resulting substrate and did not consider the overall sugar recovery from the starting material (Rosgaard et al., 2007). In general, the xylose recovery increased in the WSF as the pretreatment severity factor increased until the conditions were harsh enough to cause extensive hemicellulose degradation, which is reflected in the decrease of xylose yields (Figure 8). The xylan derived sugars in the water soluble fraction also underwent further conversion to monomers with the increase in the combined severity factor, thus indicating that in addition to solubilizing xylan the higher severity was also hydrolyzing the sugars in the water soluble fraction to monomers (Figure 9, Appendix A).

In the current work, the glucose recovery ranged from 81-100%. Thus, as was anticipated, the xylose recovery was more sensitive to pretreatment conditions and the yields varied between 16-80% (Table 11). However, it was evident that the glucose recovery was influenced by the higher acid loading as at the 1.5% acid loading the glucose recovery decreased as shown by the total glucose recovery of the substrates (Table 11). Although a portion of the glucose and xylose in the biomass were degraded when employing the increased pretreatment severities, one of the main objectives of this

study was to determine the influence of these higher severity conditions on the total recovery of soluble sugars from the wheat straw biomass after pretreatment and hydrolysis. It was expected that higher severities would facilitate enzymatic hydrolysis at low enzyme loadings which could increase the total soluble sugar recovery compared to when "compromise" pretreatment conditions are used which aim to preserve the hemicellulose component.

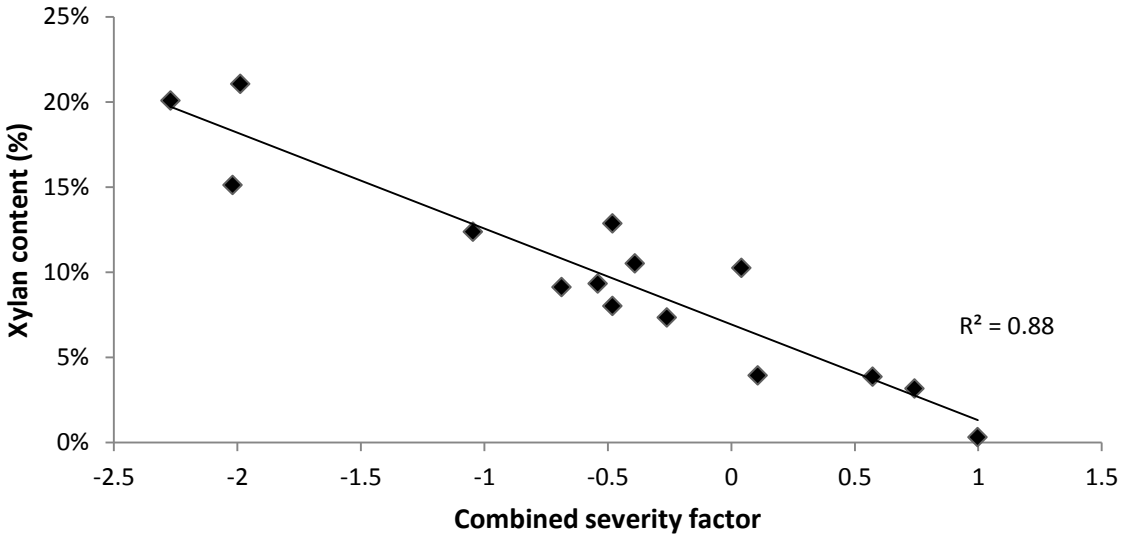

Figure 7 Relationship between the xylan content in the WIF and the combined severity factor

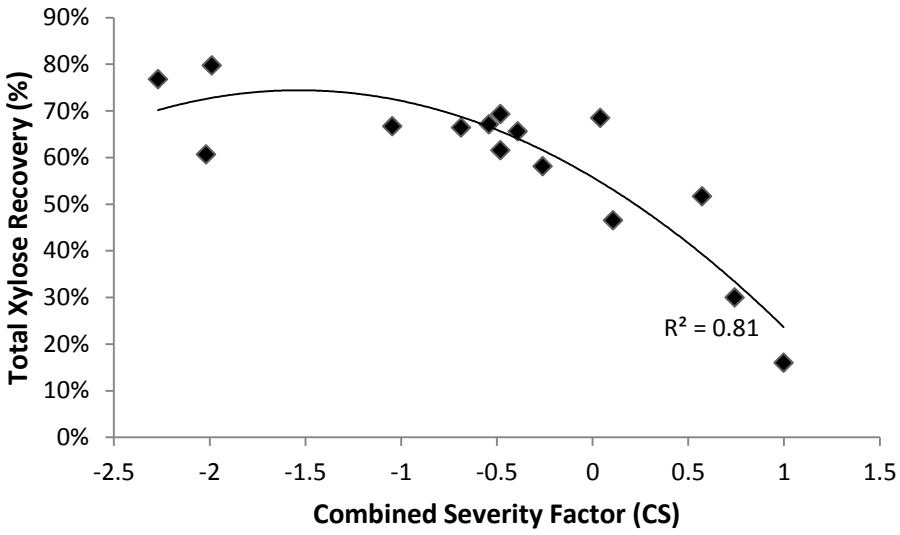

Figure 8 Relationship between total xylose recovery (WIF and WSF combined) and the combined severity (CS) factor

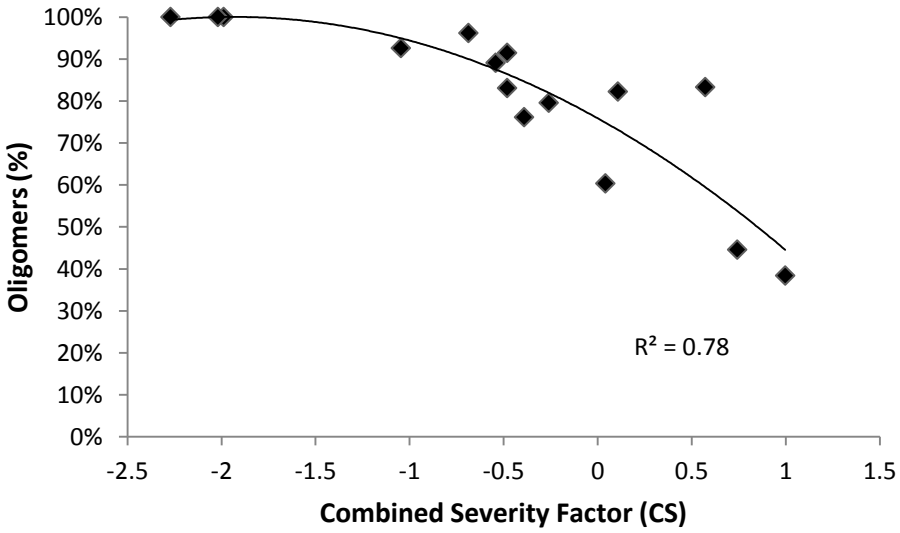

Figure 9 Relationship between the percent of oligomers present in the water soluble fraction (WSF) and the combined severity (CS) factor

Table 10 Pretreatment conditions, combined severity, solids yield and chemical composition of the steam pretreated wheat straw substrates. Recovery of mannan was beyond detectable limit (bdl).

	Conditions			CS	pH Liquor	Solids Yield	Klason							
	Time (°C)	Temp (min)	H_2SO_4 (% w/w)				Arabinan	Galactan	Glucan	Xylan	Mannan	AIL	ASL	Ash
1	170	2	0.75	-1.99	4.35	87%	1.46%	0.31%	50%	21%	bdl	18%	1%	8%
2	170	8	0.75	-1.05	4.01	79%	0.85%	0.39%	50%	12%	bdl	23%	1%	7%
3	210	2	0.75	-0.26	3.8	72%	0.60%	0.33%	58%	7%	bdl	32%	1%	9%
4	210	8	0.75	0.74	3.4	61%	0.25%	0.24%	62%	3%	bdl	29%	1%	8%
5	190	2	0	-2.02	4.97	88%	1.40%	0.50%	48%	15%	bdl	22%	1%	6%
6	190	2	1.5	0.04	2.91	82%	0.87%	0.36%	55%	10%	bdl	23%	1%	8%
7	190	8	0	-0.69	4.24	77%	0.55%	0.31%	57%	9%	bdl	23%	1%	9%
8	190	8	1.5	0.57	2.98	66%	0.26%	0.24%	58%	4%	bdl	27%	1%	10%
9	170	5	0	-2.27	5.03	89%	1.45%	0.20%	46%	20%	bdl	18%	2%	7%
10	210	5	0	0.11	3.83	67%	0.29%	0.26%	58%	4%	bdl	27%	1%	10%
11	170	5	1.5	-0.39	3.15	80%	0.76%	0.37%	50%	11%	bdl	23%	1%	10%
12	210	5	1.5	1.00	2.94	60%	0.00%	0.00%	61%	0.3%	bdl	27%	1%	11%
13	190	5	0.75	-0.54	3.89	79%	0.62%	0.32%	54%	9%	bdl	23%	1%	8%
14	190	5	0.75	-0.48	3.83	83%	0.58%	0.00%	57%	13%	bdl	24%	1%	7%
15	190	5	0.75	-0.48	3.83	77%	0.51%	0.30%	55%	8%	bdl	24%	1%	10%

Table 11 Glucose and xylose recovery after steam pretreatment of wheat straw substrates

Conditions				WIF		WSF		TOTAL	
	Time (°C)	Temp (min)	H_2SO_4 (% w/w)	Glucose	Xylose	Glucose	Xylose	Glucose	Xylose
1	170	2	0.75	88%	74%	2%	5%	90%	80%
2	170	8	0.75	81%	40%	5%	27%	86%	67%
3	210	2	0.75	84%	21%	6%	37%	90%	58%
4	210	8	0.75	76%	8%	5%	22%	81%	30%
5	190	2	0	85%	54%	4%	7%	89%	61%
6	190	2	1.5	92%	34%	4%	34%	96%	68%
7	190	8	0	88%	29%	6%	38%	94%	66%
8	190	8	1.5	78%	10%	8%	41%	86%	52%
9	170	5	0	83%	72%	5%	4%	88%	77%
10	210	5	0	78%	11%	4%	36%	82%	46%
11	170	5	1.5	80%	34%	6%	32%	86%	66%
12	210	5	1.5	74%	0.3%	8%	16%	82%	16%
13	190	5	0.75	86%	30%	6%	37%	93%	67%
14	190	5	0.75	95%	43%	5%	26%	100%	69%
15	190	5	0.75	85%	25%	8%	37%	92%	62%

3.3.2. Hydrolysis at elevated solids concentration and low enzyme loadings

Earlier work has shown that enzyme loadings utilized during the enzymatic hydrolysis step of the bioconversion of lignocellulosics to ethanol should be reduced to improve the economic viability of the process (Merino & Cherry, 2007). A promising option for the cost reduction of biochemical conversion would be to reduce the residence time during enzymatic hydrolysis while increasing the hydrolysis rate since it has been observed that after 48 hours marginal increases in the enzymatic hydrolysis yields are obtained (Stephen et al., 2012). Therefore, the enzymatic hydrolysis experiments in this portion of the study were conducted for 48 hours rather than previous work which has employed a minimum of 72 hours (Ballesteros et al., 2006; Bura et al., 2009) and up to 96 hours (Kahr et al., 2012; Marcos et al., 2012; Öhgren et al., 2005).

Initially, an enzyme loading of 10 mg/g glucan was tested which was equivalent to approximately 4.8 FPU/g glucan. The enzyme loadings utilized in this work were significantly lower than those employed previously for the hydrolysis of steam pretreated agricultural residues, which typically used enzyme loadings between 15 to 20 FPU/g solid matter (Ballesteros et al., 2006; Horn et al., 2011; Linde et al., 2008; Petersen et al., 2009; Tucker et al., 2003). These enzyme loadings, based on the amount of added protein or protein activity per grams of oven dry weight solids are estimated to be as high as 40-50 FPU/g glucan which is fivefold greater than the enzyme loadings used in this study. We anticipated that the lower enzyme loading and shorter enzymatic hydrolysis reaction times would reduce the enzymatic hydrolysis yields significantly. As well as using lower enzyme loadings, the substrates were not subjected to extensive washing, which could

further decrease the enzymatic hydrolysis yields. It has been shown that the soluble products liberated during steam and dilute acid pretreatment play a critical role in decreasing the ease of hydrolysis of pretreated substrates (Alfani et al., 2000; Carrasco et al., 2010; Kahr et al., 2012; Merino & Cherry, 2007).

It was also likely that the ease of the hydrolysis of the substrates would be predominantly influenced by the xylan content of the resulting pretreated water insoluble fraction as has been shown in previous studies of steam and dilute acid pretreated agricultural residues (Kabel et al., 2007; Mussatto et al., 2008; Öhgren et al., 2007). Therefore, the pretreated substrates at elevated severities, which had lower xylan content in the solid fraction, were anticipated to be hydrolyzed to a greater extent at the low enzyme loadings. However, it should be noted, that at the higher acid loadings and temperatures, it is possible that the lignin component can undergo condensation that can hinder subsequent enzymatic hydrolysis due to hydrophobic non-productive binding of the enzymes (Nakagame et al., 2010). Therefore, maximizing the selective removal of xylan during the pretreatment without imparting a significant amount of lignin condensation would likely constitute an ideal scenario.

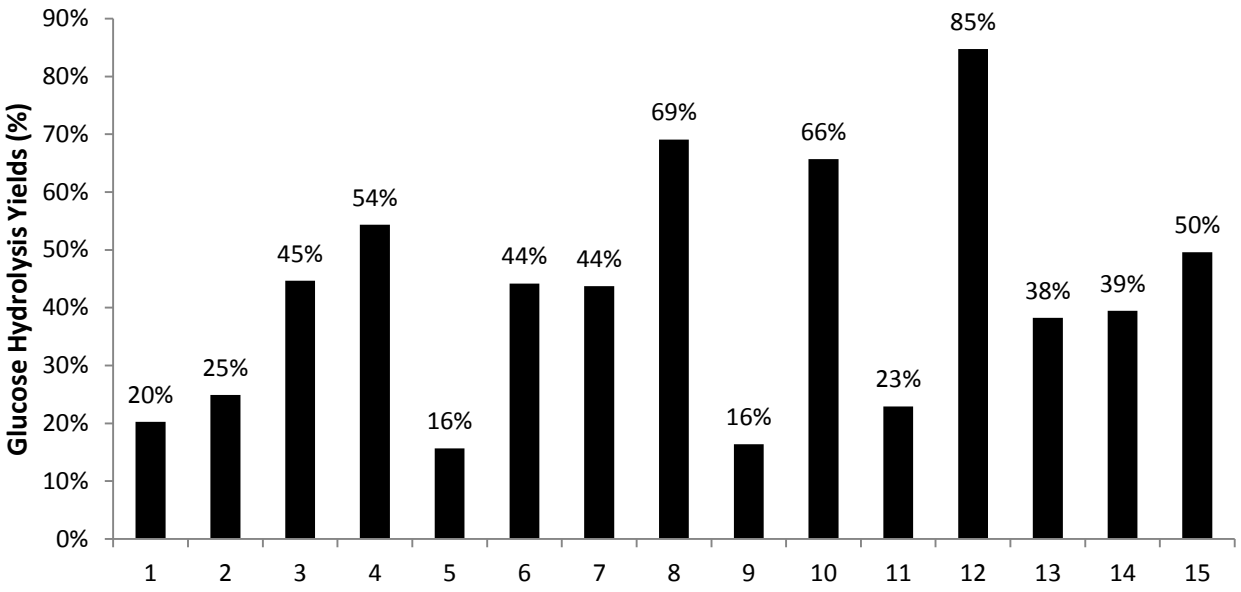

Figure 10 Glucose hydrolysis yields at 10% solids loading.
Enzymatic hydrolysis was conducted at 50°C, 48 hours and with 10 mg protein/g glucan (4.7 FPU/g glucan)

A solids loading of 10% was employed as solids loadings in excess of 12% have been regarded as inaccurate for measuring sugar yields due to the density of the solid substrate and the inadequate mixing in the Erlenmeyer flask. (Kristensen, Felby, & Jørgensen, 2009). Although higher solids loadings have been shown to be detrimental toward enzymatic hydrolysis due to issues with mixing, at the 10% solids loading the glucose hydrolysis yields were as high as 85% for condition 12 (Figure 10). Condition 12 employed the highest combined severity factor. Thus, within the range of pretreatment conditions tested, the higher severity enhanced the hydrolysis yields, likely due to the removal of xylan from the water insoluble substrate. Using the 10 mg/g glucan enzyme loading, the substrates obtained from the lowest severity pretreatments, such as samples 1, 2, 5 and 9 only reached cellulose conversions of 16-20%. It was apparent that the highest hydrolysis yields were obtained using the higher severities where only 16% of the xylose was recovered (Table 11). The effect of xylan in the WIF on the enzymatic

hydrolysis yields was quite evident as the amount of xylan removed from the water insoluble component reflected the ease of hydrolysis of the substrate, which was also directly related to the combined severity of the steam pretreatment (Figure 11). Another factor that could have contributed to the lower hydrolysis yields at the 10 mg/g enzyme loading on substrates with higher xylan recovery was the fact that the substrates were not subjected to washing prior to enzymatic hydrolysis.

