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Characterization of Cellulase Enzyme Inhibitors Formed During the Chemical Pretreatments of Rice Straw

Kalavathy Rajan
University of Arkansas, Fayetteville

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by

Kalavathy Rajan
Tamil Nadu Agricultural University,
Bachelor of Science in Agriculture, 2008
Cornell University,
Master of Professional Studies in Food Science, 2010
Tamil Nadu Agricultural University,
Master of Technology in Food Processing and Marketing, 2011

May 2015
University of Arkansas

This dissertation is approved for the recommendation to the graduate council.

_____
Dr. Danielle Julie Carrier
Dissertation Director

_____
Dr. Ed Clausen
Committee member

_____
Dr. Thomas A. Costello
Committee member

_____
Dr. Navam S. Hettiarachchy
Committee member

_____
Dr. Ruben O. Morawicki
Committee member

_____
Dr. Steven C. Ricke
Committee member

ABSTRACT

Production of fuels and chemicals from a renewable and inexpensive resource such as lignocellulosic biomass is a lucrative and sustainable option for the advanced biofuel and bio-based chemical platform. Agricultural residues constitute the bulk of potential feedstock available for cellulosic fuel production. On a global scale, rice straw is the largest source of agricultural residues and is therefore an ideal crop model for biomass deconstruction studies. Lignocellulosic biofuel production involves the processes of biomass conditioning, enzymatic saccharification, microbial fermentation and ethanol distillation, and one of the major factors affecting its techno-economic feasibility is the biomass recalcitrance to enzymatic saccharification. Preconditioning of lignocellulosic biomass, using chemical, physico-chemical, mechanical and biological pretreatments, is often practiced such that biomass becomes available to downstream processing. Pretreatments, such as dilute acid and hot water, are effective means of biomass conversion. However, despite their processing importance, preconditioning biomass also results in the production of carbohydrate and lignin degradation products that are inhibitory to downstream saccharification enzymes.

The saccharification enzyme cocktail is made up of *endo*-cellulase, *exo*-cellulase and β-glucosidase enzymes, whose role is to cleave cellulose polymers into glucose monomers. Specifically, *endo*-cellulase and *exo*-cellulase enzymes cleave cellulose chains in the middle and at the end, resulting in cellobiose molecules, which are hydrolyzed into glucose by β-glucosidase. Unfortunately, degradation compounds generated during pretreatment inhibit the saccharification enzyme cocktail. Various research groups have identified specific classes of inhibitors formed during biomass pretreatment and have studied their inhibitory effect on the saccharification cocktail. These various research groups prepared surrogate solutions in an

attempt to mimic pretreatment hydrolyzates. No group has yet attempted to elucidate the inhibitory action of compounds isolated from pretreatment hydrolyzates. Elucidating the inhibition of cellulases using actual biomass hydrolyzates would offer insights as to which inhibitors, formed during a pretreatment, are key in causing inhibition. Knowing the key inhibitor(s) would allow for the development of processing conditions that minimize their production or of their removal through hydrolyzate detoxification methods.

This research has characterized various chemical compounds released during dilute acid and hot water pretreatment of rice straw and has evaluated their inhibitory effects on *endo*-cellulase, *exo*-cellulase and β-glucosidase enzymes. The hot water pretreatment hydrolyzate, generated at 220 °C and 52 min, was found to be particularly inhibitory to *exo*- and *endo*-cellulases, and was chosen for further evaluation. This hot water hydrolyzate was fractionated using centrifugal partition chromatography (CPC) and grouped into furans, organic acids, phenolics, monomeric and oligomeric sugars. When these fractions were incubated with *exo*-cellulase, it was determined that fractions containing acetic acid and phenolics were highly inhibitory, resulting in 92 % and 87 % inhibition of initial hydrolysis rates, respectively. This study proposes a new approach for identifying key inhibitory compounds in biomass prehydrolyzates, eventually paving the way for developing strategies to the improve the enzymatic saccharification efficiency of lignocellulosic biomass.

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DEDICATION

Dedicated to my Mom

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I. Introduction

The global production of bioethanol in 2013 was 23.43 million gallons and USA was the world leader with a total production of 13.3 million gallons of bioethanol.[1] The major feedstock for bioethanol production has been cornstarch (USA) and sugarcane molasses (Brazil). However, bioethanol production from food sources poses threat to food security. As per the Energy Security and Independence Act, (EISA) 2007, fuel ethanol production from cornstarch has been mandated not to exceed 16 million gallons per year, in order to avoid inflation of corn grain costs.[2] Fuel ethanol can also be derived from lignocellulosic biomass, thereby providing a cheaper alternative to food and animal feed–based feedstock. Use of lignocellulosic biomass for fuel ethanol production also paves way to increase sustainability and reduce green house gas emissions.[3]

The estimated global production of agricultural residues (mainly rice, wheat, corn, barley, oat and sorghum) was 1.48 billion ton in 2004.[4] These residues could serve as potential feedstock for the production of 116.76 billion gallons of bioethanol.[4] Rice straw solely constitutes a single major source of lignocellulosic biomass for bioethanol production. Global rice straw production in 2009 was approximately 731 million tons, and furthermore had the potential to produce 57 billion gallons of bioethanol at 30 % conversion.[5] Rice straw produced in the Asian continent alone was 667.6 million tons, in 2009 and could support the production of 91% of the estimated cellulosic ethanol.[5] In the USA, Arkansas is the largest producer of rice at 3.67 million tons, in 2013[6] and has the potential to supply 3.81 million tons of rice straw annually for biofuel production. Therefore, rice straw was chosen as a model substrate for conducting pretreatment and enzymatic saccharification studies.

The Environmental Protection Agency (EPA), issued the revised Renewable Fuel Standard (RFS) in August 2013, such that the 2013 mandate for cellulosic biofuel production is now 6 million gallons.[7] Despite the ruling, the actual cellulosic biofuel production in 2013, was less than 0.5 million gallons, and this was only a fraction of the originally projected 1 billion gallon (RFS–EISA, 2007).[8] The cellulosic ethanol industry has to overcome several bottlenecks in i) feedstock availability, ii) biomass deconstruction, iii) fermentation and iv) consolidated processing, in order to achieve economic feasibility and sustainability.[9] The commercialization of cellulosic ethanol production depends significantly on the improvement of technologies in biomass deconstruction to sugar. This is because cellulosic ethanol prices will depend heavily on the cost of the saccharification enzyme cocktail used to break down the cellulosic biomass into fermentable sugars. On average, cellulase and xylanase enzymes contribute to 14% of the total production cost of cellulosic ethanol.[10] Therefore, the total enzyme used in the process has to be reduced by improving the pretreatment technology and as well as the enzyme efficiency.

Agricultural residues, such as rice straw, wheat straw and corn stover, are approximately made up of 35 to 40% of cellulose, 15 to 27% of hemicellulose and 7 to 12% of lignin.[11,12,13] During pretreatment, the lignin layer is fractured, hemicellulose is solubilized and removed, and the surface area of crystalline cellulose becomes available for enzymatic hydrolysis increases.[14] There are several methods of pretreatments; mechanical, physico–chemical, chemical and biological, available for the deconstruction of biomass to fermentable sugars.[15] Total yield of monomeric sugars were higher for pretreatments such as steam explosion with sulfur di-oxide (79%) and dilute acid hydrolysis (76%).[16] However, the degradation products of cellulose and hemicellulose formed during these pretreatments, such as weak acids (acetic acid, formic acid) and furan derivatives (furfural and 5-hydroxymethyl furfural), have been determined to inhibit

the saccharification enzyme cocktail.[17,18,19,20] There are several by-products formed as a result of lignin degradation during pretreatment, such as tannic acid, gallic acid, cinnamic acid, vanillin, coumaric acid, and these compounds are also inhibitory to cellulolytic, also termed saccharification, enzymes.[21,22,23] The production of inhibitors varies with the lignocellulosic feedstock used, pretreatment method employed, as well as the pretreatment severity.[24,25] Rinsing pretreated biomass with large volumes of water has been commonly used to remove such inhibitors and improve the enzymatic saccharification on a laboratory scale.[23,26] However, the cellulosic ethanol industry is estimated to consume 23 to 38 L of water per liter of ethanol and detoxification with large volumes of water would increase its production cost and as well as decrease its sustainability.[16]

Thus, in order to increase the saccharification enzyme efficiencies without compromising the sustainability of cellulosic fuel production, it is necessary to reduce the amount of degradation products formed during pretreatment. Pretreatment parameters may be optimized, such that lower concentrations of the inhibitors are formed.[24,25] However, the kinetics of formation of enzyme inhibitors is complex and difficult to control by optimizing only a select few pretreatment parameters.[27] Therefore, it is essential to first identify the major inhibitor of cellulolytic enzymes, such that it is possible to determine its kinetics of formation and the pretreatment parameters can be optimized to reduce its production. The objectives of this research was therefore:

1. To characterize the inhibitors formed during the physico-chemical pretreatment of rice straw
2. To analyze the inhibition of *exo*–cellulase, *endo*–cellulase and β-glucosidase enzymes in the presence of these inhibitors and,

3. To determine the major inhibitors in the pretreatment hydrolyzates of rice straw that are severely inhibitory to model cellulase enzyme systems.

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II. Review of Literature

Lignocellulosic biofuels: Issues and trends

About 84% of bioethanol in the world was produced by the USA and Brazil, in 2013, with the USA being the leader at 57%.[1] In the USA, bioethanol is produced from sugar and starch–based feedstock, primarily corn (first-generation biomass).[28] However, it also an important food source and competition with bioethanol industry will have significant impact on food prices and food security.[2] In contrast, lignocellulosic biomass, residues from wood or dedicated energy crops (second generation) are an attractive alternative because there is no competition with food and animal feed production, and these materials are also cheaper than first-generation biomass.[29,30,31] Additionally, the use of lignocellulosic materials for fuel ethanol production can aid in reducing greenhouse gas (GHG) emissions.[3, 32]

The Renewable Fuel Standard (RFS)-II called for 36 billion gallons of renewable fuel to be blended with traditional fuels by 2022, and the vast bulk of this will come from corn ethanol. It also called for the volume requirement of cellulosic biofuels for the year 2012 to be 6 billion gallons, and for an increase in advanced biofuels to 16 billion gallons by the year 2022.[7] However, despite the projections made by the U.S. Energy Information Agency, only 20,069 gallons of cellulosic ethanol was produced in 2012.[8] The start–up industries were behind schedule mainly due to limited feedstock supply and the economic cost of conversion technologies that were 2 to 5 times more than first generation ethanol.[9]

Lignocellulosic biomass is the largest source of hexose and pentose sugars, which can be used for the production of bioethanol.[33] Unlike first-generation biomass, in second-generation lignocellulosic substrates, cellulose is encased within hemicellulose and lignin matrix, and thus accessibility of cellulose is a major problem in bioethanol production from such sources. Thus,

the cost of biomass conversion is high due to intensive labor and increased processing steps. The single major cost–contributing factor in biomass conversion, is the cost of cellulolytic enzymes. There was a report indicating that the cost of cellulolytic enzymes was $0.50 per gallon and that significant work has to be done to reduce the enzyme cost to $0.10 per gallon.[34] In 2011, DuPont purchased Genencor with the goal to reduce enzyme costs via onsite production.[35] However, off site production of cellulases was determined to be more cost competitive and environmental friendly, due to higher economies of scale and lower GHG emissions per gallon ethanol, respectively.[36] Also, there has to be trade-offs between the use of chemical catalyst and enzymes, because, even though chemical catalysts (acids, alkali) increase sugar yields, there is formation of degradation compounds, which inhibit cellulolytic enzymes.

Other obstacles to be overcome by the advanced biofuel industry are a negative energy balance and sustainability issues related to water usage. Preliminary life cycle assessments of lignocellulosic ethanol production have shown that the GHG emissions will be reduced by 60% when compared to conventional liquid fuel production. This is accordance with the mandate of Energy Independence and Security Act (EISA), 2007. Energy credits earned from the production of valuable co–products, electricity and heat, would also improve the energy balance. Efforts have been made to recycle water in order to reduce the water usage and improve sustainability.[37] Despite these advancements, technical obstacles related to biomass deconstruction still have to be overcome for efficient and cost effective conversion of lignocellulosic biomass into biofuels.

Potential for sustainable rice straw supply in Arkansas

The major issue with achieving the EISA, 2007 mandate for cellulosic bioethanol production was the low availability of feedstock. The U.S. Department of Energy study estimated that if 4% of arable lands were dedicated to energy crops production and if the farm-

gate price was $60 per ton, 1,009 million ton of feedstock would be available for bioethanol production, in 2022.[38] The projected sources of feedstock, under high yield conditions were forestry, wood and wastes resources (10%), agricultural and crop residues (30%) and dedicated bioenergy crops (56%). However, currently no dedicated energy crops are available and the feedstock choices are restricted to agricultural residues and forestry wastes.

Globally, rice straw alone represents 23% of all agricultural wastes and is a major feedstock source for cellulosic biofuel production in countries, such as India, where extensive land use changes would be unsustainable.[32] The global distribution of rice straw is 2.85 % in Africa, 91.32 % in Asia and 0.53 % in Europe.[39] In USA, Arkansas is the leading producer of rice, at 43.4 % and has the potential to supply 3.81 million tons of rice straw annually for biofuel production. The top 20 rice-producing counties of Arkansas and their relative rice yields are given in Table 1. It is the largest amount from a single feedstock source in the state of Arkansas. Presently, high value utilization of this biomass remains largely untapped.

In Arkansas, rice is planted during April – May and harvested in the months of September to early November. Different residue management practices have been conventionally followed in Arkansas, such as burning (25.5 %), soil incorporation by tilling (38.5 %) or rolling (22.5 %) and winter flooding (18.0 %).[41] A combination of these methods, such as winter flooding and later rolling into the soil, has also been practiced. Rice straw may be mixed into relatively dry soil using conventional ploughs, discs and tillers. Or it may be pressed and poked into wet soil using cage rollers. Burning the straw is another cost effective method to dispose the residues.[42] While the rice straw incorporation into soil demands additional agronomic practices to control the weeds and pests, burning of residues increases GHG emissions and also leads to

soil carbon losses. Another alternative is to bale and remove the rice straw and sell it as a high value product.

Table 1: County-wise rice acreage, total and sustainable rice straw production in Arkansas, based on 2013 estimates.