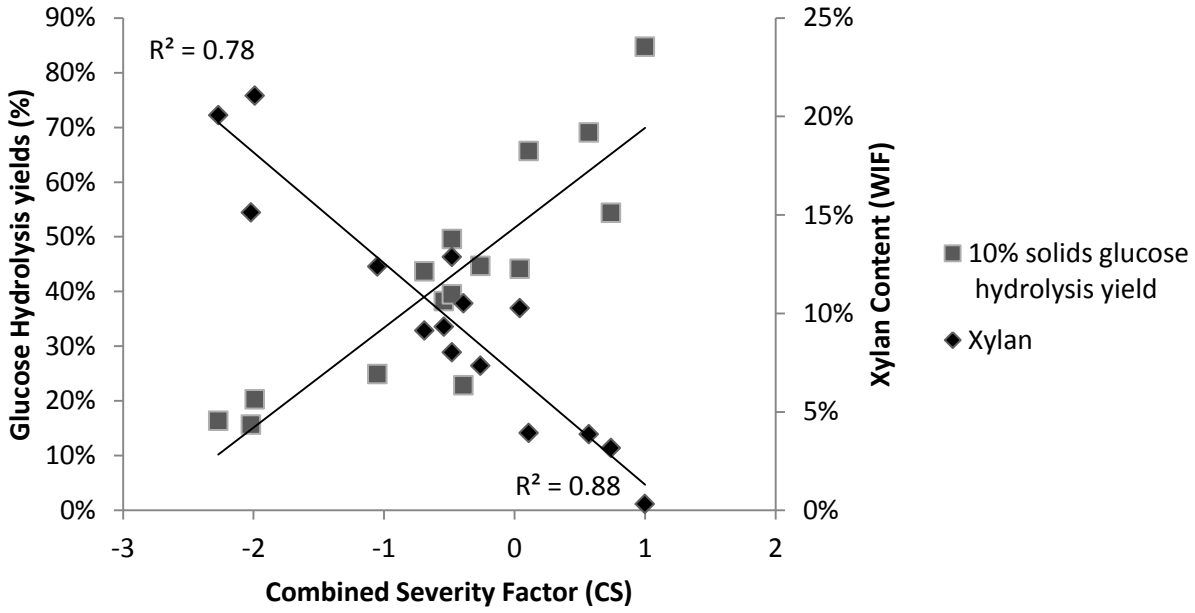

Figure 11 Relationship between the combined severity factor, xylan content in the WIF (♦) and glucose enzymatic hydrolysis yields (■)

3.3.3. The potential of substrate washing and increasing enzyme loadings to enhance enzymatic hydrolysis yields

Recent work has shown that substrate components that are released from biomass during dilute acid pretreatment can play a critical role in decreasing enzymatic hydrolysis yields of substrates that have not undergone washing prior to enzymatic hydrolysis (Ximenes et al., 2010; Ximenes et al., 2011). These components include lignin degradation products such as low molecular weight phenols and liberated sugars such as cellobiose, glucose and xylo-oligomers (Qing et al., 2010; Ximenes et al., 2010). In addition to performing the enzymatic hydrolysis reactions at high solids loadings, another strategy for maintaining higher sugar concentrations during the processing of pretreated substrates is to perform the enzymatic hydrolysis on unwashed water insoluble fractions to limit the dilution of sugars. However, during pretreatments such as dilute acid, it has been shown that inhibitory material such as low molecular weight phenolics can be released. They have been shown to inhibit the activity of cellulases during hydrolysis (Kothari & Lee, 2011; Sewalt et al., 1997). It should be noted that most previous work has assessed the enzymatic hydrolysis of pretreated agricultural residues such as corn stover and wheat straw after washing with up to 10 volumes of additional water (10-20 L) (Ballesteros et al., 2006; Bura et al., 2009). For example, one previous study on steam pretreated corn stover reported washing the substrate for 30 minutes with hot water followed by five rounds of filtration with water at 40°C which was repeated again with deionized water at 60°C (Tucker et al., 2003). In other cases the washing conditions were not reported in detail, making it difficult to determine the amount of water used in the process. To see if washing had a significant influence on the effectiveness of hydrolysis,

three representative substrates from conditions 9, 12 and 15, which correspond to the low, medium and high severity conditions in the experimental design, were used. These substrates were filtered after pretreatment and then rinsed once again in a Buchner funnel using the water soluble stream (the filtrate) without the addition of fresh water. We anticipated that the washing of the substrates from lower severity pretreatments would have a less significant effect on the resulting ease of hydrolysis of the substrate, as it was likely that lower amounts of potentially inhibitory materials toward the cellulases would be produced at the lower severity (Alfani et al., 2000; Carrasco et al., 2010).

Approximately 1g ODW of each substrate was washed thoroughly with 500 mL of distilled water and then filtered to a moisture content of approximately 80%. Washing increased the ease of enzymatic hydrolysis of the substrates by up to 18% (Figure 12). It is probable that by washing the substrate, inhibitors such as lignin residues or low molecular weight xylan are removed. The increase in enzymatic hydrolysis yields after washing is in accordance to several reports on steam pretreated agricultural residues (Alfani et al., 2000; Carrasco et al., 2010; Merino & Cherry, 2007). The increase was the most pronounced for the substrate pretreated at the medium severity. It was apparent the substrate pretreated at the medium severity was inhibited to the greatest extent by the soluble components liberated during pretreatment, while the substrate pretreated at low severity had a limited accessibility to the enzyme and thus was not aided by the washing. It is also evident that the use of the higher severity outweighed the positive effects of substrate washing as only an 8% increase in hydrolysis yield was obtained upon washing the substrate pretreated at high severity. This marginal increase of hydrolysis yields was also seen in previous work on steam pretreated wheat straw (Alfani et al., 2000).

Alternatively, rather than dilution of the substrate sugars through washing, the use of a higher enzyme loading may also be an effective approach to increase enzymatic hydrolysis yields of the unwashed substrates.

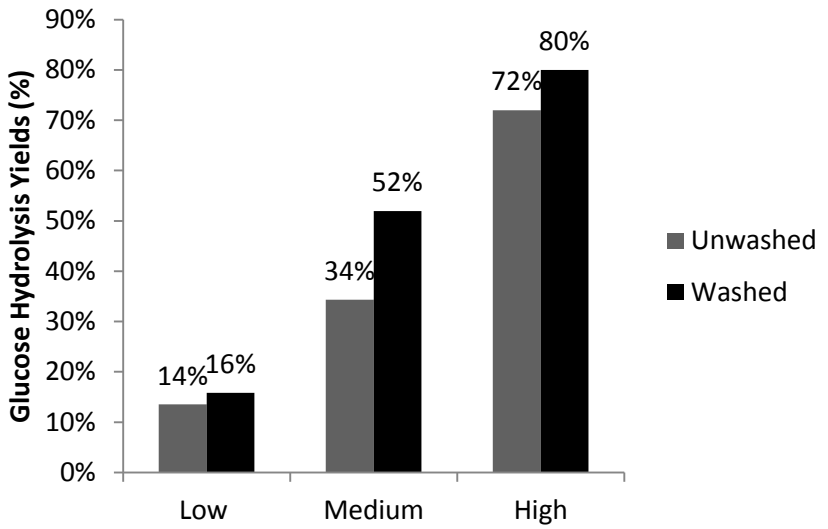

Figure 12 Glucose hydrolysis yields of washed and unwashed substrates pretreated at low (170°C, 5 min, 0% H_2SO_4), medium (190°C, 5 min, 0.75% H_2SO_4) and high (210°C, 5 min, 1.5% H_2SO_4) severity

The unwashed substrates were hydrolyzed at a high solids concentration (10%) for 48 hours using an enzyme loading of 20 mg protein/g glucan. It was thought that by increasing the enzyme loading better hydrolysis yields would be obtained with substrates pretreated at less severe conditions. Since the less severely pretreated substrates inherently have a higher sugar recovery in the solid and liquid fractions, this would result in a higher overall recovery of soluble sugars when both pretreatment and enzymatic hydrolysis are considered. A comparison of the glucose enzymatic hydrolysis yields at 10 mg/g glucan and 20 mg/g glucan showed that there were significant increases in the yields once the protein loading was increased (Figure 13). At a higher enzyme loading, substrates from pretreatment conditions 4, 8 and 10 reached glucose conversion yields of 100%. Combining the xylose recovery with the 100% conversion of the cellulose

component at the 20 mg/g enzyme loading could result in a higher total sugar yield after both pretreatment and hydrolysis.

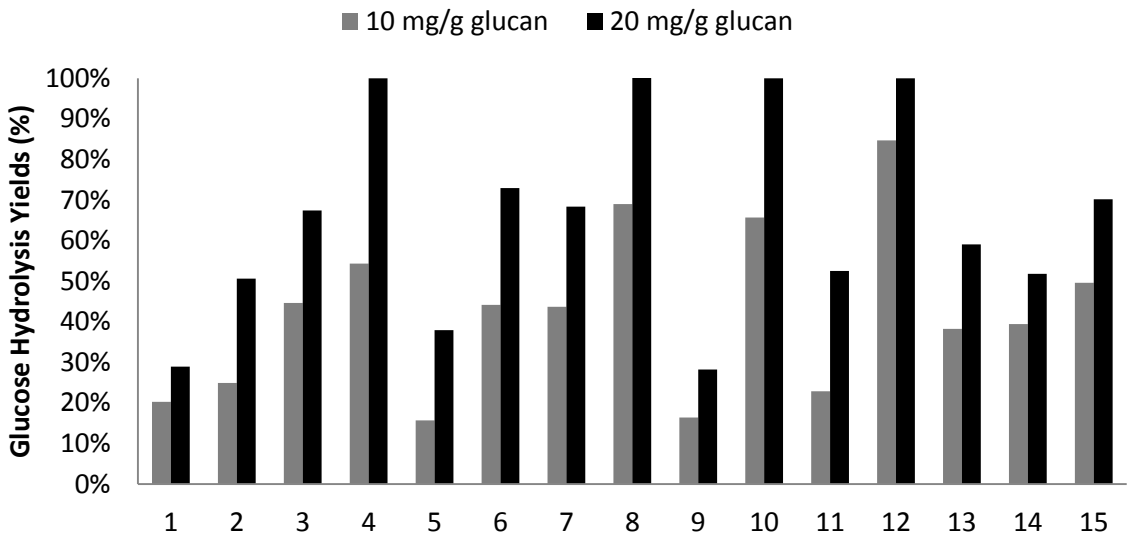

Figure 13 Enzymatic hydrolysis of steam pretreated wheat straw substrates at 10% solids using 10 mg/g glucan and 20 mg/g glucan protein loadings.

3.3.4. Total Soluble Sugar Yield

One of the benefits of utilizing an acid catalyzed steam pretreatment is the relatively selective solubilization of hemicellulosic sugars which improves the accessibility of the cellulose to cellulases and decreases the need for hemicellulases in downstream enzymatic hydrolysis (Bura et al., 2009; Hu et al., 2011). Ideally, subjecting biomass such as wheat straw to stream pretreatment and enzymatic hydrolysis should recover and solubilize all of the biomass carbohydrate constituents at the highest possible concentration using the lowest possible enzyme loadings. Therefore, the solubilization and complete recovery of hemicellulose during steam pretreatment combined with a complete conversion of the cellulose and remaining hemicellulose at low enzyme loadings and high substrate concentrations would be ideal. Much of the previously reported work has aimed to determine the highest possible severity that could be applied during pretreatment that would preserve >50% of the hemicellulose component with subsequent hydrolysis of the substrates obtained at these "compromise conditions" using relatively high enzyme loadings (30-100 mg/g glucan).

This type of approach was recently applied when processing corn stover biomass during the Consortium for Applied Fundamentals project (CAFI) (Elander et al., 2009). However, in the CAFI project, enzyme loadings ranging from 30-60 mg/g glucan and solids loadings of only 1-2% were used which elevated the perceived performance of all the pretreatment technologies that were compared. In addition it was unclear, with the reporting structure that was used, if the results from steam pretreatment included the hemicellulose recovery in both the water soluble and water insoluble fractions. As mentioned earlier, acidic pretreatments such as steam pretreatment selectively solubilize

a portion of the hemicellulose component. Therefore, it is challenging to assess the effectiveness of steam pretreatment by reporting the hemicellulose recovery in both the water soluble and water insoluble streams as it is unclear if these sugars are accessible to enzymes during subsequent hydrolysis. It is imperative to both solubilize and recover the hemicellulose component during steam pretreatment to maximize the total soluble sugar yield (TSSY) from the biomass.

It was anticipated that the TSSY would simultaneously encompass the effects of both the sugars solubilized during pretreatment and those that are solubilized during the enzymatic hydrolysis. The amount of solubilized sugars, particularly of the hemicellulose fraction, is also indicative of the severity of the pretreatment until the severity is elevated to a level where the carbohydrates become degraded. For example, a high severity pretreatment that results in the degradation of xylose and glucose but enhances enzymatic hydrolysis yields may be equivalent to an intermediate severity that solubilizes and recovers hemicellulose but is less amenable to enzymatic hydrolysis in terms of total soluble sugar recovery. Most of the previous work on the applications of steam and other pretreatments to agricultural residues has focused on the ease of hydrolysis of the pretreated substrate at high enzyme loadings (>20 FPU/g cellulose) without considering the total soluble sugar yields after the process. As mentioned above, these studies also list the total sugar recovery in both the water soluble and water insoluble streams thus inflating the overall sugar recovery of the process (Elander et al., 2009). However, in the work reported here the total soluble sugar yield (TSSY) was assessed. This value reflects the total usable sugars in the water soluble stream that can be provided to downstream fermentative processes.

The Total Soluble Sugar Yield (TSSY) was defined as the sum of the sugars solubilized in the WSF during pretreatment and the sugars that would be obtained from the enzymatic hydrolysis if the entire WIF fraction were to be hydrolyzed at a 10% solids loading. The oligomeric sugars were counted in the total solubilized sugar yields as previous work has shown that a mild treatment can be utilized to hydrolyze the oligomeric sugars obtained in WSF's (Schevchenko et al., 2000). The total soluble sugars were calculated for the enzymatic hydrolysis experiments conducted at 10% solids at both 10 mg protein/g glucan and 20 mg protein/g glucan loading. The analysis of variance for the TSSY was performed for both the 10 and 20 mg/g glucan enyme loadings to determine the most influential factors on the TSSY.

At the 10 mg protein/g glucan, the highest TSSY of 56% was obtained with the substrate pretreated at 190°C, 8 min and 1.5% H_2SO_4 which corresponded to a combined severity factor of 0.57 (condition 8). The second highest yield of 54% was obtained with the highest combined severity factor of 1.00 (condition 12), which was 210°C, 5 min, 1.5% H_2SO_4 (Table 12). It was apparent that, at the low enzyme loadings, similar to the results of the enzymatic hydrolysis experiments above, the TSSY was highest for the substrates pretreated at the higher severities regardless of the fact that under these conditions, 48% and 84% of the xylose was lost for conditions 8 and 12 respectively.

The highest TSSY values were obtained in the few substrates that were treated at higher severities and reached >70% yields during hydrolysis. The results at the low enzyme loading again suggested that some hemicellulose sugar loss must occur during pretreatment to result in a digestible material and obtain higher overall yields after pretreatment and enzymatic hydrolysis. The substrate from condition 8, where the highest

TSSY yields were obtained, had a sugar recovery of 86% glucose and 52% xylose. As discussed earlier, there was a trend between xylan content in the water insoluble fraction which is heavily influenced by the pretreatment severity, and the ease of enzymatic hydrolysis of the cellulose component (r^2=0.72, Figure 11). For example, similar levels of total xylose recovery were observed at conditions 5 and 6 (Table 11) which included both the water soluble and water insoluble xylose after pretreatment, but condition 6 had significantly higher TSSY than condition 5 (Table 12) since in the case of condition 5 much of the recovered xylose was in the water insoluble fraction (Table 11). Our results suggest that the overall soluble sugar recovery after pretreatment and enzymatic hydrolysis can be maximized by recovering most of the hemicellulose fraction in the WSF stream after pretreatment.

The ANOVA of the statistical design at the 10 mg/g enzyme loading suggested that, within the ranges tested, the temperature was the most influential factor on the TSSY, having the lowest p-values at a 95% confidence interval (Table 13). An increase in both the temperature and acid catalyst loading resulted in an increase in the TSSY as it was evident that the use of low enzyme loadings favored the use of a substrate that was more amenable to enzymatic hydrolysis but sacrificed the recovery of nearly 50% of the xylose (Figure 17). The optimization of the model based on the statistical design also determined that an improved TSSY yield of 61% could be achieved at 10 mg/g loading by changing the pretreatment conditions to 190°C, 6 min and 0.75% H_2SO_4.

It was evident that an enzyme loading of 10 mg/g glucan was too low to achieve a high TSSY values. Thus, the enzyme loading was raised to 20 mg/g glucan to improve the cellulose conversion for all of the samples. The TSSY was also increased, with many

conditions resulting in >70% TSSY. It was hypothesized that a change in the enzymatic hydrolysis conditions might lead to a shift in the pretreatment condition that resulted in the highest total fermentable sugars since a higher enzyme loading might be able to hydrolyze a substrate pretreated at a lower severity, which would most likely have a higher xylose recovery. However, despite the higher enzyme loading, we did not observe a change in the condition that resulted in the highest total fermentable sugars. The highest TSSY of 75% was still obtained at the pretreatment condition of 190°C, 8 min and 1.5% H_2SO_4 and the second highest yield of 71% was still obtained at 210°C, 5 min, 1.5% H_2SO_4. The ANOVA of the statistical design at the 20 mg/g enzyme loading confirmed that, within the ranges tested, the temperature, time and acid loading were the most influential factors on the TSSY, having the lowest p-values at a 95% confidence interval (Table 15).

The results observed at the 20 mg/g enzyme loading at a 10% solids loading compare quite favorably to previous work (Ballesteros et al., 2006) that used steam pretreatment of wheat straw using sulfuric acid as the catalyst. The TSSY value of the optimal condition determined by Ballesteros et al. (2006) was calculated to be 76%.This value was obtained using an enzyme loading of 23 FPU/g cellulose and 10% solids loading (Ballesteros et al., 2006). As a comparison, a TSSY value of 75% was obtained in this work at an enzyme loading of approximately 9.7 FPU/g cellulose or 20 mg/g cellulose.

Previous optimization studies using un-catalyzed steam or hydrothermal pretreatment at similar conditions to those utilized in this study showed TSSY values in the 68-81% range. However, these values were obtained by using up to 400 mg enzyme

loadings/g substrate compared to the 20 mg/g glucan utilized in the current study (Ballesteros et al., 2006; Palmarola-Adrados et al., 2004; Petersen et al., 2009). It should also be noted that in the previous work reported for steam pretreatment of wheat straw, the entire hemicellulose fraction was included rather than the xylan and glucan components exclusively. The substrates used in all of the previous studies also employed washed substrates while unwashed substrates were used in this study.

In contrast to the results at the 10 mg/g enzyme loading where only temperature played a significant role, the ANOVA of the TSSY data at the 20 mg/g enzyme loading showed that all three variables were significant at a 95% confidence interval (Table 15). It was apparent that the higher enzyme loading improved the correlation between the variables time, temperature and time and the TSSY, which was the response variable (Figure 16). Similar to the results at 10 mg/g, the amount of residual xylan in the water insoluble fraction played a key role in influencing the ease of hydrolysis of the substrates (Figure 15).

The centrepoint condition (conditions 13, 14, and 15), which had an intermediate recovery of xylan in the water insoluble fraction, was hydrolyzed using a cellulase cocktail supplemented with xylanases while keeping the protein loading of the enzyme cocktail at 20 mg/g. Since the xylan content in the water insoluble fraction seemed to correlate with a decrease in enzymatic hydrolysis yields, it was anticipated that the addition of xylanases would solubilize the xylan in the water insoluble fraction and work synergistically with cellulases to improve the conversion of the cellulose component (Hu et al., 2011). Condition 14, which had 13% xylan content was used for the hydrolysis experiments and it was expected that the supplementation of the enzyme cocktail with

xylanases would allow for a substantial improvement since previous work has shown that the effects of xylanase supplementation are more amplified with substrates that contain a greater amount of xylan (Bura et al., 2009).

Since the 20 mg/g protein loadings employed in this study were substantially lower than the 35 mg/g loading utilized by Hu et al. (2011) it was anticipated that the ratio of the xylanase protein to cellulase of 7:1 would need to be altered to ensure sufficient cellulase activity was present in the enzyme cocktail. Therefore ratios of cellulase to xylanase protein were varied from 4 mg:16 mg, 10 mg:10 mg and 16 mg: 4 mg. It was found that using a ratio of 16 mg of cellulase and 4 mg of xylanase resulted in glucose and xylose hydrolysis yields of 72% and 98%, respectively, which represents an increase of 22% and 55% in the enzymatic hydrolysis of these sugars compared to the control that used 20 mg of cellulase (Figure 14). The total soluble sugars yield was 72%, which is comparable to the maximum soluble sugars that were obtained with condition 8 (190°C, 8 min and 1.5% H_2SO_4) using 20 mg of cellulase. The substrate at condition 8 underwent 46% xylose degradation during pretreatment compared to 69% recovery of xylose obtained with condition 14. Therefore, optimizing the enzymatic hydrolysis with xylanases would be an alternative approach to increase sugar recovery since the pretreatment severity can be reduced while achieving a good hydrolysis conversion yield at moderately low enzyme loadings.

However, it should be noted that the TSSY of 72% did not surpass the TSSY of 75% that was obtained for the substrate from condition 8 that was pretreated at a high severity. It was apparent that, although the addition of xylanases allows for lower severity pretreatment conditions to be utilized, the overall soluble sugar recovery remains

approximately the same since the cellulose conversion decreases at the expense of increased xylan recovery (Figure 14).

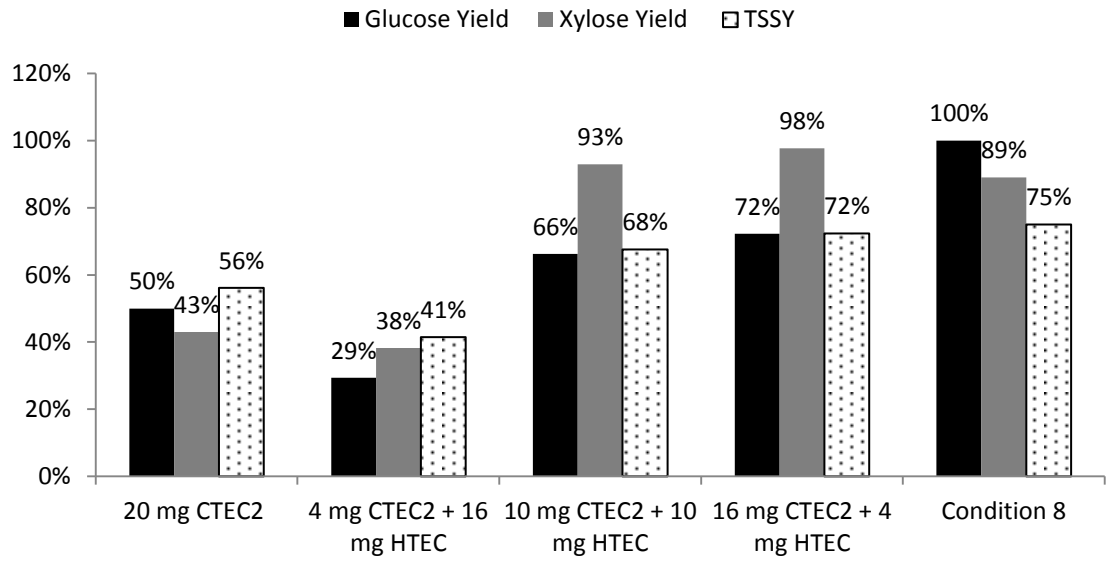

Figure 14 Enzymatic hydrolysis of wheat straw substrate pretreated at a medium severity (190°C, 5 min, 0.75% H_2SO_4) using a combination of Ctec2 and Htec ratios.
Black and grey bar graphs shows glucose yield and xylose yield after enzymatic hydrolysis for 48 hours at 10% solids and a total protein loading of 20 mg protein/g glucan. Experiments were done with three different cellulase (Ctec2) to xylanase (Htec) ratios (4:16, 10:10, 16:4). Condition 8 is included for comparison. Dotted bar graph shows TSSY (total soluble sugar yields).

Table 12 Total Soluble Sugar Yields (TSSY) at 10% solids hydrolysis at a 10 mg protein/g glucan loading

Conditions	Temp (°C)	Time (min)	H_2SO_4 (% w/w)	Glucose and Xylose from raw material (g)	Glucose and Xylose after pretreatment and enzymatic hydrolysis (g)	TSSY (%)
1	170	2	0.75	50.4	9.2	18%
2	170	8	0.75	50.4	14.7	29%
3	210	2	0.75	50.4	23.4	46%
4	210	8	0.75	50.4	20.0	40%
5	190	2	0	50.4	7.7	15%
6	190	2	1.5	50.3	24.2	48%
7	190	8	0	50.3	24.0	48%
8	190	8	1.5	50.4	28.3	56%
9	170	5	0	48.9	8.5	17%
10	210	5	0	48.8	24.7	51%
11	170	5	1.5	50.4	14.2	28%
12	210	5	1.5	50.4	27.0	54%
13	190	5	0.75	50.4	20.5	41%
14	190	5	0.75	50.4	22.6	45%
15	190	5	0.75	50.4	25.2	50%

Table 13 ANOVA for Total Soluble Sugar Yields (TSSY) at 10 mg protein/g glucan

	Df	Sum of Squares	Mean Square	F-ratio	p-Value
Temp	1	0.12	0.12	16.75	0.009
Time	1	0.02	0.02	3.50	0.120
H_2SO_4	1	0.04	0.04	5.41	0.068
Temp*Temp	1	0.02	0.02	3.44	0.123
Temp*Time	1	0.01	0.01	1.11	0.341
Temp*H_2SO_4	1	0.00	0.00	0.23	0.653
Time*Time	1	0.01	0.01	0.73	0.432
Time* H_2SO_4	1	0.01	0.01	2.11	0.207
H_2SO_4* H_2SO_4	1	0.00	0.00	0.01	0.941
Error	5	0.0351	0.0070		
Total	14	0.27			

Table 14 Total soluble sugar yields (TSSY) at 10% solids hydrolysis at a 20 mg protein/g glucan

Conditions	Temp (°C)	Time (min)	H_2SO_4 (% w/w)	Glucose and Xylose from raw material (g)	Glucose and Xylose after pretreatment and enzymatic hydrolysis (g)	TSSY (%)
1	170	2	0.75	50.4	12.8	25%
2	170	8	0.75	50.4	27.2	54%
3	210	2	0.75	50.4	32.1	64%
4	210	8	0.75	50.4	32.6	65%
5	190	2	0	50.4	16.9	34%
6	190	2	1.5	50.3	33.7	67%
7	190	8	0	50.3	33.2	66%
8	190	8	1.5	50.4	37.8	75%
9	170	5	0	48.9	13.6	28%
10	210	5	0	48.8	34.6	71%
11	170	5	1.5	50.4	24.3	48%
12	210	5	1.5	50.4	36.9	73%
13	190	5	0.75	50.4	29.1	58%
14	190	5	0.75	50.4	35.6	71%
15	190	5	0.75	50.4	32.9	65%

Table 15 ANOVA for Total Soluble Sugar Yields (TSSY) at 20 mg protein/g glucan

	Df	Sum of Squares	Mean Square	F-ratio	p-Value
Temp	1	0.18	0.18	41.57	0.001
Time	1	0.03	0.03	7.68	0.039
H_2SO_4	1	0.04	0.04	9.88	0.026
Temp*Temp	1	0.02	0.02	3.54	0.119
Temp*Time	1	0.00	0.00	0.96	0.372
Temp* H_2SO_4	1	0.00	0.00	0.57	0.485
Time*Time	1	0.01	0.01	1.18	0.327
Time* H_2SO_4	1	0.01	0.01	2.50	0.174
H_2SO_4* H_2SO_4	1	0.01	0.01	1.34	0.299
Error	5	0.022	0.0044		
Total	14	0.33			

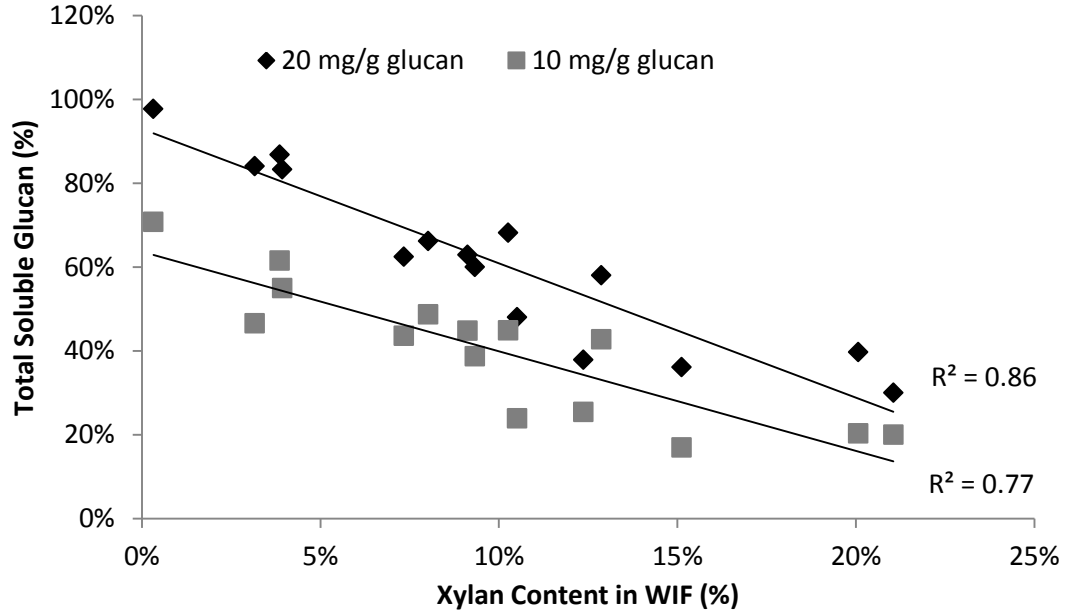

Figure 15 Relationship between xylan content in the solid and total soluble glucan yields at 10 mg protein/g glucan and 20 mg/g glucan, 10% solids

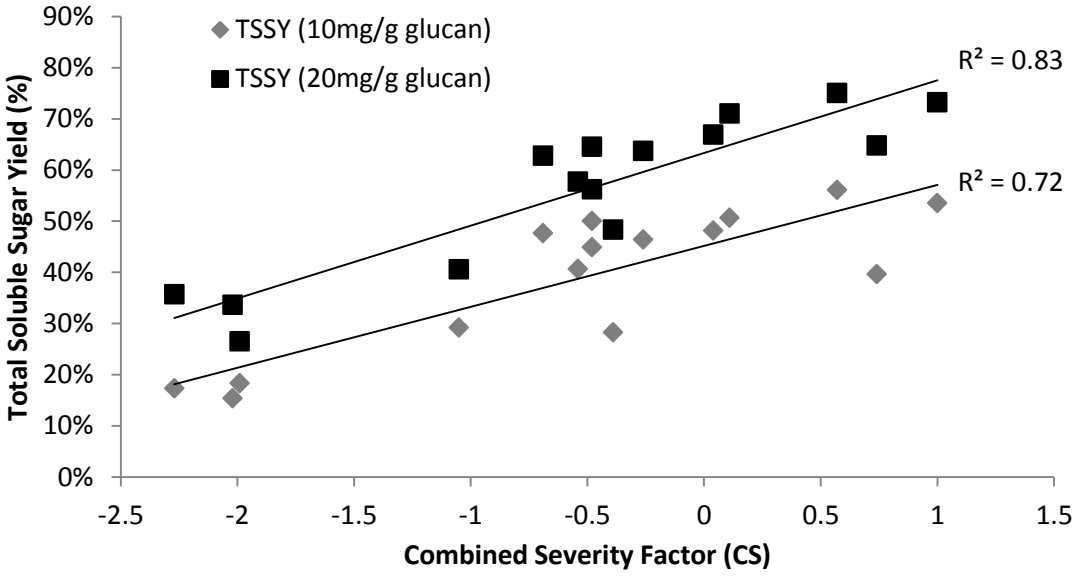

Figure 16 Relationship between total soluble sugar yield and combined severity factor (CS)

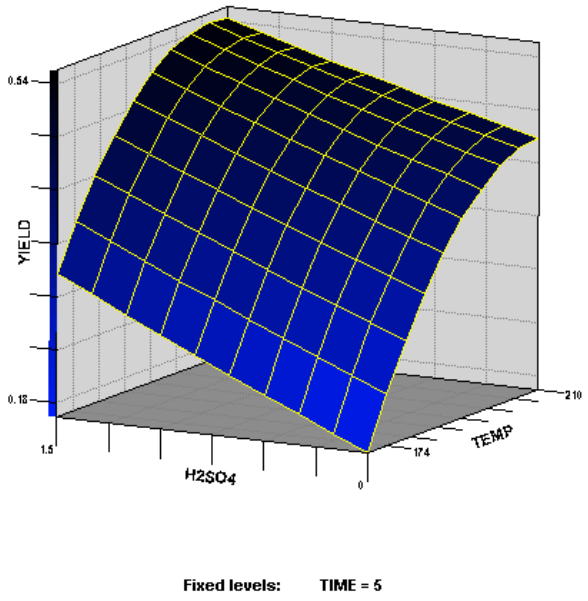

Figure 17 Effect of process variables on the total soluble sugar yields after pretreatment and enzymatic hydrolysis at 10 mg protein/g glucan.
The fixed variable is time at 5 minutes.