County	Area harvested (acres)	Total straw production* (kg)	Sustainable straw production# (kg)
1. Poinsett	89,300	379,064,448	132,672,557
2. Arkansas	75,700	332,035,200	116,212,320
3. Jackson	75,800	292,463,136	102,362,098
4. Lonoke	67,600	291,211,200	101,923,920
5. Lawrence	77,000	289,578,240	101,352,384
6. Cross	66,500	282,828,672	98,990,035
7. Greene	66,000	269,547,264	94,341,542
8. Prairie	55,400	241,133,760	84,396,816
9. Craighead	58,100	232,968,960	81,539,136
10. Jefferson	52,400	215,822,880	75,538,008
11. Woodruff	44,800	183,708,000	64,297,800
12. Mississippi	27,300	158,342,688	55,419,941
13. Monroe	37,000	153,988,128	53,895,845
14. Randolph	29,800	115,286,976	40,350,442
15. Chicot	26,700	108,265,248	37,892,837
16. Lee	17,800	75,660,480	26,481,168
17. Phillips	17,800	70,761,600	24,766,560
18. Lincoln	12,200	50,186,304	17,565,206
19. Desha	10,400	43,164,576	15,107,602
20. Independence	8,500	33,475,680	11,716,488
Total	**916,100**	**3,819,493,440**	**1,336,822,704**

* Straw to grain ratio was assumed to be 1.2[40]

Calculated at 35 % of total rice straw harvest

Source: U.S. Department of Agriculture, National Agricultural Statistical Services.

Soil incorporation is a widely followed method for stubble management. Rice straw upon decomposition increases the nutrient content of the soil. The typical nutrient content of rice straw on a dry weight basis is 5.5 % silica, 0.7 % nitrogen, 1.7 % potassium, 0.5 % chloride, 0.3 % calcium, 0.2 % magnesium, 0.2 % sodium, 0.1 % phosphorous and 0.1 % sulfur.[43] Rice straw also contains 24 % carbon, fixed from the atmosphere during photosynthesis. Technically, removal of 1 ton of straw leads to an average loss of 240 kg of carbon, 7 kg of nitrogen, 2 kg of phosphorous (P_2O_5), 17 kg of potassium (K_2O), 0.75 kg of sulfur and 55 kg of silica from the soil.[44] Crop residue cover also plays an important role in preventing wind and water erosion of the topsoil. Studies have reported that removal of 35 to 50 % of the rice straw residues would be optimal to prevent soil erosion and to enrich the soil organic matter content.[40, 45] Assuming that 35 % of the total rice straw was harvested, then the annual feedstock available for cellulosic fuel production in Arkansas, would be 1.34 million tons (Table 1). The theoretical maximum ethanol yield from rice straw was estimated to be 63.8 gallons per ton and therefore the potential for cellulosic ethanol production in Arkansas, using rice straw as feedstock would be approximately 85.3 million gallons per year.

Biomass recalcitrance and significance of pretreatment

Cellulose forms the crystalline backbone of a plant cell wall and hemicellulose forms a sheath around cellulose. Cellulose is a linear polymer of multiple glucose units joined by β 1→4 glycosidic linkages. Hemicellulose is a branched heteropolymer and in agricultural residues it is primarily made up of arabinoxylan. These arabinoxylan units are acetylated and the degree of acetylation varies with biomass type. Hemicellulose and lignin form the secondary cell wall, and in agricultural residues the lignification of secondary cell wall is approximately 40 % lower than woody biomass.[46] Lignin in herbaceous biomass is made up of monomer residues incorporated

with *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) aromatic rings.[47] The abundance and distribution of these aromatic rings vary for each biomass and thus create heterogeneity in lignocellulosic feedstock.

Rice straw, like any lignocellulosic biomass, is also composed of cellulose, hemicellulose and lignin. Because cellulose is embedded in a hemicellulose–lignin matrix, pretreatment of the lignocellulosic biomass is needed to enhance the accessibility of this substrate for the conversion of cellulose to glucose.[48] Removal of lignin and hemicellulose, reduction of cellulose crystallinity, and increase of porosity in pretreatment processes can significantly improve the subsequent hydrolysis.[14]

Sustainability issues of biomass pretreatment

There are a number of biological, mechanical, physico-chemical and chemical technologies available for the pretreatment of lignocellulosic biomass, including use of fungi, ball milling, steam explosion, ammonia fiber expansion (AFEX), wet oxidation, acid, alkali, lime, and ionic liquids pretreatment.[49] All these pretreatment technologies have their own advantages and disadvantages. Their general modes of action are to increase the accessible area for enzymatic hydrolysis, decrease cellulose crystallinity, disrupt the lignin structure, hydrolysis and removal of hemicellulose.[49] Some major disadvantages of the above said technologies are lower conversion efficiency, high input cost, long process duration, formation of degradation products and other technical difficulties. The nature of these difficulties are complicated by the heterogeneous composition of biomass feedstock.

Pretreatments such as sulfur dioxide steam explosion, dilute acid and AFEX yielded more than 76% sugar recoveries. These treatments are efficient in solubilizing and removal of hemicellulose, alteration of lignin structure and improvement in the accessible area of cellulose

for subsequent enzymatic hydrolysis.[15] A techno-economic analysis showed that the cost of dilute acid pretreatment and AFEX were the closest to the ideal low production costs.[50] The capital cost of AFEX pretreatment is high due to the requirement of specialized equipment. In the end, dilute acid pretreatment is likely to be adopted for large–scale lignocellulosic bioethanol production, due to its low cost and simpler technology.

Despite its several advantages, dilute acid pretreatment has major sustainability issues with respect to the formation of degradation products, decrease in enzyme efficiency and increase in water usage for detoxification.[18,51] Therefore, for commercialization of lignocellulosic biofuels, it is important to rectify these problems and develop an efficient and environmentally friendly pretreatment method.

By-products of lignocellulosic biomass pretreatments

Degradation of carbohydrates, during physico-chemical pretreatments, leads to the production of inhibitors such as acetic acid, formic acid, levulinic acid, furfural, 5−hydroxymethylfurfural (HMF) and xylo–oligosaccharides, which are inhibitory to the saccharification enzymes and to fermentation microorganisms. [19,20,23,52,53] The enzymatic conversion of dilute acid pretreated poplar substrate, after 24 h incubation, was reduced by 93% in the presence of a 5 mg/mL combination of both furfural and formic acid.[18] The inhibitory effects of pure xylobiose and higher DP xylooligosaccharides have been elucidated against the commercial cellulases on avicel substrate. The initial glucose yield was reduced by 82% in the presence of 12.5 g/L of xylo-oligomers due to the competitive inhibition of cellulases.[54] Degradation of lignin also leads to the formation of phenolic compounds such as tannic acid, gallic acid, vanillin, cinnamic acid, ferulic acid, p-coumaric acid, etc.[21] Pure phenolic compounds (tannic acid, gallic acid, vanillin, cinnamic acid, ferulic acid, p-coumaric acid, sinapic acid,

syringaldehyde and p-hydroxybenzoic acid) have been shown to inhibit cellobiose activity of β-glucosidases from various microbial sources and also CMC-ase and filter paper activities of commercial cellulase cocktails.[22,55,56] Condensation of the solubilized hemicellulose also leads to the formation of lignin-like compounds called humins, that are also inhibitory to cellulolytic enzymes.[57] A schematic of the formation of inhibitors during biomass deconstruction is given in Figure 1. By correlating the parameters of steam explosion of corn stover and the production of inhibitors, it has been elucidated that the formation of weak acids and furan derivatives follow first-order reactions and the formation of phenolic compounds showed typical characteristics of a continuous reaction kinetic.[27] Degradation of hemicellulose to xylo-oligosaccharides was also determined to follow the first order reaction kinetics during hot water hydrolysis of birchwood xylan.[58]