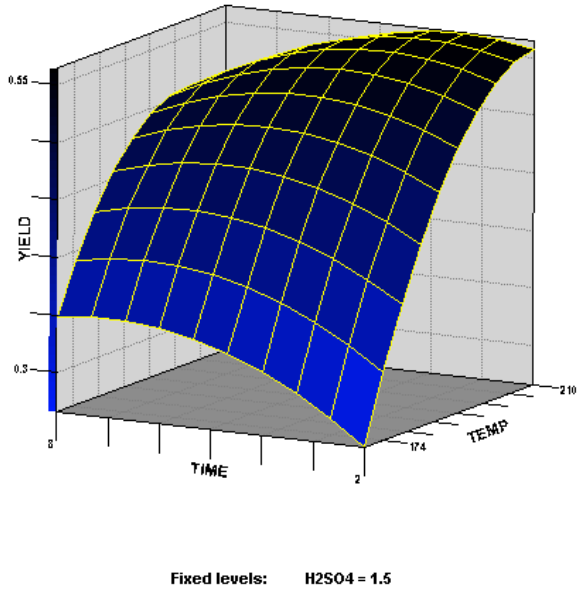

Figure 18 Effect of process variables on the total soluble sugar yields after pretreatment and enzymatic hydrolysis at 10 mg protein/g glucan.
The fixed variable is sulfuric acid loading at 1.5%.

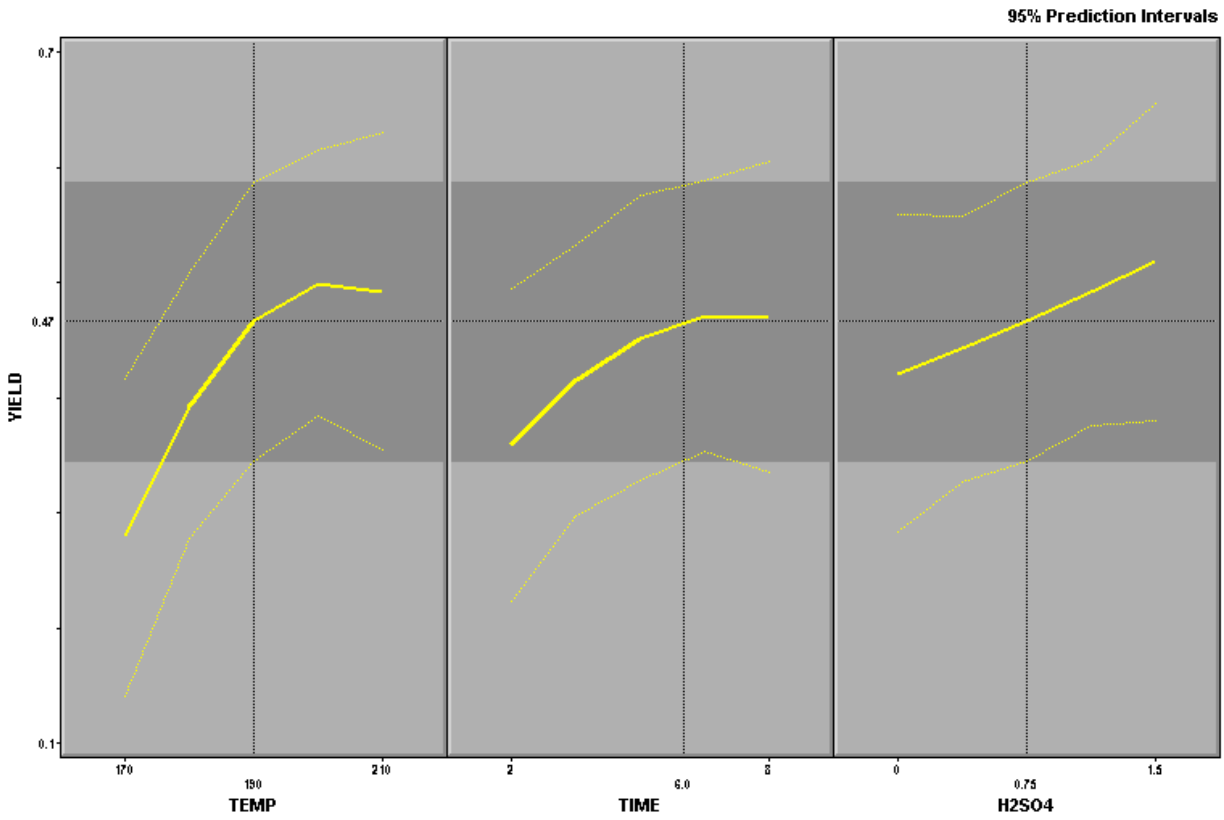

Figure 19 Effect of process variables on the total soluble sugar yields after pretreatment and enzymatic hydrolysis at 10 mg protein/g glucan.

3.3.5. Discussion

Although many studies have looked at the effectiveness of enzymatic hydrolysis yields and sugar recoveries after pretreatment and enzymatic hydrolysis (Ballesteros et al., 2006; Horn et al., 2011; Palmarola-Adrados et al., 2004; Petersen et al., 2009; Rosgaard et al., 2007), there are only a few that have used the total amount of sugars solubilized during the pretreatment and enzymatic hydrolysis processes to assess the effectiveness of the pretreatment conditions. Several of these previous studies equate a high sugar recovery in the combined water insoluble/soluble fractions as a so-called effective pretreatment, but high enzyme loadings are typically used to hydrolyze the resulting substrates and the actual amount of total soluble sugars are typically not clearly quantified. Ultimately, especially during acidic pretreatments such as steam which specifically solubilize hemicellulose, it is the soluble sugars after pretreatment which can be utilized for downstream processes. Therefore, in this study, the use of the total soluble sugar yield (TSSY) was shown to be a useful indicator to assess both the effectiveness of pretreatment and enzymatic hydrolysis in extracting the maximum possible amount of sugar from the biomass at low enzyme loadings. The various severities employed for the pretreatment showed significant differences in TSSY as the highest severity pretreatment that solubilized the xylan component also maximized enzymatic hydrolysis yield.

The steam pretreatment of the wheat straw conducted at the various conditions in this study were a clear illustration of the compromise between maximizing hemicellulose recovery and enabling efficient enzymatic hydrolysis at low enzyme loadings. It was apparent that the removal of hemicellulose from the water insoluble fraction during the steam pretreatment to improve enzymatic hydrolysis required conditions sufficiently

severe to also cause the degradation of the hemicellulose component. Although increasing the cellulase loading aided in increasing the total soluble sugar yield after pretreatment and enzymatic hydrolysis, the yields remained heavily influenced by the xylan content of the water insoluble fraction. Therefore, it may be beneficial to perform pretreatments at lower severities to maximize sugar recovery while using a combination of hemicellulases and cellulases to maximize the total soluble sugar yields after the pretreatment and hydrolysis processes.

The subsequent work in this thesis was performed prior to the work described thus far on wheat straw to gain insights into the alteration of biomass properties when sulfur dioxide is used as a catalyst. Although the limitations of experimental comparisons between sulfur dioxide and sulfuric acid as catalysts for steam pretreatments have been well described in the thesis thus far, SO_2 has also been shown to be an effective steam pretreatment catalyst. It was hypothesized that one of the possible reasons that previous work has demonstrated that SO_2 is an effective catalyst is its ability to potentially sulfonate the lignin component. Although sulfonation with SO_2 has been shown to be limited in the absence of an added base salt such as sodium, calcium, or magnesium, small amounts of sulfonation have been shown to have significant enhancing effects on hydrolysis yields (Sjöström, 1993). It has been suggested that the sulfonation of lignin increases hydrophilicity and swelling of the substrate thereby reducing hydrophobic interactions between cellulase enzymes and lignin and improving cellulose accessibility (Del Rio et al., 2011). It is also possible that the abundant ash component (5-10% ash content) of agricultural residues can provide adequate pH buffering capacity to facilitate sulfonation of the lignin component similar to a sulfite pulping reaction.

3.4. Effect of SO_2-catalyzed steam pretreatment on the physiochemical properties of wheat straw substrates

3.4.1. Rationale

There is limited information available regarding the mode of action of SO_2 during steam pretreatment. It has been suggested that, during pretreatment, SO_2 converts into HSO_3^- or SO_3 as in acid sulfite pulping (Sjöström, 1993) where the resulting SO_3 can ultimately result in the generation of H_2SO_4 (Schevchenko et al., 2000). However, it should be noted that several investigations comparing H_2SO_4 and SO_2 have shown that their effects on the substrates are different (Söderström et al., 2003; Tengborg et al., 1998). In general, SO_2-catalyzed steam pretreatment solubilizes the hemicellulose component and alters the lignin structure, while there is an increase in cellulose crystallinity (Kokta & Ahmed, 1998; Negro et al., 2003) and a decrease in the degree of polymerization (Ramos et al., 1992). In particular, it is evident in the case of agricultural residues such as corn stover and wheat straw that the solubilization of hemicellulose during steam pretreatment has been shown to result in an increase in enzymatic hydrolysis yields (Bura et al., 2009; Mussatto et al., 2008; Öhgren et al., 2007).

In addition to solubilizing hemicellulose, steam pretreatment with SO_2 imparts changes to the lignin structure including the initial cleavage of the β-O-4 bonds resulting in the formation of free phenolic hydroxyl groups with a concomitant condensation and flow of lignin above its glass transition temperature that results in the redistribution of lignin droplets at the surface of steam pretreated substrate fibers (Donaldson et al., 1988; Hemmingson, 1986; Toussaint et al., 1991). There have been several studies showing the

changes in the relative amounts of hemicellulose, lignin and cellulose that the biomass undergoes during SO_2 catalyzed steam pretreatment of agricultural residues. However, there have been far fewer studies on the changes in the physiochemical characteristics imparted by SO_2 that have been implicated in affecting the ease of enzymatic hydrolysis of steam pretreated substrates such as, swelling, porosity, fiber charge, cellulose crystallinity and degree of polymerization. Understanding the substrate characteristics that must be altered during pretreatment to maximize enzymatic hydrolysis yields at the lowest possible enzyme loadings could allow for a targeted pretreatment approach geared to yield specific substrate characteristics without having to sacrifice product recovery. It is recognized that the measurement of the chemical composition of the substrates alone without assessing the physiochemical characteristics of the substrate does not usually reflect the ease of hydrolysis of pretreated lignocellulosic substrates. For example, a purely cellulosic substrate such as Avicel has been shown to reach lower enzymatic hydrolysis yields than those obtained during the enzymatic hydrolysis of organosolv pretreated softwood which contains up to 20% lignin (Del Rio et al., 2009; Nakagame et al., 2010).

As previously mentioned, it has been shown that the removal of xylan during the pretreatment of agricultural biomass increases substrate digestibility. However, the impact of hemicellulose removal on the physiochemical properties of the substrate and the mechanism for the resulting enhancement of enzymatic hydrolysis remains unclear. For example, increases in fiber swelling as measured by the water retention value are usually accompanied by an enhancement in enzymatic hydrolysis yields presumably due to the swelling-induced increases in substrate accessibility to the enzymes (Ogiwara &

Arai, 1968). Considering the beneficial effects of fiber swelling to enzymatic hydrolysis it would be expected that the presence of hemicellulose, which is a hydrophilic polymer shown to contribute to fiber swelling properties of lignocellulosic substrates (Pejic et al., 2008), would enhance enzymatic hydrolysis (Vansteenkiste et al., 2004). However, hemicellulose removal has actually been shown to result in increases in cellulose accessibility, possibly due to hemicellulose acting as a physical barrier to the cellulose during enzymatic hydrolysis (Bura et al., 2009; Chandra et al., 2011). Therefore, in addition to quantifying the amounts of hemicellulose, cellulose and lignin in pretreated substrates, it is also important to measure the physiochemical properties of the substrate to gain further insight into the changes to the biomass that occur during pretreatment.

The actual effects of sulfur dioxide during steam pretreatment on the characteristics of the substrate have also yet to be elucidated. In addition to facilitating the removal of hemicellulose at lower severities to improve overall sugar recoveries, it has been hypothesized that the SO_2 catalyst may also act to sulfonate the lignin component of the substrate (Clark et al., 1989). The sulfonation of substrate lignin has been shown to be beneficial to the subsequent enzymatic hydrolysis likely due to reductions in non-productive binding of cellulases to lignin (Del Rio et al., 2011; Kumar et al., 2011). In the pulp and paper industry, sulfonation has been shown to occur through the utilization of a base salt in combination with sulfur dioxide to bring the pH of the cooking liquor above 3 to prevent a so-called "black cook" which results in lignin condensation. However, sulfonation has been shown to occur at a pH as low as 1-2 (Fengel & Wegener, 1984) so it is entirely possible that the addition of SO_2 could result in lignin sulfonation which has prompted previous investigations of the acetone soluble

lignin component (Hemmingson, 1986) and the liquid streams after SO_2 catalyzed steam pretreatment (Clark et al., 1989).

As well as previous work on SO_2 catalyzed steam pretreatment, novel pulping strategies such as SO_2-ethanol-water have shown that the biomass undergoes sulfonation and the generation of soluble lignosulfonates in the resulting process streams (Iakovlev & van Heiningen, 2011). Clark et al. (1989) suggested that sulfur may be incorporated in the lignin after steam pretreatment but the potential sulfonation of the substrate was assessed through the measurement of the sulfonate content of the water soluble fraction rather than a measurement of the sulfonation of the residual lignin of the water insoluble fraction. Hemmingson (1986) examined the properties of isolated lignin from steam pretreated *Pinus radiata* wood and did not find evidence of sulfonation during the SO_2 catalyzed steam pretreatment. However, these workers employed acetone to extract the lignin from the steam pretreated substrate and thus may have measured a smaller sub-fraction of the total lignin component of the substrate rather than a quantification of the bulk sulfonic acid groups on the substrate.

Both the occurrence of sulfonation during the SO_2 catalyzed steam pretreatment of biomass and the resulting effects of SO_2 itself on the physiochemical properties of the substrate remain unclear. Therefore, the objective of this study was to employ varying SO_2 loadings during the steam pretreatment of wheat straw to elucidate the changes undergone by the biomass during the SO_2 facilitated removal of hemicellulose, and the relationship between the resulting substrate characteristics and the ease of subsequent enzymatic hydrolysis of the substrate cellulose component.

3.4.2. Effect of SO_2 on substrate properties

As mentioned earlier, it is likely that the removal of hemicellulose and potential sulfonation of the substrate upon addition of SO_2 will alter the physical and chemical substrate characteristics such as accessibility and charge. The SO_2 induced changes on the physical and chemical properties of the substrates where then related to the ease of enzymatic hydrolysis to determine the key attributes of the substrate responsible for the enhancement of enzymatic hydrolysis yields. We initially varied the level of sulfur dioxide loading from 0-10% to determine if high loadings of sulfur dioxide changed the overall sugar and lignin recovery and if it could result in substrate changes and lignin sulfonation (Table 16 and Table 17).

Table 16 Chemical composition and enzymatic hydrolysis yields of the water insoluble fraction of steam pretreated wheat straw substrates.
Recovery of galactan and mannan was beyond detectable limits (bdl).

% SO_2	Arabinan	Glucan	Xylan	Galactan	Mannan	AIL	Ash	24 h Glucose Hydrolysis Yields
0%	0.43%	61%	5.90%	bdl	bdl	28.20%	3.46%	42%
1%	0.18%	68%	2.66%	bdl	bdl	28.90%	4.88%	53%
3%	0.18%	65%	2.35%	bdl	bdl	28.90%	4.65%	56%
5%	0.15%	62%	0.38%	bdl	bdl	29.90%	5.52%	62%
10%	0.13%	66%	0.26%	bdl	bdl	32.68%	4.38%	75%

It was evident that, as the SO_2 loading on the substrate prior to steam pretreatment was increased, the xylan content of the substrate decreased (Table 16). Consequently, the removal of hemicellulose also resulted in an increase in enzymatic hydrolysis yields which has also been observed previously during the steam pretreatment of agricultural residues such as corn stover (Bura et al., 2009; Chandra et al., 2011; Öhgren et al., 2007). It is likely that the enzymatic hydrolysis yields increased as the SO_2 loading during the

pretreatment was raised due to the increased acidity of the pretreatment condition. When acidity is taken into account, the increase in SO_2 loading results in an elevated combined pretreatment severity (Table 16) (Chum et al., 1990; Kabel et al., 2007). Arabinoxylan and glucuronoarabinoxylan are the predominant hemicelluloses present in agricultural residues such as corn stover and wheat straw (Scheller & Ulvskov, 2010). Consistent with this, both the xylan and arabinan underwent the most significant changes as the SO_2 loading was raised. Although the hemicellulose component of agricultural residues such as wheat straw contain acetyl groups which can participate in "autocatalysis" reactions during steam pretreatment, the addition of SO_2 to steam pretreatment has been shown to improve the selectivity of the pretreatment enabling the removal of hemicellulose at less severe conditions (Chandra et al., 2007; Toussaint et al., 1991), thus improving both hemicellulose recovery and the ease of hydrolysis of the resulting substrate.