Enzymatic saccharification and factors affecting efficiency

Saccharification cocktail composed of *endo*-cellulase, *exo*-cellulase, β–glucosidase and xylanase are widely used for enzymatic hydrolysis of lignocellulosic biomass. Xylanase hydrolyze the β 1→4 and β 1→6 linkages in arabinoxylan polymer and increase the accessibility of cellulose polymer. Endo-cellulases (endo-1→4-β-D-glucanase or EG) randomly hydrolyzes the β 1→4 glycosidic linkages of cellulose and generate β–limit dextrins.[59] Exo-cellulases (Cellobiohydrolase or CBH) hydrolyze every second β 1→4 glycosidic linkage from the reducing (CBH-1) and non-reducing (CBH-II) end of the cellulose polymer, resulting in the production of cellobiose. Cellobiose thus produced, is the substrate for conversion by β–glucosidases, that which hydrolyzes it to glucose. The enzyme loading and the composition of cellulases would therefore affect the efficiency of enzymatic saccharification of lignocellulosic biomass.[60]

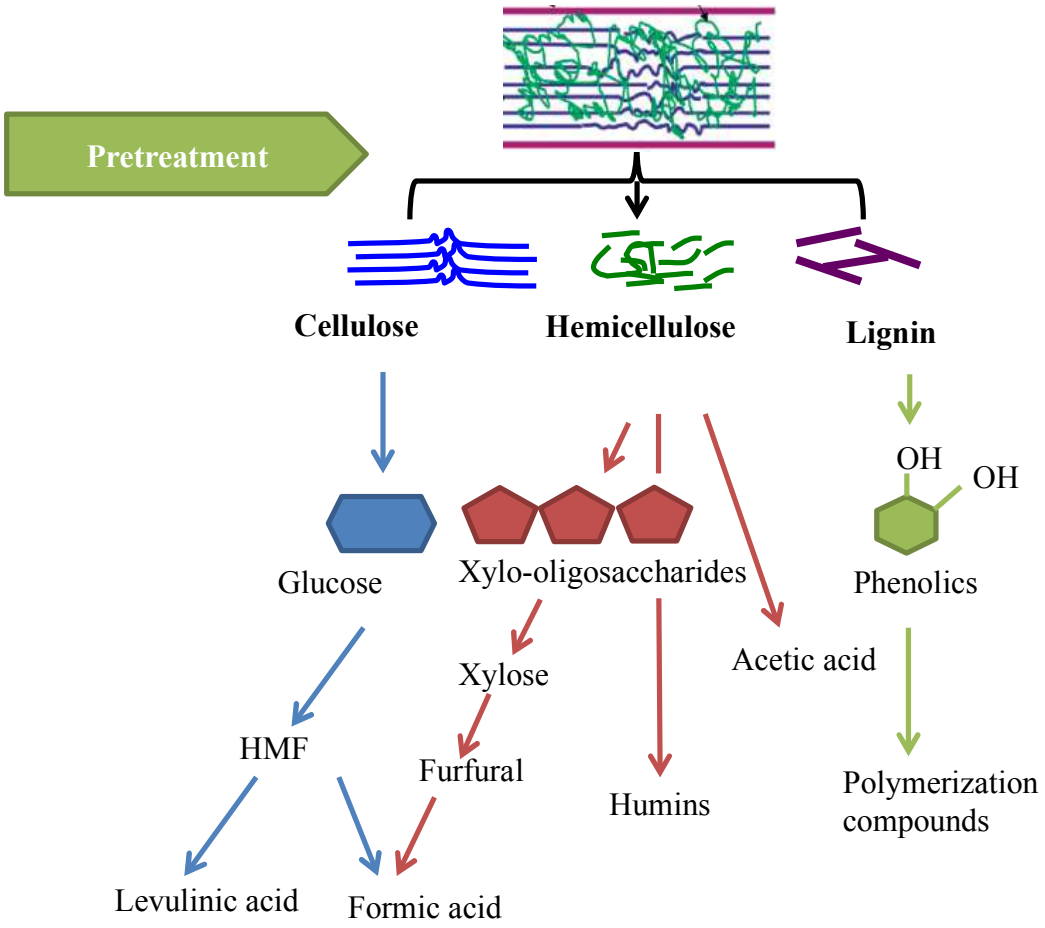

Figure 1. A schematic representation of degradation products formed during typical physico-chemical deconstruction of lignocellulosic biomass.[19,52,53]

Enzymatic saccharification of structural carbohydrates is a heterogeneous phenomenon, which unlike homogeneous reactions involves two major steps: enzyme adsorption on the substrate surface and hydrolysis of the polymers to form shorter chained molecules. Most saccharification cocktails include cellulases and xylanases that are composed of two distinct domains connected by a peptide linker: a cellulose or carbohydrate binding domain and a catalytic domain.[61] The efficiency of enzymatic hydrolysis depends on the accessibility of cellulose binding domains to cellulose and the activity of enzyme catalytic domains, once it has adsorbed on the substrate.

Modes of enzyme inhibition

Lignin–derived phenolic compounds are known to deactivate and inhibit the saccharification enzymes by precipitation and competitive binding, respectively.[55,56] Polyphenolic inhibitors also reduce the accessibility to cellulose by preventing the solubilization of hemicelluloses.[62,63]

Hemicellulose can obstruct the accessibility of cellulases by forming a sheath around cellulose. Therefore pretreatment and supplementation of the saccharification cocktail with xylanases will improve the macro-accessibility to the cellulose binding domains of EG and CBH1.[61] Humins formed during depolymerization and condensation of xylan, can precipitate the cellulases and thereby deactivate the enzymes.[64]

Crystalline cellulose is highly hydrophobic and irreversibly binds to CBH1, thus reducing its enzyme activity.[65] Crystallinity of cellulose, also greatly affects the micro-accessibility of the cellulase binding domains. The current solution, to overcome the problems with accessibility and reduction in cellulase activity due to biomass recalcitrance and the production of inhibitors, is to increase the enzyme loading. As discussed earlier, this will lead to increase in total production cost and also increase in GHG emissions, thus reducing the commercialization potential of cellulosic biofuels.

Despite having elucidated the formation of various inhibitory compounds and proposing their modes of inhibition, no efforts have been made to identify the component of cellulases that were most inhibited and the major inhibitor of the cellulases. A schematic of the proposed enzymatic hydrolysis studies is given in Figure 2.