The increase in AIL (acid insoluble lignin) at 10% SO_2 may be due to the severity of the pretreatment condition (Table 16) as it has been noted (Negro et al., 2003; Ramos, 2003; Sannigrahi et al., 2011) that at higher severities, condensation and degradation reactions of lignin and hemicellulose produce acid insoluble polymers referred to as "pseudo-lignin" that increase the amount of AIL in the Klason analysis. Indeed, it has been shown that dilute acid pretreatment of pure cellulose results in the formation of "pseudo-lignin" structures that are measured as acid insoluble lignin during the Klason lignin analysis (Sannigrahi et al., 2011).

In addition to changes to the chemical composition of the substrate, the pretreatment induces alterations to the biomass structure that can influence subsequent enzymatic hydrolysis. These physiochemical properties of the substrate include porosity,

swelling, charge, particle size, cellulose crystallinity and degree of polymerization. The crystallinity of the substrates as measured by FT-IR did not change significantly with the increased SO_2 loading (Table 17). It seems that the measured crystallinity did not correlate with enzymatic hydrolysis yields. The effects of crystallinity on enzymatic hydrolysis of lignocellulosic substrates remain inconclusive, whereas there seems to be a correlation between crystallinity and extent of hydrolysis in artificial pure cellulosic substrates (Hall et al., 2010). Unlike crystallinity, the particle size and the degree of polymerization of cellulose as estimated by cellulose viscosity underwent substantial changes. Particle size as measured by the Fibre Quality Analyzer (FQA) decreased as the SO_2 loading was increased (Table 17), which is comparable to previous work (Chandra et al., 2009).

Viscosity, which is an indirect measurement of the degree of polymerization, also decreased with increased SO_2 loadings (Table 17). For the samples with higher hemicellulose contents (5.9% to 2.4% xylan content), we extracted the hemicellulose from the samples so as to limit the potential skewing of the viscosity measurements toward lower values due to the presence of low molecular weight hemicellulose. The reduction in cellulose viscosity was also accompanied by an increase in yields during subsequent enzymatic hydrolysis. Although other substrate properties related to cellulose accessibility were not measured, previous work on the organosolv pretreatment of lodgepole pine has shown that decreases in cellulose viscosity were indicative of increased hydrolysis yields (Pan et al., 2007). Indeed, it is conceivable that the increase in free cellulose chain ends as a result of decreases in the degree of polymerization may actually increase reaction sites for exoglucanases such as Cel 7a (Wood, 1975). However,

it is unlikely that the decrease in cellulose viscosity alone can be considered a key indicator of cellulose accessibility. Cellulolytic enzymes require access to the cellulose macromolecule to catalyze hydrolysis and previous studies using substrates of similar degree of polymerization have shown the increases in substrate accessibility and decreases in crystallinity were controlling the ease of hydrolysis of cellulose (Hall et al., 2010; Jeoh et al., 2007).

It is interesting to note that the reduction in particle size of the substrates was also accompanied by a decrease in the viscosity of cellulose which correlated linearly with an R^2 value of 0.88 (Figure 20). The relationship between the degree of polymerization of cellulose and fiber length remains unclear and is counterintuitive as the degree of polymerization of cellulose is a property influenced at the molecular level whereas the particle/fiber size is a macroscopic characteristic. A similar relationship between fiber length and viscosity has been found for sulfite and kraft pulps (Lapierre et al., 2006; Lapierre et al., 2009; Lapierre et al., 2009). However, this relationship has yet to be reported in the case of steam pretreated substrates. It has been suggested that either acid induced kinks in fibers result in fiber breakage and degradation of cellulose or that the localized acidic attack of the cellulose results in decreases in DP and results in breakage of fibers when they are exposed to physical stress (Lapierre et al., 2009).

It is likely that in addition to reducing the particle size and decreasing the cellulose viscosity, the increased SO_2 loading also contributed to the increased values obtained for the adsorption of the Direct Orange dye of the Simon's Stain indicating that the higher severity pretreatments increased substrate accessibility (Table 17). Similar to previous work utilizing the Simon's staining technique, the adsorption of the dye also

reflected the ease of hydrolysis of the pretreated substrates (Chandra et al., 2009; Esteghlalian et al., 2001). Although the SS test involves the competitive adsorption between the large >100kDa Direct Orange dye and the small 992 Da Direct Blue dye, only the Direct Orange dye was utilized in this study as it has been estimated that the size of the Direct Orange dye is in the same range (7-32 Å) of the rate limiting pore size of 51 Å for cellulose hydrolysis (Grethlein, 1985). Another characteristic that may have contributed to the increase in Direct Orange dye adsorption is the decrease in xylan content of the substrates, which has been shown to both increase accessibility to cellulases and to the Direct Orange dye (Chandra et al., 2011) which results in higher cellulose hydrolysis yields (Table 17).

It is evident that the increases in accessible surface area of the pretreated substrates as measured by the adsorption of the Direct Orange dye were likely influenced by increases in substrate porosity (internal surface area), decreases in particle size (external surface area) and the extent of hemicellulose removal that results in an increased exposure of cellulose. Hemicellulose may have a dual role as increases in hemicellulose content can increase substrate swelling yet reduce access to cellulose thereby decreasing enzymatic hydrolysis yields. To test this hypothesis, the swelling capacity of the substrates was assessed using the Water Retention Value (WRV) method to determine the relationship between hemicellulose removal and swelling. The WRV of the substrates decreased as xylan was removed from the substrate. However, it was shown that the enzymatic hydrolysis yields tended to decrease with increased WRV (Figure 21). These findings agree with some observations in the literature which suggest that substrate swelling as measured by the WRV can be used to predict the ease of

enzymatic hydrolysis (Luo & Zhu, 2011; Ogiwara & Arai, 1968). For example, in the study by Ogiwara and Arai (1968) the substrates used were bleached pulps used for paper making. The chemical composition of the substrates was not provided, so it was not possible to determine the effect of hemicellulose on the WRV measurements. Luo et al. (Luo & Zhu, 2011) also found that WRV was a good method to predict the ease of hydrolysis but in their study the same substrate was subjected to different degrees of hornification by drying. Hornification is the irreversible loss of the swelling capacity of the fibers when they are rewetted (Hubbe et al., 2007). Therefore, it is expected that the WRV would be sensitive enough to detect changes in swelling due to hornification especially since the WRV has been used to extensively characterize the ease of recycling of pulps utilized in papermaking (Hubbe et al., 2007; Wan et al., 2010).

Our results show that hemicellulose removal decreases the swelling capacity of the fibers. Swelling depends on several fiber properties such as the type of chemical treatment, ionic interactions, lignin content, hemicellulose content, the pH of the medium and the nature of the solvent (Carlsson et al., 1983; Eriksson et al., 1991; Luukko & Maloney, 1999; Ogiwara & Arai, 1968). Hemicellulose can be considered as an absorbent material which has resulted in intensified investigations for the potential utilization in hydrogels for wound dressings, as an additive in paper-making, as food gums, gelatin replacement and fat substitute in cheese (Ebringerová, 2006). Therefore, the hydrophilic nature of hemicellulose and its partial solubility have an important role in increasing swelling. It has also been demonstrated previously that the removal of hemicellulose results in a decrease in WRV values with hemp fibers (Pejic et al., 2008) as well as in other studies (Eriksson et al., 1991; Wan et al., 2010).

It is evident that the utility of the WRV for predicting the ease of hydrolysis was highly dependent on the presence of hemicellulose. Although increased swelling has been shown to be beneficial for enhanced hydrolysis yields, the manner in which the increased swelling is achieved seems to be a key factor. In the case of hemicellulose such as xylan, increased xylan can increase substrate swelling. However, the increased hemicellulose can also actually decrease the accessibility of enzymes to the cellulose component. Similarly, during organosolv pretreatment of lodgepole pine, it was shown that the substrates that retained the greatest amount of hemicellulose during pretreatment also had the highest WRV and consequently also had reduced hydrolysis yields compared to substrates with lower xylan content and WRV (Del Rio et al., 2009). These results strongly suggest that the "swelling" of the substrate as measured by its water retaining capacity may not be equivalent to increased accessibility to the cellulose component. The WRV estimates the adsorption of water on the substrate and this can be influenced by the presence of hydrophilic polymers such as hemicellulose, whereas methods like Simon's Stain rely on the adsorption of a probe to cellulose, which is a better indicator of cellulose accessibility.

Another factor that could possibly influence the hydrophilicity and enzyme-substrate interaction during hydrolysis is the possible increases in acid catalyzed lignin condensation occurring during the pretreatment as the sulfur dioxide loading was raised. Increased lignin condensation as a result of raising pretreatment severity has been shown to increase the likelihood of non-productive binding of cellulases to lignin during enzymatic hydrolysis (Nakagame et al., 2011b). Regardless of the increased lignin condensation, these workers observed that the substrates pretreated at higher severity

achieved greater yields during enzymatic hydrolysis likely due to the removal of hemicellulose and increases in cellulose accessibility during pretreatment (Nakagame et al., 2011b). However, in this work the increases in substrate accessibility generated at higher pretreatment severity were not measured. It should also be noted that the lignin component in agricultural residues may be quite different than that of softwoods, as unlike softwoods (Kumar et al., 2011), the acid groups in the substrates of this study also increased at higher pretreatment severities.

Table 17 Substrate characteristics of steam pretreated wheat straw substrates. LOI = Lateral Order Index, ratio of the 1428 cm^{-1} and 898 cm^{-1} peaks.

SO_2 loading (%)	Sulfonic Acid Content (meq/g)	Carboxylic Acid content (meq/g)	Crystallinity LOI	Viscosity mPa·s	Particle size (mm)	Simon's Stain (mg dye/g fibre)
0%	0	39	0.91	12.50	0.76	54
1%	0	59	0.95	6.17	0.64	50
3%	0	55	1.01	4.32	0.61	74
5%	0	57	1.04	3.19	0.51	68
10%	0	77	1.00	2.57	0.47	81

In addition to assessing the effects of SO_2 on the characteristics of the pretreated substrates that may influence their ease of subsequent enzymatic hydrolysis, one of the key questions that we wanted to address in this study that has thus far remained unclear in the literature was if the substrate undergoes sulfonation during sulfur dioxide catalyzed pretreatment. Previous studies either measured only the liquid fraction after pretreatment (Clark et al., 1989) or only an acetone-extracted fraction of the lignin component (Hemmingson, 1986). When compared to sulfuric acid, sulfur dioxide has been shown to improve enzymatic hydrolysis while sulfuric acid has been shown to improve hemicellulose extraction (Söderström et al., 2003) while sulfonation post treatment has

been shown to result in pronounced improvements to enzymatic hydrolysis yields for both steam and organosolv pretreated substrates (Del Rio et al., 2011; Kumar et al., 2011). Indeed, in a partially aqueous system, the sulfonation of lignin has been shown to occur during sulfur dioxide-ethanol-water biomass fractionation. The conductometric titration method was used to measure bulk sulfonic and carboxylic acid groups on the substrate. Despite increases in the sulfur dioxide loading to 10% on the biomass during pretreatment, sulfonate groups were not found on the substrate. However, the carboxylic acid content of the substrate increased as the SO_2 loading was raised, which may partially explain the increase in hydrolysis yields with SO_2 addition. This is an interesting result since carboxylic acid groups have been shown to both increase substrate swelling (Carlsson et al., 1983; Chandra et al., 2011; Chandra et al., 2004; Gellerstedt & Gatenholm, 1999), and hydrophilicity and reduce the non-productive binding of cellulases to lignin (Nakagame et al., 2011a).

Wheat straw contains coumaric and ferulic acids which cross link the hemicellulose component and lignin via ester or ether bonds (Buranov & Mazza, 2008; Scheller & Ulvskov, 2010). Therefore, the increases in the carboxylic acid groups as the sulfur dioxide loading was increased may be a result of the acidic cleavage of these hemicellulose-lignin linkages thus leaving a carboxylic acid group as part of the pendant coumaric and ferulic acids that remain bound to the lignin component. In fact, IR analysis of isolated lignin from steam exploded wheat straw confirmed that the lignin component possessed both *p*-coumaric acid and ester-linked ferulic acid (Hongzhang & Liying, 2007). Previous work has also shown that the hydrophilic character imparted by the increased carboxylic acid functionalities of the lignin component of steam pretreated

agricultural residues compared to softwood lignin may be a significant factor responsible for the far less detrimental effect of the lignin component from corn stover on enzymatic hydrolysis compared to the lignin from steam pretreated softwood (Nakagame et al., 2011a). Thus, it is possible that the increase in carboxylic acid group content was beneficial to the enzymatic hydrolysis. It was evident that as the SO_2 loading was raised for the pretreatment of wheat straw in this study, hemicellulose removal, increases in cellulose accessibility, decreases in cellulose viscosity and increases in carboxylic acid groups were all likely contributors to the increased overall susceptibility of the substrate to enzymatic hydrolysis.

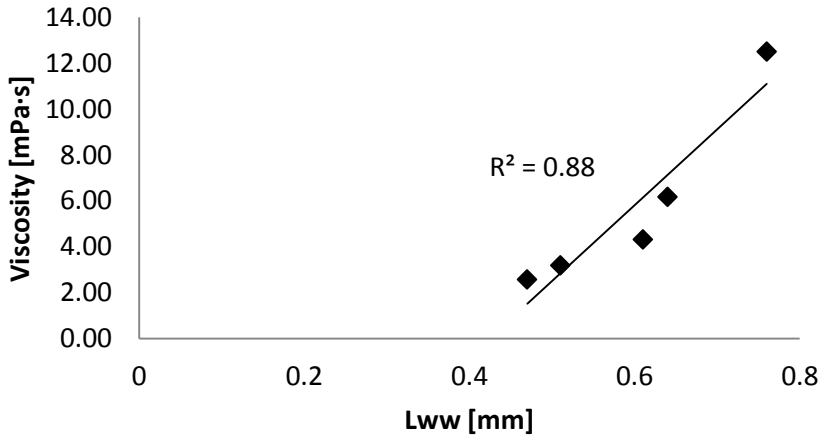

Figure 20 Relationship between cellulose viscosity and particle size of the fibers (length weighted average) of steam pretreated wheat straw substrates.

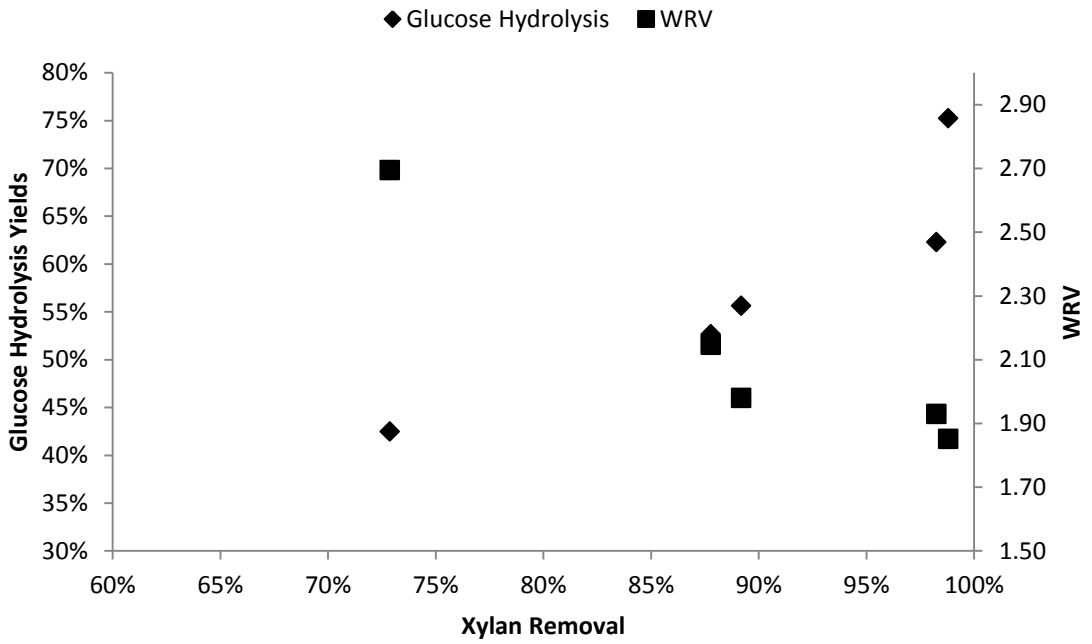

Figure 21 The effect of xylan removal (x-axis) on the swelling capacity of the substrates, as measured by the water retention value (WRV) (■) and on the glucose hydrolysis yields (♦) after 24 hours.
Reactions performed at 2% solids in a constant reaction volume of 1 mL and a 2:1 ratio of β-glucosidase to cellulase activity (IU:FPU), at pH 4.8 in 50 mM sodium acetate buffer, 50°C, 20 mg cellulase protein/g glucan (7.5 FPU/g glucan).

3.4.3. Discussion

This work was performed to elucidate the effects of adding sulfur dioxide as a catalyst during steam pretreatment on the properties of the resulting substrate. Increasing the SO_2 loading for steam pretreatment of wheat straw was not shown to result in sulfonation but rather increased the amount of bulk carboxylic acid groups likely due to an exposure of carboxylic functionalities from ferolylated and coumaric acid groups on lignin thus suggesting that SO_2 acts mainly to remove the hemicellulose from the substrate at lower steam pretreatment severities. The use of the WRV as an estimate of cellulose accessibility may be complicated when measuring substrates that contain a significant amount of hemicellulose such as those pretreated at autocatalyzed or alkaline conditions. The manner in which increased swelling of a cellulosic substrate is achieved should always be considered since swelling of the entire substrate and enhancement of cellulose accessibility to cellulolytic enzymes may not be correlated in every case.

4. Conclusions

One of the challenges in the bioconversion of agricultural residues is to achieve a compromise between sugar recovery, particularly of the labile hemicellulose fraction, and enzymatic hydrolysis of the water insoluble, cellulosic component at low enzyme loadings. In order to increase the sugar recovery from steam pretreated wheat straw we tested different biomass handling strategies that would allow us to increase the sugar recovery in the pretreatment step. It was found that increasing the moisture content and subjecting the biomass to explosive decompression led to a high recovery of glucose and xylose while maintaining a high glucan digestibility of 78% after a 72 hour enzymatic hydrolysis. We were able to obtain glucose and xylose recoveries of 91% and 86%, respectively. The use of a steel basket to prevent explosive decompression of the biomass resulted in a loss of approximately 50% of the hemicellulose sugars regardless of the moisture content treatment, so it was concluded that this method of biomass handling is not suitable for agricultural residues.

Previous works comparing SO_2 and H_2SO_4 as acid catalysts for steam pretreatment have based their comparisons at different pretreatment conditions or at equal catalyst loadings. However, the way in which the comparisons were made resulted in H_2SO_4 being a less effective catalyst because the conditions were much more severe with H_2SO_4 than for the pretreatments performed with SO_2. Thus, our comparison looked at both catalysts at the same pretreatment and enzymatic hydrolysis conditions while the only parameter that was altered was the amount of acid catalyst used. We found that 1.5% H_2SO_4 could produce a comparable sugar recovery of 90% glucose and 84% xylose

and a glucose enzymatic hydrolysis yield of 82% versus 87% glucose and 88% xylose recovery and an enzymatic hydrolysis yield of 83% for the substrate pretreated with 3% SO_2. This result is significant as it suggests that the mode of action of SO_2 and H_2SO_4 on the biomass might be different likely due to the lower pKa of the H_2SO_4 and that less acid could potentially be utilized for the steam pretreatment of agricultural residues.

Once it was determined that pre-wetting the substrate while subjecting it to explosive decompression with H_2SO_4 as an acid catalyst was favorable to increase sugar recovery during pretreatment, a Box-Behnken statistical design was used to evaluate a range of pretreatment conditions to determine the compromise between sugar recovery during pretreatment and enzymatic hydrolysis and overall yields. At an enzymatic hydrolysis of 10% solids and 10 mg protein/g glucan we were able to obtain 54% of the glucose and xylose (based on the raw material) at the expense of losing 18% of the glucose and 48% of the xylose during pretreatment. We were able to increase the overall sugar recovery to 74% by doubling the enzyme loading to 20 mg protein/g glucan. Thus, in order to achieve high overall sugar recovery after pretreatment and enzymatic hydrolysis some sugar hemicellulose loss is likely to occur. It was also evident that the enzymatic hydrolysis of the glucose was greatly influenced by the xylose content in the WIF. Therefore, it is beneficial to the overall process if most of the hemicellulose fraction can be solubilized during pretreatment so that the enzymatic hydrolysis step can be conducted at low enzyme loadings while obtaining high conversion yields. Since the recovery of hemicellulose in the WSF is difficult to achieve without degrading the sugars, a strategy that could be tested is the use of a two-stage pretreatment. In this approach the first step would target the hemicellulose fraction by pretreating the biomass at a low

temperature, with added acid catalyst, and the second stage would fractionate and increase the accessible surface area to cellulose by pretreating at a higher temperature. Alternatively, it would be possible to increase the recovery of the hemicellulose fraction in a one-stage pretreatment by decreasing the pretreatment severity and tailoring the enzyme cocktail so that there is a greater proportion of xylanases to solubilize the hemicellulose during enzymatic hydrolysis. It was demonstrated that a medium severity pretreatment could achieve soluble sugar yields of 72% after enzymatic hydrolysis with 16 mg of cellulase and 4 mg of xylanase, which was comparable to the 75% of soluble sugars achieved using a high severity pretreatment. Further improvement of the pretreatment and enzyme ratios could result in even higher soluble sugar yields since the medium severity pretreatment recovered 100% of the cellulose and 70% of the xylan fraction.

It proved difficult to make a comparison of our results with the yields reported for steam pretreated wheat straw in the literature as our measure of yield is based on glucose and xylose and others have reported their yields based on glucose alone or a combination of glucose, xylose and arabinose. However, a total yield of 75% sugars after a 48 hour hydrolysis is one of the better results that has been observed considering that 20 mg protein/g glucan was used for the enzymatic hydrolysis on unwashed substrates. This enzyme loading is significantly lower than what has been previously reported in the literature (Ballesteros et al., 2006; Palmarola-Adrados et al., 2004; Petersen et al., 2009) and to our knowledge this is the only statistical design in which the substrates were not washed prior to hydrolysis, therefore providing a more realistic assessment of the potential of wheat straw for bioconversion.

Finally, we conducted some preliminary work on the effects of SO_2 on the physiochemical properties of steam pretreated wheat straw substrates. We hoped to determine whether sulfonation occurred at the conditions regularly used for steam pretreatment. Although no sulfonation was detected, even at SO_2 loadings of 10%, it was found that the carboxylic acid content increased while other changes in the biomass such as a decrease in the viscosity and an increase in accessible surface area also occurred. As the SO_2 loading was increased the removal of hemicellulose increased, which could be one of the reasons as to why the enzymatic hydrolysis yields increased as the SO_2 loading went from 0-10%. The increased removal of hemicellulose was inversely related with the water retention values (WRV).

Bibliography

Aden, A., & Foust, T. (2009). Technoeconomic analysis of the dilute sulfuric acid and enzymatic hydrolysis process for the conversion of corn stover to ethanol. *Cellulose, 16*(4), 535-545.

Alfani, F., Gallifuoco, A., Saporosi, A., Spera, A., & Cantarella, M. (2000). Comparison of SHF and SSF processes for the bioconversion of steam-exploded wheat straw. *Journal of Industrial Microbiology and Biotechnology, 25*, 184-192.

Alvira, P., Tomás-Pejó, E., Ballesteros, M., & Negro, M. J. (2010). Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review. *Bioresource Technology, 101*(13), 4851-4861.

Balat, M., Balat, H., & Öz, C. (2008). Progress in bioethanol processing. *Progress in Energy and Combustion Science, 34*(5), 551-573.

Ballesteros, I., Negro, M. J., Oliva, J. M., Cabañas, A., Manzanares, P., & Ballesteros, M. (2006). Ethanol production from steam-explosion pretreated wheat straw. *Applied Biochemistry and Biotechnology, 129-132*, 496-508.

Bansal, P., Hall, M., Realff, M. J., Lee, J. H., & Bommarius, A. S. (2010). Multivariate statistical analysis of X-ray data from cellulose: A new method to determine degree of crystallinity and predict hydrolysis rates. *Bioresource Technology, 101*(12), 4461-4471.

Bardi, U. (2009). Peak oil: The four stages of a new idea. *Energy, 34*(3), 323-326.

Beringer, T., Lucht, W., & Schaphoff, S. (2011). Bioenergy production potential of global biomass plantations under environmental and agricultural constraints. *GCB Bioenergy, 3*(4), 299-312.

Berlin, A., Balakshin, M., Gilkes, N., Kadla, J., Maximenko, V., Kubo, S., & Saddler, J. (2006). Inhibition of cellulase, xylanase and ß-glucosidase activities by softwood lignin preparations. *Journal of Biotechnology, 125*(2), 198-209.

Berndes, G. (2010). *Bioenergy, land use change and climate change mitigation.*International Energy Agency (IEA).

Berrang-Ford, L., Ford, J. D., & Paterson, J. (2011). Are we adapting to climate change? *Global Environmental Change, 21*(1), 25-33.

Biermann, C. J. (1996). *Handbook of pulping and papermaking* (2nd ed.). London: Academic Press.

Bin, Y., & Hongzhang, C. (2010). Effect of the ash on enzymatic hydrolysis of steam-exploded rice straw. *Bioresource Technology, 101*(23), 9114-9119.

Bosch, P., Wallberg, O., Joelsson, E., Galbe, M., & Zacchi, G. (2010). Impact of dual temperature profile in dilute acid hydrolysis of spruce for ethanol production. *Biotechnology for Biofuels, 3*(15), 1-12.

Brownell, H. H., & Saddler, J. N. (1987). Steam pretreatment of lignocellulosic material for enhanced enzymatic hydrolysis. *Biotechnology and Bioengineering, 29*(2), 228-235.

Brownell, H. H., Yu, E., & Saddler, J. N. (1986). Steam-explosion pretreatment of wood: Effect of chip size, acid, moisture content and pressure drop. *Biotechnology and Bioengineering, 28*(6), 792-801.

Bura, R., Chandra, R., & Saddler, J. (2009). Influence of xylan on the enzymatic hydrolysis of steam-pretreated corn stover and hybrid poplar. *Biotechnology Progress, 25*(2), 315-322.

Buranov, A. U., & Mazza, G. (2008). Lignin in straw of herbaceous crops. *Industrial Crops and Products, 28*, 237-259.

Carlsson, G., Kolseth, P., & Lindström, T. (1983). Polyelectrolyte swelling behavior of chlorite delignified spruce wood fibers. *Wood Science and Technology, 17*(1), 69-73.

Carrasco, C., Baudel, H. M., Sendelius, J., Modig, T., Roslander, C., Galbe, M., Hahn-Hägerdal, B., Zacchi, G., & Lidén, G. (2010). SO2-catalyzed steam pretreatment and fermentation of enzymatically hydrolyzed sugarcane bagasse. *Enzyme and Microbial Technology, 46*(2), 64-73.

Carvalheiro, F., Silva-fernandes, T., Duarte, L. C., & Gírio, F. M. (2009). Wheat straw autohydrolysis : Process optimization and products characterization. *Applied Biochemistry and Biotechnology, 153*, 84-93.

Chandra, R. P., Au-Yeung, K., Chanis, C., Roos, A. A., Mabee, W., Chung, P. A., Ghatora, S., & Saddler, J. (2011). The influence of pretreatment and enzyme loading on the effectiveness of batch and fed-batch hydrolysis of corn stover. *Biotechnology Progress, 27*(1), 77-85.

Chandra, R. P., Bura, R., Mabee, W. E., Berlin, A., Pan, X., & Saddler, J. N. (2007). Substrate pretreatment: The key to effective enzymatic hydrolysis of lignocellulosics? *Advances in biochemical Engineering/Biotechnology* (pp. 67-93)

Chandra, R. P., Ewanick, S. M., Chung, P. A., Au-Yeung, K., Del Rio, L., Mabee, W., & Saddler, J. N. (2009). Comparison of methods to assess the enzyme accessibility and hydrolysis of pretreated lignocellulosic substrates. *Biotechnology Letters, 31*, 1217-1222.

Chandra, R., Lehtonen, L., & Ragauskas, A. (2004). Modification of high lignin content kraft pulps with laccase to improve paper strength properties. 1. laccase treatment in the presence of gallic acid. *Biotechnology Progress, 20*(1), 255-261.

Chang, V. S., & Holtzapple, M. T. (2000). Factors affection biomass enzymatic reactivity. *Applied Biochemistry and Biotechnology, 84-86*, 5-37.

Chum, H., Johnson, D., Black, S., & Overend, R. (1990). Pretreatment-catalyst effects and the combined severity parameter. *Applied Biochemistry and Biotechnology, 24-25*(1), 1-14.

Clark, T. A., Mackie, K. L., Dare, P. H., & McDonald, A. G. (1989). Steam explosion of the softwood pinus radiata with sulphur dioxide addition. *Journal of Wood Chemistry and Technology, 9*(2), 373-403.

Cullis, I. F., Saddler, J. N., & Mansfield, S. D. (2004). Effect of initial moisture content and chip size on the bioconversion efficiency of softwood lignocellulosics. *Biotechnology and Bioengineering, 85*(4), 413-421.

De Bari, I., Nanna, F., & Braccio, G. (2007). SO_2-catalyzed steam fractionation of aspen chips for bioethanol production: optimization of the catalyst impregnation. *Industrial & Engineering Chemistry Research, 46*(23), 7711-7720.

Del Rio, L. F., Chandra, R. P., & Saddler, J. N. (2009). The effect of varying organosolv pretreatment chemicals on the physicochemical properties and cellulolytic hydrolysis of mountain pine beetle-killed lodgepole pine. *Applied Biochemistry and Biotechnology, 161*(1-8), 1-21.

Del Rio, L., Chandra, R., & Saddler, J. (2011). The effects of increasing swelling and anionic charges on the enzymatic hydrolysis of organosolv-pretreated softwoods at low enzyme loadings. *Biotechnology and Bioengineering, 108*(7), 1549-1558.

Donaldson, L. A., Wong, K. K. Y., & Mackie, K. L. (1988). Ultrastructure of steam-exploded wood. *Wood Science and Technology, 22*, 103-114.

Dunn, J. P., Stenger Jr., H. G., & Wachs, I. E. (1999). Oxidation of sulfur dioxide over supported vanadia catalysts: Molecular structure – reactivity relationships and reaction kinetics. *Catalysis Today, 51*(2), 301-318.

Ebringerová, A. (2006). Structural diversity and application potential of hemicelluloses. *Macromolecular Symposium, 232*, 1-12.

Eggeman, T., & Elander, R. T. (2005). Process and economic analysis of pretreatment technologies. *Bioresource Technology, 96*, 2019-2025.

Eklund, R., Galbe, M., & Zacchi, G. (1995). The influence of SO_2 and H2SO4 impregnation of willow prior to steam pretreatment. *Bioresource Engineering, 52*, 225-229.

Elander, R. T., Dale, B. E., Holtzapple, M., Ladisch, M. R., Lee, Y. Y., Mitchinson, C., Saddler, J. N., & Wyman, C. E. (2009). Summary of findings from the biomass refining consortium for applied fundamentals and innovation (CAFI): Corn stover pretreatment. *Cellulose, 16*, 649-659.

Eriksson, I., Haglind, I., Lidbrandt, O., & Salmén, L. (1991). Fiber swelling favoured by lignin softening. *Wood Science and Technology, 25*, 135-144.

Esteghlalian, A. R., Bilodeau, M., Mansfield, S. D., & Saddler, J. N. (2001). Do enzymatic hydrolyzability and simon's stain reflect the changes in the accessibility of lignocellulosic substrates to cellulase enzymes? *Biotechnology Progress, 17*, 1049-1054.

Ewanick, S., & Bura, R. (2011). The effect of biomass moisture content on bioethanol yields from steam pretreated switchgrass and sugarcane bagasse. *Bioresource Technology, 102*(3), 2651-2658.

Fan, L. T., Lee, Y., & Gharpuray, M. M. (1982). The nature of lignocellulosics and their pretreatments for enzymatic hydrolysis. *Advances in Bichemical Engineering/Biotechnology, 23*, 157-187.

Fengel, D., & Wegener, G. (1984). *Wood: Chemistry, ultrastructure, reactions*. Berlin ; New York: W. de Gruyter.

Galbe, M., Sassne, P., Wingren, A., & Zacchi, G. (2007). Process engineering economics of bioethanol production. *Advances in Bichemical Engineering/Biotechnology, 108*, 303-327.

García-Aparicio, M. P., Ballesteros, M., Manzanares, P., Ballesteros, I., González, A., & Negro, M. J. (2007). Xylanase contribution to the efficiency of cellulose enzymatic hydrolysis. *Applied Biochemistry and Biotechnology, 136-140*, 353-365.

Gellerstedt, F., & Gatenholm, P. (1999). Surface properties of lignocellulosic fibers bearing carboxylic groups. *Cellulose, 6*, 103-121.

Ghose, T. K. (1987). Measurement of cellulase activities. *Pure and Applied Chemistry, 59*(2), 257-268.

Gírio, F. M., Fonseca, C., Carvalheiro, F., Duarte, L. C., Marques, S., & Bogel-Lukasik, R. (2010). Hemicelluloses for fuel ethanol: A review. *Bioresource Technology, 101*, 4775-4800.

Gnansounou, E. (2010). Production and use of lignocellulosic bioethanol in europe: Current situation and perspectives. *Bioresource Technology, 101*, 4842-4850.

Gosling, S. N., Warren, R., Arnell, N. W., Good, P., Caesar, J., Bernie, D., Lowe, J. A., van der Linden, P., O'Hanley, J. R., & Smith, S. M. (2011). A review of recent developments in climate change science. part II: The global-scale impacts of climate change. *Progress in Physical Geography, 35*(4), 443-464.