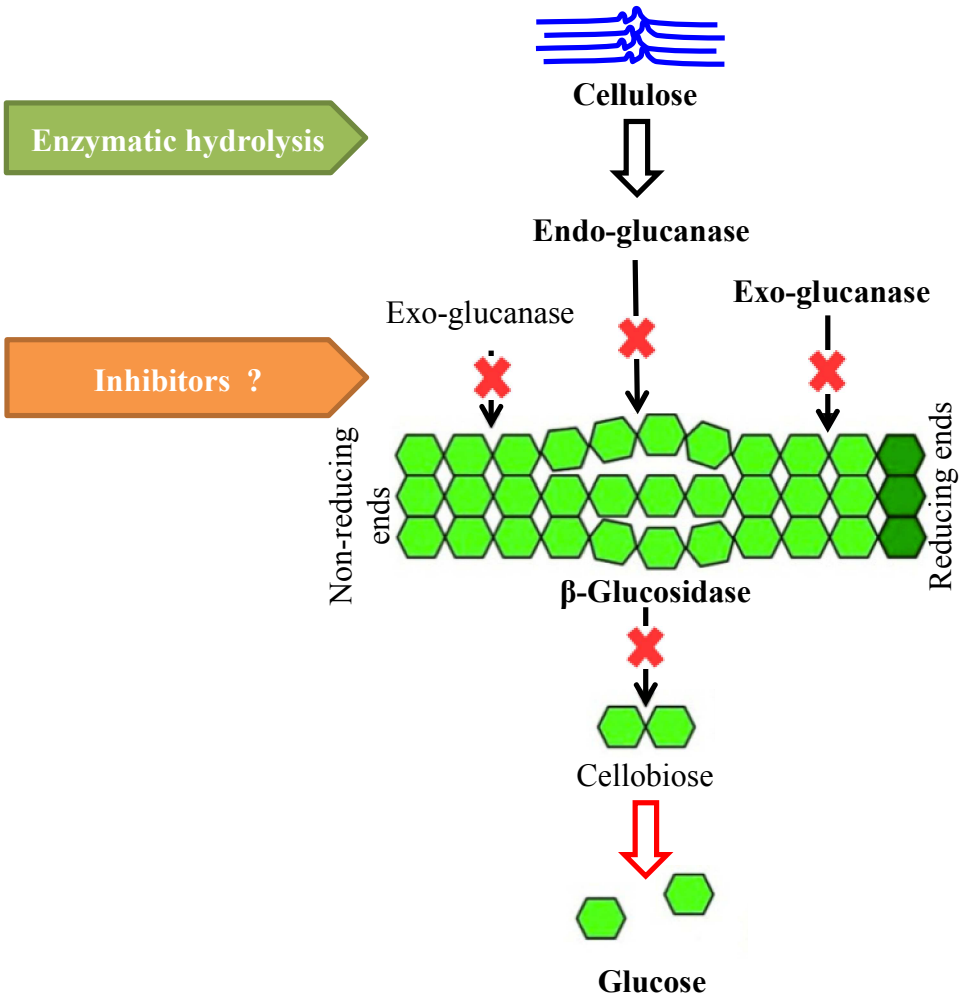

Figure 2. A schematic representation of the goals of this study: 1) to elucidate the component of saccharification mixture most inhibited by the presence of inhibitors and 2) to identify the most inhibitory compound that inhibits the activity of select cellulases.

Optimization of biomass pretreatment

Physico-chemical pretreatment, such as dilute acid, involves deconstructing the biomass using 1 to 5 % v/v of sulfuric acid, at high temperatures of 120 to 200°C and reaction times ranging from 30 to 90 min. High (30 %) or low (10 %) solid loading is also an important parameter that influences the process design and overall efficiency. After pretreatment the pH of biomass is neutralized to approximately 4.8 and then subjected to enzymatic saccharification, at approximately 50°C and at varying enzyme loadings and incubation times. Agricultural residues

(namely rice straw, wheat straw and corn stover) have been widely studied for determining their optimal pretreatment and enzyme saccharification conditions for maximal sugar recovery. Wheat straw was pretreated at 160 °C, 10 min and 0.5 % sulfuric acid concentration and 83 % of monosaccharides were recovered, after 72 h of enzyme hydrolysis.[66] Conventional two step acid hydrolysis of corn stover at a 30% solid loading, 1% acid concentration and 158 °C, resulted in 85 % removal of hemicellulose.[67] Further enzymatic saccharification, at a 20 % solid loading and 20 mg protein/g cellulose, provided 90 % glucose conversion after 84 h.[67] Selecting the best pretreatment conditions, depends on the biomass type and the pretreatment goals of either maximizing hemicellulose conversion or minimizing cellulose conversion. Statistical modeling may be used to maximize the xylose and glucose yields, after pretreatment and enzymatic hydrolysis, respectively. In a study, using response surface methodology, the optimal pretreatment conditions to solubilize 91 % of xylose from wheat straw were determined to be 147 °C, 30 min reaction time and at a 1.6 % acid concentration.[25] Using similar statistical modeling, the optimal conditions for dilute acid pretreatment of rice straw, were determined to be 1.2 % sulfuric acid, 11.6 min and 142°C.[68] At a 20 % solid loading, 73 % of xylose was recovered using the above said pretreatment conditions. Another possibility is to use the combined severity factor, which combines the reaction time, pretreatment temperature, and sulfuric acid concentration into a single variable.[69] Using this factor, the optimal pretreatment conditions for 83 % recovery of monosaccharides, from rice straw, were determined to be 1% sulfuric acid concentration, 160 or 180 °C and at 1 to 5 min reaction times. Cellulase loadings of 10 FPU/g dry matter and β–glucosidase loading of 15 IU/g dry matter were used in the same study and the incubation time was 72 h.[24]

Some of the optimization studies have also accounted for the production of inhibitors specifically furfural, HMF, acetic acid and formic acid.[68,69] Using statistical modeling, it was demonstrated that degradation of glucose to HMF can be prevented during dilute acid pretreatment of wheat straw.[25] It was also demonstrated that by determining the optimal combined severity factor, minimal concentrations of acetic acid, furfural and HMF (1.6, 1.1, 0.2 g/L, respectively) were produced during dilute acid pretreatment of rice straw.[24] Similarly, by altering the pretreatment severity with respect to time and temperature, the production of furan derivatives and weak acids was controlled during the dilute acid pretreatment of corn stover.[70] In another study, by optimizing the dilute acid pretreatment conditions of wheat straw at 140 °C, 30 min and a1.0% sulfuric acid concentration, the amount of formic acid, furfural, acetic acid and HMF were reduced to 32.37 ± 4.91, 12.08 ± 1.69, 7.98 ± 1.02 and 1.14 ± 0.22 g kg^{-1}, respectively.[11] Such optimization studies have also demonstrated a reduction in water usage, for detoxification, by at least 1.5 times. [11,51]

For the fractionation and removal of phenolic inhibitors, the use of absorptive resins has been recommended.[71,72] Since the cost of installation of packed resin columns for the removal of a single class of inhibitors would be prohibitory, it is logical to develop an optimization strategy to prevent the formation of such inhibitors.

Conclusion

Two major bottlenecks faced by the lignocellulosic biofuel industry are the reduction in enzyme saccharification efficiency and the increase in water usage. Both problems are caused by the production of inhibitors during the pretreatment of biomass. Even though dilute acid pretreatment is a relatively inexpensive and simpler pretreatment technology, it leads to the production of inhibitors. There are several degradation products that inhibit the cellulases, such

as, furan derivatives, weak acids, phenolic compounds, xylo-oligosaccharides and humins, that are formed during dilute acid pretreatment of lignocellulosic biomass. Of these inhibitors, it is still unclear, which are most inhibitory and which component of the saccharification cocktail is most inhibited. In order to improve enzymatic conversion efficiency, it is necessary to optimize the pretreatment conditions, such that the production of the most-inhibitory compound is minimized. Hence, it is essential to first identify the major inhibitor of cellulolytic enzymes such that, it is possible to determine its kinetics of formation and the pretreatment parameters can be optimized to reduce its production. The objectives of this research was therefore:

1. To characterize the inhibitors formed during the physico-chemical pretreatment of rice straw
2. To analyze the inhibition of *exo*–cellulase, *endo*–cellulase and β-glucosidase enzymes in the presence of these inhibitors and,
3. To determine the major inhibitors in the pretreatment hydrolyzates of rice straw that are severely inhibitory to model cellulase enzyme systems.

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III. Effect of Dilute Acid Pretreatment Conditions and Washing on the Production of Inhibitors and on Recovery of Sugars During Wheat Straw Enzymatic Hydrolysis

Kalavathy Rajan[a], Danielle Julie Carrier[b*]

[a] Department of Food Science, University of Arkansas, Fayetteville, AR.