Grethlein, H. E. (1985). The effect of pore size distribution on the rate of enzymatic hydrolysis of cellulosic substrates. *Biotechnology, 3*, 155-160.

Hahn-Hägerdal, B., Galbe, M., Gorwa-Grauslund, M. F., Lidén, G., & Zacchi, G. (2006). Bio-ethanol – the fuel of tomorrow from the residues of today. *Trends in Biotechnology, 24*(12), 549-556.

Hahn-HäGerdal, B., Lindén, T., Senac, T., & Skoog, K. (1991). Ethanolic fermentation of pentoses in lignocellulose hydrolysates. *Applied Biochemistry and Biotechnology, 28-29*(1), 131-144.

Hall, M., Bansal, P., Lee, J. H., Realff, M. J., & Bommarius, A. S. (2010). Cellulose crystallinity - a key predictor of the enzymatic hydrolysis rate. *FEBS Journal, 277*, 1571-1582.

Hamelinck, C. N., Hooijdonk, G. v., & Faaij, A. P. (2005). Ethanol from lignocellulosic biomass: Techno-economic performance in short-, middle- and long-term. *Biomass and Bioenergy, 28*(4), 384-410.

Han, M., Moon, S., Kim, Y., Kim, Y., Chung, B., & Choi, G. (2009). Bioethanol production from ammonia percolated wheat straw. *Biotechnology and Bioprocess Engineering, 14*(5), 606-611.

Hemmingson, J. A. (1986). Exploded wood lignins from pinus radiata wood. the effect of SO2 pretreatment on structure. *Journal of Wood Chemistry and Technology, 6*(1), 113-125.

Hindrichs, R. A., & Kleinbach, M. (2013). *Energy: Its use and the environment* (5th ed.). Boston, MA: Brooks/Cole, Cengage Learning.

Hodge, D. B., Karim, M. N., Schell, D. J., & McMillan, J. D. (2008). Soluble and insoluble solids contributions to high-solids enzymatic hydrolysis of lignocellulose. *Bioresource Technology, 99*, 8940-8948.

Hongzhang, C., & Liying, L. (2007). Unpolluted fractionation of wheat straw by steam explosion and ethanol extraction. *Bioresource Technology, 98*(3), 666-676.

Horn, S. J., Nguyen, Q. D., Westereng, B., Nilsen, P. J., & Eijsink, V. G. H. (2011). Screening of steam explosion conditions for glucose production from non-impregnated wheat straw. *Biomass and Bioenergy, 35*(12), 4879-4886.

Hu, J., Arantes, V., & Saddler, J. N. (2011). The enhancement of enzymatic hydrolysis of lignocellulosic substrates by the addition of accessory enzymes such as xylanase: Is it an additive or synergistic effect? *Biotechnology for Biofuels, 4*(36), 1-13.

Huang, H., Ramaswamy, S., Al-Dajani, W., Tschirner, U., & Cairncross, R. A. (2009). Effect of biomass species and plant size on cellulosic ethanol: A comparative process and economic analysis. *Biomass and Bioenergy, 33*(2), 234-246.

Hubbe, M. A., Venditti, R. A., & Rojas, O. J. (2007). What happens to cellulosic fibers during papermaking and recycling? A review. *BioResources, 2*(4), 739-788.

Iakovlev, M., & van Heiningen, A. (2011). SO_2-ethanol-water (SEW) pulping: I. lignin determination in pulps and liquors. *Journal of Wood Chemistry and Technology, 31*(3), 233.

International Energy Agency. (2011). *Technology roadmap: Biofuels for transport.* Paris, France: IEA.

Jeoh, T., Ishizawa, C., Davis, M. F., Himmel, M. E., Adney, W. S., & Johnson, D. K. (2007). Cellulase digestibility of pretreated biomass is limited by cellulose accessibility. *Biotechnology and Bioengineering, 98*(1), 112-122.

Jørgensen, H., Vibe-Pedersen, J., Larsen, J., & Felby, C. (2007). Liquefaction of lignocellulose at high-solids concentrations. *Biotechnology and Bioengineering, 96*(5), 862-870.

Judt, M. (1993). Non-wood plant fibres, will there be a come-back in paper-making? *Industrial Crops and Products, 2*, 51-57.

Kabel, M. A., Bos, G., Zeevalking, J., Voragen, A. G. J., & Schols, H. A. (2007). Effect of pretreatment severity on xylan solubility and enzymatic breakdown of the remaining cellulose from wheat straw. *Bioresource Technology, 98*, 2034-2042.

Kahr, H., Jäger, A., & Lanzerstorfer, C. (2012). Bioethanol production from steam explosion pretreated straw. *Bioethanol,* Retrieved from http://www.intechopen.com/books/bioethanol/bioethanol-production-from-steam-explosion-pretreated-straw

Katz, S., Beatson, R. P., & Scallon, A. M. (1984). The determination of strong and weak acidic groups in sulfite pulps. *Svensk Papperstidning, 87*(6), 48-53.

Kerstetter, J. D., & Lyons, J. K. (2001). *Wheat straw for ethanol production in washington: A resource, technical, and economic assessment.* No. WSUCEEP201084 Olympia, WA: Washington State University Cooperative Extension Energy Program.

Kim, S., & Dale, B. E. (2004). Global potential bioethanol production from wasted crops and crop residues. *Biomass and Bioenergy, 26*(4), 361-375.

Kokta, B. V., & Ahmed, A. (1998). Steam explosion pulping. In R. A. Young, & M. Akhtar (Eds.), *Environmentally friendly technologies for the pulp and paper industry* (pp. 191-214). NY: John Wiley & Sons.

Kothari, U., & Lee, Y. (2011). Inhibition effects of dilute-acid prehydrolysate of corn stover on enzymatic hydrolysis of solka floc. *Applied Biochemistry and Biotechnology, 165*(5-6), 1391-1405.

Kristensen, J. B., Thygesen, L. G., Felby, C., Jørgensen, H., & Elder, T. (2008). Cell-wall structural changes in wheat straw pretreated for bioethanol production. *Biotechnology for Biofuels, 1*(1), 1-9.

Kristensen, J., Felby, C., & Jorgensen, H. (2009). Yield-determining factors in high-solids enzymatic hydrolysis of lignocellulose. *Biotechnology for Biofuels, 2*(1), 11.

Kristensen, J., Felby, C., & Jørgensen, H. (2009). Determining yields in high solids enzymatic hydrolysis of biomass. *Applied Biochemistry and Biotechnology, 156*(1), 127-132.

Kumar, L., Chandra, R., & Saddler, J. (2011). Influence of steam pretreatment severity on post-treatments used to enhance the enzymatic hydrolysis of pretreated softwoods at low enzyme loadings. *Biotechnology and Bioengineering, 108*(10), 2300-2311.

Kumar, R., & Wyman, C. E. (2009a). Effect of enzyme supplementation at moderate cellulase loadings on initial glucose and xylose release from corn stover solids pretreated by leading technologies. *Biotechnology and Bioengineering, 102*(2), 457-467.

Kumar, R., & Wyman, C. E. (2009b). Does change in accessibility with conversion depend on both the substrate and pretreatment technology? *Bioresource Technology, 100*(18), 4193-4202.

Lapierre, L., Bouchard, J., & Berry, R. (2006). On the relationship between fibre length, cellulose chain length and pulp viscosity of a softwood sulfite pulp. *Holzforschung, 60*(4), 372-377.

Lapierre, L., Bouchard, J., & Berry, R. (2009). The relationship found between fibre length and viscosity of three different commercial kraft pulps. *Holzforschung, 63*(4), 402-407.

Laser, M., Schulman, D., Allen, S. G., Lichwa, J., Antal, M. J., & Lynd, L. R. (2002). A comparison of liquid hot water and steam pretreatments of sugar cane bagasse for bioconversion to ethanol. *Bioresource Technology, 81*(1), 33-44.

Lashof, D. A., & Ahuja, D. R. (1990). Relative contributions of greenhouse gas emissions to global warming. *Nature, 344*(6266), 529-531.

Li, J., Gellerstedt, G., & Toven, K. (2009). Steam explosion lignins; their extraction, structure and potential as feedstock for biodiesel and chemicals. *Bioresource Technology, 100*, 2556-2561.

Li, J., Henriksson, G., & Gellerstedt, G. (2007). Lignin depolymerization/repolymerization and its critical role for delignification of aspen wood by steam explosion. *Bioresource Technology, 98*(16), 3061-3068.

Li, X., Mupondwa, E., Panigrahi, S., Tabil, L., Sokhansanj, S., & Stumborg, M. (2012). A review of agricultural crop residue supply in canada for cellulosic ethanol production. *Renewable and Sustainable Energy Reviews, 16*(5), 2954-2965.

Linde, M., Galbe, M., & Zacchi, G. (2006). Steam pretreatment of acid-sprayed and acid-soaked barley straw for production of ethanol. *Applied Biochemistry and Biotechnology, 130*(1), 546-562.

Linde, M., Jakobsson, E., Galbe, M., & Zacchi, G. (2008). Steam pretreatment of dilute H2SO4-impregnated wheat straw and SSF with low yeast and enzyme loadings for bioethanol production. *Biomass and Bioenergy, 32*(4), 326-332.

Luo, X., & Zhu, J. Y. (2011). Effects of drying-induced fiber hornification on enzymatic saccharification of lignocelluloses. *Enzyme and Microbial Technology, 48*(1), 92-99.

Luukko, K., & Maloney, T. C. (1999). Swelling of mechanical pulp fines. *Cellulose, 6*, 123-135.

Lynd, L. R., Weimer, P. J., van Zyl, W., & Pretorius, I. S. (2002). Microbial cellulose utilization: Fundamentals and biotechnology. *Microbiology and Molecular Biology Reviews, 66*, 506-577.

Mabee, W. E., & Saddler, J. N. (2010). Bioethanol from lignocellulosics: Status and perspectives in canada. *Bioresource Technology, 101*(13), 4806-4813.

Mansfield, S. D., Mooney, C., & Saddler, J. N. (1999). Substrate and enzyme characteristics that limit cellulose hydrolysis. *Biotechnology Progress, 15*, 804-816.

Marcos, M., García-Cubero, M. T., González-Benito, G., Coca, M., Bolado, S., & Lucas, S. (2012). Optimization of the enzymatic hydrolysis conditions of steam-exploded wheat straw for maximum glucose and xylose recovery. *Journal of Chemical Technology & Biotechnology, in press*

Martín, C., Galbe, M., Nilvebrant, N., & Jönsson, L. J. (2002). Comparison of the fermentability of enzymatic hydrolyzates of sugarcane bagasse pretreated by steam explosion using different impregnating agents. *Applied Biochemistry and Biotechnology, 98-100*, 699-716.

Merino, S. T., & Cherry, J. (2007). Progress and challenges in enzyme development for biomass utilization. *Advances in Biochemical Engineering/Biotechnology, 108*, 95-120.

Mes-Hartree, M., Dale, B. E., & Craig, W. K. (1988). Comparison of steam and ammonia pretreatment for enzymatic hydrolysis of cellulose. *Applied Microbiology and Biotechnology, 29*(5), 462-468.

Mohagheghi, A., Tucker, M., Grohmann, K., & Wyman, C. (1992). High solids simultaneous saccharification and fermentation of pretreated wheat straw to ethanol. *Applied Biochemistry and Biotechnology, 33*(2), 67-81.

Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y. Y., Holtzapple, M., & Ladisch, M. (2005a). Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresource Technology, 96*(6), 673-686.

Mosier, N., Hendrickson, R., Ho, N., Sedlak, M., & Ladisch, M. R. (2005b). Optimization of pH controlled liquid hot water pretreatment of corn stover. *Bioresource Technology, 96*(18), 1986-1993.

Mussatto, S. I., Fernandes, M., Milagres, A. M. F., & Roberto, I. C. (2008). Effect of hemicellulose and lignin on enzymatic hydrolysis of cellulose from brewer's spent grain. *Enzyme and Microbial Technology, 43*(2), 124-129.

Nakagame, S., Chandra, R. P., Kadla, J. F., & Saddler, J. N. (2011a). Enhancing the enzymatic hydrolysis of lignocellulosic biomass by increasing the carboxylic acid content of the associated lignin. *Biotechnology and Bioengineering, 108*(3), 538-548.

Nakagame, S., Chandra, R. P., Kadla, J. F., & Saddler, J. N. (2011b). The isolation, characterization and effect of lignin isolated from steam pretreated douglas-fir on the enzymatic hydrolysis of cellulose. *Bioresource Technology, 102*(6), 4507-4517.

Nakagame, S., Chandra, R. P., & Saddler, J. N. (2010). The effect of isolated lignins, obtained from a range of pretreated lignocellulosic substrates, on enzymatic hydrolysis. *Biotechnology and Bioengineering, 105*(5), 871-879.

National Research Council. (2001). *Climate change science. an analysis of some key questions.* Washington, D.C.: National Academy Press.

Negro, M. J., Manzanares, P., Oliva, J. M., Ballesteros, I., & Ballesteros, M. (2003). Changes in various physical/chemical parameters of pinus pinaster wood after steam explosion pretreatment. *Biomass and Bioenergy, 25*(3), 301-308.

Nidetzky, B., Steiner, W., Hayn, M., & Esterbauer, H. (1993). Enzymatic hydrolysis of wheat straw after steam pretreatment: Experimental data and kinetic modelling. *Bioresource Technology, 44*(1), 25-32.

Ogiwara, Y., & Arai, K. (1968). Swelling degree of cellulose materials and hydrolysis rate with cellulase. *Textile Research Journal, 38,* 885-891.

Öhgren, K., Bura, R., Saddler, J., & Zacchi, G. (2007). Effect of hemicellulose and lignin removal on enzymatic hydrolysis of steam pretreated corn stover. *Bioresource Technology, 98,* 2503-2510.

Öhgren, K., Galbe, M., & Zacchi, G. (2005). Optimization of steam pretreatment of SO_2-impregnated corn stover for fuel ethanol production. *Applied Biochemistry and Biotechnology, 124*(1), 1055-1067.

Overend, R., & Chornet, E. P. (1987). Fractionation of lignocellulosics by steam-aqueous pretreatments. *Philosophical Transactions of the Royal Society A, 321*(1561), 523-536.

Palmarola-Adrados, B., Galbe, M., & Zacchi, G. (2004). Combined steam pretreatment and enzymatic hydrolysis of starch-free wheat fibers. *Applied Biochemistry and Biotechnology, 115*(1), 989-1002.

Palmqvist, E., & Hahn-Hägerdal, B. (2000). Fermentation of lignocellulosic hydrolysates. I: Inhibition and detoxification. *Bioresource Technology, 74*(1), 17-24.

Pan, X., Xie, D., Yu, R. W., Lam, D., & Saddler, J. N. (2007). Pretreatment of lodgepole pine killed by mountain pine beetle using the ethanol organosolv process: Fractionation and process optimization. *Industrial & Engineering Chemistry Research, 46*(8), 2609-2617.

Pejic, B. M., Kostic, M. M., Skundric, P. D., & Praskalo, J. Z. (2008). The effects of hemicelluloses and lignin removal on water uptake behavior of hemp fibers. *Bioresource Technology, 99*(15), 7152-7159.

Petersen, M. Ø., Larsen, J., & Thomsen, M. H. (2009). Optimization of hydrothermal pretreatment of wheat straw for production of bioethanol at low water consumption without addition of chemicals. *Biomass and Bioenergy, 33*(5), 834-840.

Phillips, S., & Norton, R. (2012). Global wheat production and fertilizer use. *Better Crops with Plant Food; Special Issue: Nutrient Management for Wheat., 96*(3), 4-6.

Piccolo, C., & Bezzo, F. (2009). A techno-economic comparison between two technologies for bioethanol production from lignocellulose. *Biomass and Bioenergy, 33*(3), 478-491.

Qi, B., Chen, X., Shen, F., Su, Y., & Wan, Y. (2009). Optimization of enzymatic hydrolysis of wheat straw pretreated by alkaline peroxide using response surface methodology. *Industrial & Engineering Chemistry Research, 48*(15), 7346-7353.

Qing, Q., Yang, B., & Wyman, C. E. (2010). Xylooligomers are strong inhibitors of cellulose hydrolysis by enzymes. *Bioresource Technology, 101*(4), 9624-9630.

Ragauskas, A. J., Williams, C. K., Davison, B. H., Britovsek, G., Cairney, J., Eckert, C. A., Frederick, W. J. J., Hallett, J. P., Leak, D. J., Liotta, C. L., Mielenz, J. R., Murphy, R., Templer, R., & Tschaplinski, T. (2006). The path forward for biofuels and biomaterials. *Science, 311*, 484-489.

Ramos, L. P., Breuil, C., & Saddler, J. N. (1992). Comparison of steam pretreatment of eucalyptus, aspen and spruce wood chips and their enzymatic hydrolysis. *Applied Biochemistry and Biotechnology, 34-35*, 37-47.