[b] Department of Biological and Agricultural Engineering, 203, Engineering Hall, 1 University of Arkansas, Fayetteville, AR 72701, USA. **Phone:** +1(479) 575-2540. **Email:** carrier@uark.edu

[*]Corresponding author

Highlights

- 89% sugar yield was obtained at 140 °C, 30 min, 10 dm^3 m^{-3} acid concentration
- Formic acid and furfural displayed the highest inhibitor concentrations
- Optimizing pretreatment conditions reduced inhibitors by 37%
- Rinsing pretreated solids with water was effective in removing 86.5% of the furans

Abstract

Pretreatment is an essential process to break down recalcitrant biomass and dilute acid hydrolysis is one of the most efficient and cost effective pretreatment technologies available today. However there are potential disadvantages in using dilute acid as a pretreatment, such as the production of degradation products, which inhibits the ensuing processing chain and limits its adoption. In this work, wheat straw was pretreated under varying dilute acid conditions; the resulting degradation products were determined and the quality of sugar stream generated via enzymatic saccharification was monitored. The dilute acid pretreatment conditions were: temperatures of 140 and 160 °C, sulfuric acid (96.5 % mass fraction) concentrations of 5, 10 and 20 dm^3 m^{-3} and reaction times of 10, 20, 30, 45 and 60 min. Pretreated wheat straw was washed with six dilutions of water and hydrolyzed with commercial cellulase enzymes for 48 h. Optimal conditions for pretreating wheat straw were determined as: 140 °C, 30 min and 10 dm^3 m^{-3} sulfuric acid concentration. At these conditions, the glucose yield from wheat straw was maximized at 89% of the theoretical maximum, while the concentrations of formic acid, furfural, acetic acid and 5-hydroxymethylfurfural were 32.37± 4.91, 12.08± 1.69, 7.98± 1.02 and 1.14± 0.22 g kg^{-1}, respectively. Increases in pretreatment severity led to increases in inhibitor generation, as well as a 27% reduction in monosaccharide yield. Rinsing with deionized water was effective in removing inhibitors, such as 86% of furfural. The formation of inhibitors was thus observed to depend on dilute acid pretreatment conditions.

Key words: Wheat, agricultural residue, dilute acid, pretreatment, inhibitors, washing

Introduction

The global consumption of crude oil and liquid fuels was 14.18 hm^3 day^{-1} in 2012, and is projected to grow at an increasing rate in the next two years [1]. Biofuels accounted for only 2 % of the total global fuel consumption [2]. Biofuels can be produced from lignocellulosic biomass, such as agricultural residues [3]. Rice, corn, or wheat residues are potential feedstock for the commercial production of lignocellulosic biofuel, especially in the Non-OECD (Organization for Economic Cooperation and Development) Asian nations, which are projected to drive the global liquid fuel consumption rate [1]. Wheat occupies the largest cropping area harvested in the world and can supply 865 Tg of dry straw annually, on a sustainable basis [4]. Wheat straw has been used to produce cellulosic ethanol via dilute acid pretreatment and enzyme saccharification, at yields of 240 g kg^{-1} [5].

Briefly, cellulosic ethanol production through the biochemical platform involves pretreating the biomass to loosen plant cell walls, saccharifying pretreated biomass with enzymes, fermenting sugar stream and recovering ethanol [3]. Dilute acid pretreatment is a relatively low cost technology for the enhanced deconstruction of plant cell wall [6, 7]. However, this method also leads to the production of inhibitors such as acetic acid, formic acid, levulinic acid, furfural, 5− hydroxymethylfurfural (HMF), which are inhibitory to enzymatic saccharification [8] and to fermentation microorganisms [9]. The produced inhibitors must be removed prior to saccharification and fermentation. Detoxification strategies include treatment with alkali or sulfite, evaporation of initial volume, anion exchange, enzymatic processing, or fungal co-cultivation [10]. Rinsing pretreated biomass with up to nine volumes of water has been shown to alleviate the effect of inhibitors [11]. However, adding this biomass-rinsing step increases water consumption in an already water intensive manufacturing process. Upcoming

cellulosic biofuels plants are projected to consume 23 to 38 m^3 of water per m^3 of ethanol, of which one third will be used directly in processing [12].

In order to promote the manufacturing and consumption of second-generation biofuels, process sustainability needs to be increased, while the cost of production has to be reduced. Therefore water usage, necessary for inhibitor removal, needs to be reduced. Prior studies reported on the presence of inhibitors in biofuel-based wheat straw systems [5, 13, 14]. However, these studies did not identify dilute acid pretreatment conditions that maximized saccharification, as well as minimized inhibitor generation. In this study, wheat straw was pretreated at combinations of dilute acid concentrations, reaction time and temperature to produce a high quality fermentable sugar stream in which inhibitor concentrations were minimized.

Materials and methods

Chemicals

Glucose, xylose and arabinose standards were purchased from Sigma-Aldrich (St. Louis, MO) and Alfa-Aesar (Ward Hill, MA). Sulfuric acid ACS reagent, 95.0 to 98.0 %, was purchased from EMD Chemicals (Gibbstown, NJ). Calcium carbonate was obtained from Fisher Scientific (Fair Lawn, NJ). Water was prepared with a Direct-Q system (Millipore, Billerica, MA) that had resistivity of 18.2 MΩ. All solvents were of HPLC grade and filtered through 0.2 μm filter assembly (Thermo Fischer Scientific, Nashville, TN). Accellerase® 1500 enzyme, with an endoglucanase activity of 2200−2800 CMC U g^{-1} (Carboxymethylcellulose units) and β-glucosidase activity of 525−775 pNPG U g^{-1} (pNP-glucoside units) was graciously donated by Genencor (Rochester, NY).

Biomass and compositional analysis

Hard red winter wheat, *Triticum aestivum* L, was grown in Girard, Kansas (longitude 94° 50' 16" W and latitude 37° 30' 40" N) and harvested in June 2011. Residue, mainly stalks, were assembled into 0.61 m by 0.91 m square bales and kept in a covered outdoor storage facility. Once purchased in January 2012, the wheat straw bale was kept in a 4 °C walk-in refrigerator, until use in summer 2012. The biomass was ground in a Wiley mill and passed through a 20 mesh screen, such that the particle size was in the range of 0.80 to 0.91 mm [15]. The total solids, extractives, ash, structural carbohydrates, including glucose, xylose, arabinose, and the lignin content of wheat straw were determined as previously reported [16-20].

Dilute acid pretreatment

Dilute acid pretreatment of wheat straw was conducted at temperatures of 140 or 160 °C; sulfuric acid concentrations of 5, 10 and 20 dm^3 m^{-3} and reaction times of 10, 20, 30, 45 or 60 min. Wheat straw samples mixed with dilute acid solution, at 100 kg m^{-3} solid loading, were loaded in stainless steel tube reactors (length 10 cm, internal diameter 1.4065 cm, thickness 639 μm and total chamber volume of 16 cm^3) and submerged in an industrial fluidized sand bath (Techne Ltd., Burlington, NJ). The sand bath was heated at least one hour prior to the experiments, such that the pretreatments were efficiently conducted at the selected reaction temperatures [21]. After pretreatment, the volume of hydrolyzate was recorded and a portion of acid hydrolyzate was collected in 15 cm^3 centrifuge tubes. The hydrolyzed aliquots were centrifuged (Clinical 200 Large capacity centrifuges, VWR International, Houston, TX) at 2912 × *g* for 180 s in order to separate the solid and liquid fractions. The solid fraction was washed with deionized water, at 167 kg m^{-3} solid loading, for detoxification. Wash water was

centrifuged and separated from the solids, at 2912 × *g*, for 600 s. Liquid and wash water fractions were analyzed for the presence of by-products, namely formic acid, acetic acid, furfural and HMF as well as for glucose and xylose.

Enzymatic hydrolysis

The solid fraction was washed and incubated with cellulase cocktail, Accellerase® 1500 (Genencor, Rochester, NY), at pH 4.8. For each experiment, 1.5 g of solid fraction was loaded in 50 cm^3 amber bottles with 5 cm^3 of sodium citrate buffer (100 mol m^{-3}), 0.5 cm^3 of the enzyme cocktail and 3 cm^3 of deionized water [22]. The mixture was heated to 55 °C in a reciprocating water bath (Thermo Scientific, Nashville, TN), agitated at 1.67 Hz for 48 h. Samples of one cm^3 were collected every 24 h and immediately immersed in an ice bath to inactivate the enzyme [23]. Afterwards, the samples were centrifuged at 12,100 × *g*, for 60 s (Eppendorf MiniSpin® plus, Sigma- Aldrich, St. Louis, MO) and the liquid enzyme hydrolyzate was separated and stored at 4 °C prior to High Performance Liquid Chromatography (HPLC) analysis.

HPLC analysis

The samples for sugar analysis, including the water soluble, wash water and enzyme hydrolyzate fractions, were neutralized to pH 7.0, by adding calcium carbonate. The neutralized samples were viscous and required successive filtration using 0.45 μm and 0.22 μm filter syringes (National Scientific Company, Rockwood, TN) in order to completely remove any particulates, prior to HPLC analysis. Protocols for HPLC detection were based on the NREL (National Renewable Energy Laboratory) report #TP-510-42618. Briefly, analysis of monomers was conducted on a Waters Alliance HPLC system (Model 2695, Waters Corporation, Milford, MA) fitted with a SP-G precolumn and a SP0810 column (Shodex, Kawasaki, Japan). Analyses

of by-products were performed using the Waters Alliance HPLC system fitted with a Bio-Rad Aminex (Life Sciences Research, Hercules, CA) HPX-87H ion exclusion column as described in [20, 21, 23, 24]. The concentrations of monomers, such as glucose, xylose and arabinose as well as acetic acid, furfural, formic acid and HMF, were determined from in-house calibration curves. The percentage recovery of each product was calculated on an extractive free basis. The maximum theoretical yields of glucose and xylose were calculated by multiplying the mass of glucan and xylan with their respective conversion factors: 1.11 (180/162) and 1.14 (150/132) [18].

Statistical analysis

A full factorial design was employed for the treatment of the wheat straw sample and the treatments were conducted in triplicates. Inferential statistical methods (ANOVA, Student's *t*-test) were used to determine the effect of individual test parameters namely, reaction time, temperature, sulfuric acid concentration and enzymatic hydrolysis duration.

Results and discussions

Effects of pretreatment temperature, reaction time and acid concentration on carbohydrate recovery

The total solids of wheat straw was determined to be 939.5 ± 15 g kg^{-1} and its composition on a dry weight basis, is given in Table 1. The glucan: xylan ratio was calculated as 2.29, which was higher than previously reported values of 1.69 and 1.75 [25, 26]. Overall carbohydrate recovery is presented in Fig.1. Hydrolyzate carbohydrate recovery is presented in Fig.1a; maximum calculated values for xylose and glucose, were 45.13 ± 2.51 % and 12.88 ± 0.81 % of the theoretical maximum, respectively. Previous studies reported that pretreatment

temperature was an important factor in pretreatment effectiveness [5, 13]. Results presented in Fig.1a indicated, however, that acid concentration also affected xylose recovery. Highest xylose recovery was obtained at a pretreatment temperature of 140 °C, for 30 min and sulfuric acid concentration of 10 dm^3 m^{-3}.

Increasing the duration of enzymatic hydrolysis from 24 to 48 h resulted in an overall increase of 59% and 46% in the yields of glucose and xylose, respectively. Fig. 1b reports the carbohydrate yields after 48 h of enzymatic hydrolysis. At different pretreatment conditions, the quantities of monomeric sugars released during enzyme saccharification were also different, as seen in Fig. 1b. The yield of xylose was significantly reduced at 20 dm^3 m^{-3} sulfuric acid concentration ($P < 0.05$) and therefore the total yield of fermentable sugars was affected. The results presented in Table 2 indicated that the highest glucose recovery of 89%, was obtained by pretreating the biomass at 140 °C, for 30 min, with a sulfuric acid concentration of 10 dm^3 m^{-3} and enzymatically hydrolyzed for 48 h. This is higher than the 83% recovery reported using the pretreatment conditions of 160 °C, 10 min, 5 dm^3 m^{-3} acid concentration and 72 h of enzyme hydrolysis [14].

Effects of pretreatment temperature, reaction time and acid concentration on inhibitor generation

Increasing temperatures, reaction time and acid concentration of the dilute acid pretreatment resulted in an increase in the concentration of degradation products, such as formic acid, acetic acid, furfural and HMF [8, 13]. Results presented in Fig. 2 also show that inhibitors generation increased with pretreatment temperatures and acid concentration. The concentration of all inhibitors increased as a function of sulfuric acid concentrations from 5 to 20 dm^3 m^{-3}. In

the case of formic acid, a significant increase was observed at acid concentrations of 20 dm^3 m^{-3} Results presented in Table 2 for 160 ˚C and 20 dm^3 m^{-3} indicated that significant differences were observed for xylose concentrations as a function of reaction time. Given that xylose degrades to furfural and formic acid [27], xylose decreases can possibly explain furfural and formic acid increases reported in Fig. 2.

Pretreating in 20 dm^3 m^{-3} dilute acid at 160 °C for 30, 45 or 60 min resulted in 161, 226 and 164 g kg^{-1}, respectively, of combined acetic acid, furfural, formic acid and HMF yields. For similar conditions, but at 140 °C, the combination concentrations were 98, 136 and 133 g kg^{-1}, respectively. At the condition that allowed for highest carbohydrate recovery, 140 °C, 30 min, 10 dm^3 m^{-3} sulfuric acid, only 37% of the maximum inhibitor concentrations were produced. Pretreating at 140 °C, 30 min, 10 dm^3 m^{-3} sulfuric acid resulted in 32.37 ± 4.91, 12.08 ± 1.69, 7.98 ± 1.02 and 1.14 ± 0.22 g kg^{-1} of formic acid, furfural, acetic acid and HMF, respectively. Decreases of 16% and 38% in respective glucose and xylose recovery were observed at pretreatment conditions of 160 °C, 20 dm^3 m^{-3} dilute sulfuric acid and 45 min, possibly mirroring increases of formic acid and furfural. Synchronizing pretreatment conditions such that maximum carbohydrate coincides with minimum inhibitor generation would be considered critical for the process.

Pretreating wheat straw with 36.7 dm^3 m^{-3} sulfuric acid has been shown to generate only 24 g kg^{-1} of acetic acid, 9 g kg^{-1} of furfural, and 1.3 g kg^{-1} of HMF [5]. Differences in nature and condition of feedstock as well as with pretreatment conditions could account for the differences in acetic acid and formic acid reported in this work and that of [5]. Previous work has shown that

formic acid inhibits enzymatic hydrolysis [24]. Thus, it is critical to establish a balance between carbohydrate recovery and acetic acid, furfural, formic acid and HMF generation.

Effect of water wash

Studies showed that inhibition might be partly overcome by the removal or detoxification of inhibitors, such as acetic acid, furfural, formic acid and HMF [28]. There are a number of methods for detoxification. The simplest, however, is to rinse the pretreated biomass with water, washing out the inhibitors prior to enzymatic hydrolysis [29]. Rinsing pretreated biomass with treated effluent water has also been reported [12]. To detoxify pretreated corn stover and obtain 85% cellulose conversion, the use of nine volumes of water has been reported at the pilot-plant scale [11]. In order to minimize water use, six volumes of rinsing water were used in this study. Analysis of the wash water indicated that, on average, 87% of formic acid and 64% of acetic acid were removed in the water-soluble fraction. However, 86% of furfural and 87% HMF were removed in the wash water upon rinsing the solid fraction. These results indicate that, although produced during pretreatment, acetic acid, furfural, formic acid and HMF are not removed by similar mechanism. These results also show that it is critical to characterize pretreatment conditions such that the production of formic acid and acetic acid is minimized at the onset.