Ramos, L. P. (2003). The chemistry involved in the steam treatment of lignocellulosic materials. *Química Nova, 26*(6), 863-671.

Rogner, H. (2012). Energy resources. *Environment and Policy, 54*(3), 149-160.

Rosgaard, L., Pedersen, S., & Meyer, A. (2007). Comparison of different pretreatment strategies for enzymatic hydrolysis of wheat and barley straw. *Applied Biochemistry and Biotechnology, 143*(3), 284-296.

Saha, B. C., Iten, L. B., Cotta, M. A., & Wu, Y. V. (2005). Dilute acid pretreatment, enzymatic saccharification and fermentation of wheat straw to ethanol. *Process Biochemistry, 40*(12), 3693-3700.

Sannigrahi, P., Kim, D., Jung, S., & Ragauskas, A. (2011). Pseudo-lignin and pretreatment chemistry. *Energy Environ.Sci., 4*(4), 1306-1310.

Sassner, P., Galbe, M., & Zacchi, G. (2005). Steam pretreatment of *salix* with and without SO_2 impregnation for production of bioethanol. *Applied Biochemistry and Biotechnology, 121-124*, 1101-1117.

Sassner, P., Galbe, M., & Zacchi, G. (2008). Techno-economic evaluation of bioethanol from three different lignocellulosic materials. *Biomass and Bioenergy, 32*, 422-430.

Sassner, P., Mårtensson, C., Galbe, M., & Zacchi, G. (2008). Steam pretreatment of H_2SO_4-impregnated salix for the production of bioethanol. *Bioresource Technology, 99*(1), 137-145.

Scheller, H. V., & Ulvskov, P. (2010). Hemicelluloses. *Annual Review of Plant Biology, 61*, 236-289.

Schevchenko, S. M., Chang, K., Robsinon, J., & Saddler, J. N. (2000). Optimization of monosaccharide recovery by post-hydrolysis of the water-soluble hemicellulose component after steam explosion of softwood chips. *Bioresource Technology, 72*, 207-211.

Selig, M. J., Viamajala, S., Decker, S. R., Tucker, M. P., Himmel, M. E., & Vinzant, T. B. (2007). Deposition of lignin droplets produced during dilute acid pretreatment of maize stems retard enzymatic hydrolysis of cellulose. *Biotechnology Progress, 23*, 1333-1339.

Sewalt, V. J. H., Glasser, W. G., & Beauchemin, K. A. (1997). Lignin impact on fiber degradation. 3. reversal of inhibition of enzymatic hydrolysis by chemical modification of lignin and by additives. *Journal of Agricultural Food Chemistry, 45*, 1823-1828.

Sims, R. E. H., Schock, R. N., Adegbulugbe, A., Fenhann, J., Konstantinaviciute, I., Moomaw, W., . . . Torres-Mart. (2007). Energy supply. In B. Metz, O. R. Davidson, P. R. Bosch, R. Dave & L. A. Meyer (Eds.), *Climate change 2007: Mitigation. contribution of working group III to the fourth assessment report of the intergovernmental panel on climate change*. Cambridge, UK and New York, USA: Cambridge Press University.

Sjöström, E. (1993). *Wood chemistry. fundamentals and applications* (2nd ed.). California, USA: Academic Press.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., & Crocker, D. (2008). *Determination of structural carbohydrates and lignin in biomass.* No. NREL/TP-510-42618

Sluiter, A., Ruiz, R., Scarlata, C., Sluiter, J., & Templeton, D. (2005). *Determination of extractives in biomass.* No. NREL/TP-510-42619

Söderström, J., Pilcher, L., Galbe, M., & Zacchi, G. (2003). Combined use of H_2SO_4 and SO_2 impregnation for steam pretreatment of spruce in ethanol production. *Applied Biochemistry and Biotechnology, 105*(1), 127-140.

Stenberg, K., Tengborg, C., Galbe, M., & Zacchi, G. (1998). Optimisation of steam pretreatment of SO2-impregnated mixed softwoods for ethanol production. *J.Chem.Technol.Biotechnol., 71*(4), 299-308.

Stephen, J. D., Mabee, W. E., & Saddler, J. N. (2012). Will second-generation ethanol be able to compete with first-generation ethanol? opportunities for cost reduction. *Biofuels, Bioproducts and Biorefining, 6*(2), 159-176.

Stumborg, M., Townley-Smith, L., & Coxworth, E. (1996). Sustainability and economic issues for cereal crop residue export. *Canadian Journal of Plant Science, 76*(4), 669-673.

Sun, X. F., Xu, F., Sun, R. C., Wang, Y. X., Fowler, P., & Baird, M. S. (2004). Characteristics of degraded lignins obtained from steam exploded wheat straw. *Polymer Degradation and Stability, 86*, 245-256.

Sun, Y., & Cheng, J. (2002). Hydrolysis of lignocellulosic materials for ethanol production: A review. *Bioresource Technology, 83*(1), 1-11.

Talebnia, F., Karakashev, D., & Angelidaki, I. (2010). Production of bioethanol from wheat straw: An overview on pretreatment, hydrolysis and fermentation. *Bioresource Technology, 101*, 4744-4753.

Tengborg, C., Stenberg, K., Galbe, M., Zacchi, G., Larsson, S., Palmqvist, E., & Hahn-Hägerdal, B. (1998). Comparison of SO_2 and H_2SO_4 impregnation of softwood prior to steam pretreatment on ethanol production. *Applied Biochemistry and Biotechnology, 70-72*(1), 3-15.

Toussaint, B., Excoffier, G., & Vignon, M. R. (1991). Effect of steam explosion treatment on the physico-chemical characteristics and enzymic hydrolysis of poplar cell wall components. *Animal Feed Science and Technology, 32*(1-3), 235-242.

Tucker, M., Kim, K., Newman, M., & Nguyen, Q. (2003). Effects of temperature and moisture on dilute-acid steam explosion pretreatment of corn stover and cellulase enzyme digestibility. *Applied Biochemistry and Biotechnology, 105*(1), 165-177.

Vansteenkiste, E., Babot, C., Rouau, X., & Micard, V. (2004). Oxidative gelation of feruloylated arabinoxylan as affected by protein. influence on protein enzymatic hydrolysis. *Food Hydrocolloids, 18*(4), 557-564.

Vasudevan, P. T., Gagnon, M. D., & Briggs, M. S. (2010). Environmentally sustainable biofuels – the case for biodiesel, biobutanol and cellulosic ethanol. In O. V. Singh, & S. P. Harvey (Eds.), *Sustainable biotechnology* (pp. 43-62) Springer Netherlands.

Viikari, L., Vehmaanperä, J., & Koivula, A. (2012). Lignocellulosic ethanol: From science to industry. *Biomass and Bioenergy, In press*

Vlasenko, E. Y., Ding, H., Labavitch, J. M., & Shoemaker, S. P. (1997). Enzymatic hydrolysis of pretreated rice straw. *Bioresource Technology, 59*(2–3), 109-119.

Vogel, J. (2008). Unique aspects of the grass cell wall. *Current Opinion in Plant Biology, 11*(3), 301-307.

Volynets, B., & Dahman, Y. (2010). Assessment of pretreatments and enzymatic hydrolysis of wheat straw as a sugar source for bioprocess industry. *International Journal of Energy and Environment, 2*(3), 427-446.

Wan, J., Wang, Y., & Xiao, Q. (2010). Effects of hemicellulose removal on cellulose fiber structure and recycling characteristics of eucalyptus pulp. *Bioresource Technology, 101*(12), 4577-4583.

Wingren, A., Söderström, J., Galbe, M., & Zacchi, G. (2004). Process considerations and economic evaluation of two-step steam pretreatment for production of fuel ethanol from softwood. *Biotechnology Progress, 20*(5), 1421-1429.

Wood, T. M. (1975). Properties and mode of action of cellulases. *Biotechnology Bioenergy Symposium, 5*, 111-137.

Wyman, C. E., Decker, S. R., Himmel, M. E., Brady, J. W., Skopec, C. E., & Viikari, L. (2005). Hydrolysis of cellulose and hemicellulose. In S. Dumitriu (Ed.), *Polysaccharides. structural diversity and functional versatility* (2nd ed., pp. 993-1033). New York: Marcel Dekker.

Ximenes, E., Kim, Y., Mosier, N., Dien, B., & Ladisch, M. (2010). Inhibition of cellulases by phenols. *Enzyme and Microbial Technology, 46*(3-4), 170-176.

Ximenes, E., Kim, Y., Mosier, N., Dien, B., & Ladisch, M. (2011). Deactivation of cellulases by phenols. *Enzyme and Microbial Technology, 48*(1), 54-60.

Yoshida, M., Liu, Y., Uchida, S., Kawarada, K., Ukagami, Y., Ichinose, H., Kaneko, S., & Fukuda, K. (2008). Effects of cellulose crystallinity, hemicellulose, and lignin on the enzymatic hydrolysis of *miscanthus sinensis* to monosaccharides. *Bioscience, Biotechnology, and Biochemistry, 3*, 805-810.

Zimbardi, F., Viola, E., Nanna, F., Larocca, E., Cardinale, M., & Barisano, D. (2007). Acid impregnation and steam explosion of corn stover in batch processes. *Industrial Crops and Products, 26*(2), 195-206.

Appendices

Appendix A. Monomers and Oligomers in the water soluble fraction (WSF) of steam pretreated wheat straw substrates

Conditions				Total Sugars in WSF (mg, Liquor + Gun wash)					Oligomer Sugars in WSF (mg, Liquor + Gun wash)				
	Time	Temp	Catalyst	Arabinose	Galactose	Glucose	Xylose	Mannose	Arabinose	Galactose	Glucose	Xylose	Mannose
1	170	2	0.75	0.4	0.2	0.8	0.9	0.0	0.2	0.2	0.7	0.9	0.0
2	170	8	0.75	0.7	0.3	1.8	4.4	0.0	0.4	0.3	1.7	4.1	0.0
3	210	2	0.75	0.5	0.3	2.0	6.1	0.0	0.2	0.2	1.8	4.8	0.0
4	210	8	0.75	0.2	0.3	1.7	3.7	0.0	0.0	0.1	1.2	1.6	0.0
5	190	2	0	0.3	0.2	1.2	1.1	0.0	0.3	0.2	1.2	1.1	0.0
6	190	2	1.5	0.6	0.3	1.4	5.6	0.0	0.1	0.2	1.2	3.4	0.0
7	190	8	0	0.6	0.3	1.9	6.2	0.0	0.3	0.3	1.9	6.0	0.0
8	190	8	1.5	0.6	0.4	2.6	6.8	0.0	0.1	0.3	2.4	5.7	0.0
9	170	5	0	0.3	0.2	1.8	0.7	0.0	0.3	0.2	1.8	0.7	0.0
10	210	5	0	0.2	0.3	1.4	5.7	0.0	0.1	0.2	1.3	4.7	0.0
11	170	5	1.5	0.8	0.4	1.9	5.2	0.0	0.1	0.2	1.7	4.0	0.0
12	210	5	1.5	0.3	0.3	2.9	2.6	0.0	0.1	0.1	1.9	1.0	0.0
13	190	5	0.75	0.6	0.3	2.2	6.1	0.0	0.2	0.3	2.0	5.5	0.0
14	190	5	0.75	0.6	0.3	1.8	4.3	0.0	0.3	0.2	1.8	3.9	0.0
15	190	5	0.75	0.6	0.4	2.6	6.0	0.0	0.2	0.3	2.4	5.0	0.0

Appendix B. Total soluble glucose obtained after steam pretreatment and enzymatic hydrolysis of wheat straw substrates at 10% solids loading and 10 mg protein/g glucan. TSS = total soluble sugars.

Conditions	WIF (g)	Glucose obtained from hydrolysis (g)	Theoretical Sugars from WIF	Sugars from WSF	TSS	Glucose in raw material (g)	Yield
1	65.4	0.09	6.0	0.8	6.8	33.9	20%
2	59.6	0.11	6.9	1.8	8.6	33.9	25%
3	54.1	0.24	12.8	2.0	14.8	33.9	44%
4	45.6	0.31	14.1	1.7	15.8	33.9	47%
5	66.3	0.07	4.5	1.2	5.8	33.9	17%
6	61.8	0.22	13.8	1.4	15.2	33.8	45%
7	57.9	0.23	13.2	1.9	15.2	33.8	45%
8	49.7	0.37	18.3	2.6	20.9	33.9	62%
9	64.7	0.08	4.9	1.8	6.7	32.9	20%
10	48.4	0.34	16.6	1.4	18.0	32.8	55%
11	59.8	0.10	6.2	1.9	8.1	33.8	24%
12	44.8	0.47	21.1	2.9	24.0	33.9	71%
13	59.5	0.18	10.9	2.2	13.1	33.9	39%
14	62.2	0.20	12.7	1.8	14.5	33.8	43%
15	57.5	0.24	13.9	2.6	16.5	33.8	49%

Appendix C. Total soluble xylose obtained after steam pretreatment and enzymatic hydrolysis of wheat straw substrates using 10% solids loading and 10 mg protein/g glucan. TSS = total soluble sugars

Conditions	WIF (g)	Xylose obtained from hydrolysis (g)	Theoretical Sugars from WIF	Sugars from WSF	TSS	Xylose in raw material (g)	Yield
1	65.43	0.0	1.5	0.9	2.4	16.5	15%
2	59.63	0.0	1.7	4.4	6.1	16.5	37%
3	54.10	0.0	2.5	6.1	8.6	16.5	52%
4	45.62	0.0	0.5	3.7	4.2	16.5	25%
5	66.25	0.0	0.9	1.1	2.0	16.5	12%
6	61.79	0.1	3.4	5.6	9.0	16.5	55%
7	57.85	0.0	2.5	6.2	8.8	16.5	53%
8	49.74	0.0	0.6	6.8	7.4	16.5	45%
9	64.73	0.0	1.1	0.7	1.8	16.0	11%
10	48.42	0.0	0.9	5.7	6.7	16.0	42%
11	59.76	0.0	0.9	5.2	6.1	16.5	37%
12	44.82	0.0	0.4	2.6	3.0	16.5	18%
13	59.53	0.0	1.2	6.1	7.4	16.5	45%
14	62.21	0.1	3.8	4.3	8.1	16.5	49%
15	57.49	0.0	2.6	6.0	8.7	16.5	53%

Appendix D. Total soluble glucose obtained after steam pretreatment and enzymatic hydrolysis of wheat straw substrates using 10% solids loading and 20 mg protein/g glucan. TSS = total soluble sugars

Conditions	Pretreated Pulp	Glucose (g)	Sugars from WIF	Sugars from WSF	TSS	Glucose in raw (g)	Yield
1	65.43	0.13	8.57	0.83	9.40	33.86	28%
2	59.63	0.23	13.67	1.76	15.43	33.87	46%
3	54.10	0.35	19.19	1.97	21.17	33.86	63%
4	45.62	0.59	26.75	1.73	28.47	33.88	84%
5	66.25	0.17	11.02	1.24	12.26	33.89	36%
6	61.79	0.35	21.71	1.36	23.08	33.83	68%
7	57.85	0.34	19.75	1.94	21.69	33.84	64%
8	49.74	0.54	26.87	2.56	29.43	33.89	87%
9	64.73	0.13	8.47	1.77	10.24	32.88	31%
10	48.42	0.54	25.91	1.41	27.32	32.80	83%
11	59.76	0.24	14.30	1.95	16.25	33.84	48%
12	44.82	0.67	30.23	2.87	33.10	33.87	98%
13	59.53	0.29	17.36	2.17	19.54	33.87	58%
14	62.21	0.40	25.16	1.80	26.96	33.84	80%
15	57.49	0.35	19.85	2.56	22.41	33.85	66%

Appendix E. Total soluble xylose obtained after steam pretreatment and enzymatic hydrolysis of wheat straw substrates using 10% solids loading and 20 mg protein/g glucan. TSS = total soluble sugars

Conditions	Pretreated Pulp	Xylose (g)	Sugars from WIF	Sugars from WSF	TSS	Xylose in raw (g)	Yield
1	65.43	0.04	2.55	0.87	3.42	16.52	21%
2	59.63	0.12	7.39	4.43	11.82	16.53	72%
3	54.10	0.09	4.87	6.06	10.93	16.52	66%
4	45.62	0.01	0.49	3.67	4.16	16.53	25%
5	66.25	0.05	3.60	1.08	4.68	16.54	28%
6	61.79	0.08	4.96	5.64	10.60	16.51	64%
7	57.85	0.09	5.26	6.25	11.51	16.51	70%
8	49.74	0.03	1.55	6.83	8.38	16.54	51%
9	64.73	0.04	2.62	0.72	3.34	16.05	21%
10	48.42	0.03	1.59	5.74	7.33	16.00	46%
11	59.76	0.05	2.86	5.22	8.07	16.51	49%
12	44.82	0.03	1.15	2.64	3.78	16.53	23%
13	59.53	0.06	3.41	6.13	9.53	16.53	58%
14	62.21	0.07	4.36	4.29	8.65	16.51	52%
15	57.49	0.08	4.47	6.04	10.51	16.52	64%