Conclusion

Thus in this study, it was determined that the pretreatment temperature, duration and acid concentrations had significant effect on the production of inhibitors. The pretreatment conditions, 140 °C, 30 min, 10 dm^3 m^{-3} acid concentration, accompanied by a six-volume rinse resulted in a 89% carbohydrate recovery and a 37% inhibitor generation yield. Favorable pretreatment conditions thus reduced the concentration of the inhibitors generated. Such

improvements would facilitate the development of effective pretreatment conditions and most likely result in decreasing production costs. Future work will concentrate on pinpointing which inhibitor or inhibitor combination, in the water soluble and wash water fractions of wheat straw, affects the enzymatic hydrolysis.

Acknowledgments

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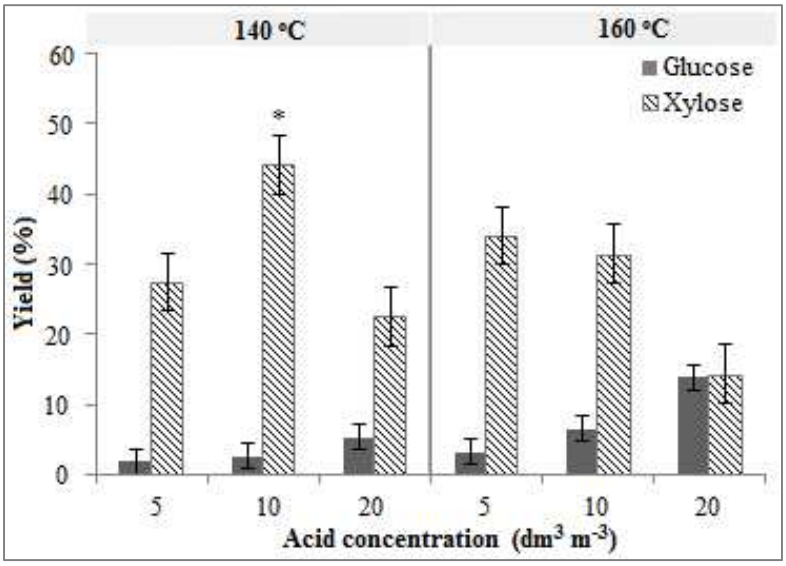

Fig.1a. Sugar recovery in pretreated hydrolyzate. Average yields of glucose and xylose (%) recovered from wheat straw pretreated at 30 min, for different acid concentrations and temperatures. Value indicated by '*' symbol was significantly different ($P < 0.05$). Each error bar was constructed using 1 standard error from the mean.

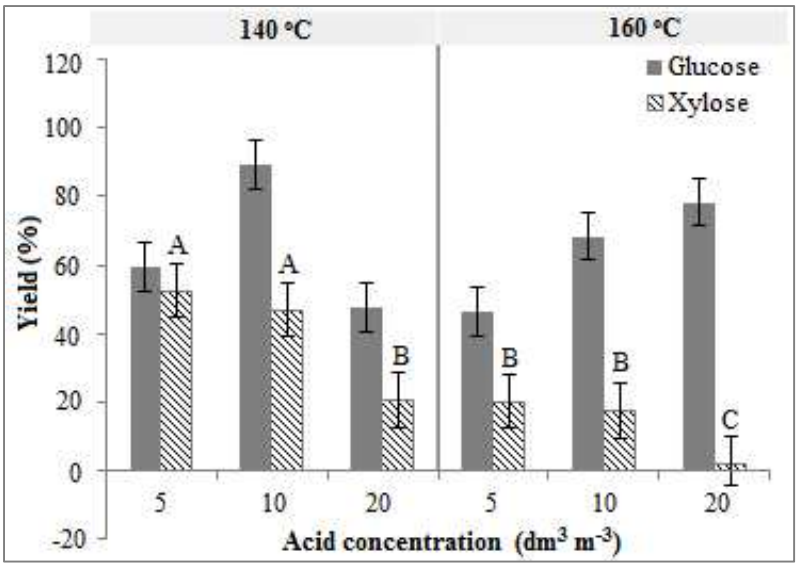

Fig. 1b. Sugar recovery in enzyme hydrolyzate. Average yields of glucose and xylose (%) after 48 h enzymatic hydrolysis, at different pretreatment temperatures, acid concentrations and at 30 min reaction time. Bars not indicated by the same letters were significantly different ($P < 0.05$). Each error bar was constructed using 1 standard error from the mean.

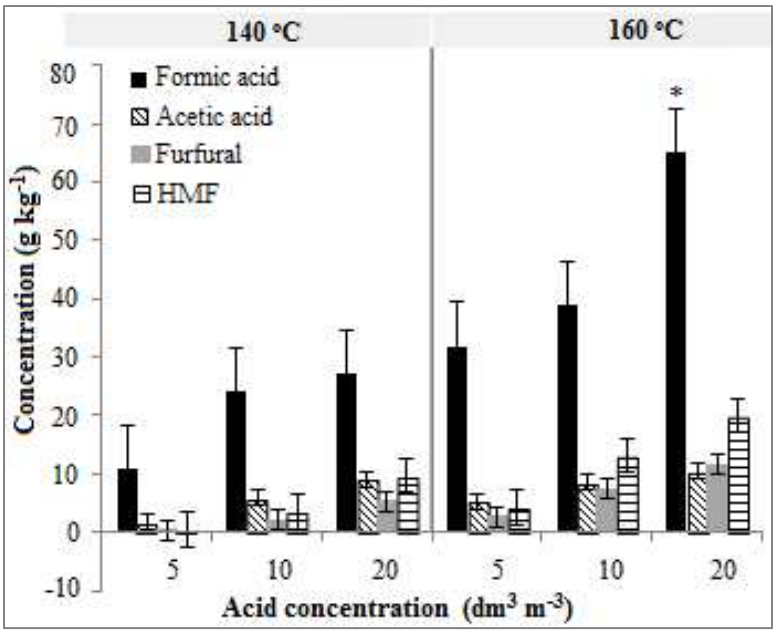

Fig. 2. Inhibitors in wheat straw. Mean concentration of the inhibitors (g kg^{-1}) recovered from water-soluble fraction, at 30 min, for different pretreatment temperatures and acid concentrations. Symbol '*' indicates significantly different value from corresponding bars of the same color ($P < 0.05$). Each error bar was constructed using 1 standard error from the mean.

Table 1 − Composition of wheat straw (mean ± standard deviation), in % Oven Dry Weight (ODW) basis.

Components	**% ODW**
Moisture	6.41 ± 0.09
Ash	5.01 ± 0.09
Total extractives	5.47 ± 0.99
Acid insoluble (Klason) lignin	16.10 ± 0.13
Acid soluble lignin	0.77 ± 0.04
Glucan	38.36 ± 3.63
Xylan	16.72 ± 1.72
Arabinan	2.99 ± 0.41
Total	**91.83 ± 7.10**

Table 2 − Average yield of sugar monomers (± standard deviation) from pretreated wheat straw, incubated at 55 °C, with Accellerase® 1500 enzyme, for 24 h & 48 h.

Duration (h)	Monosaccharide yield (% theoretical maximum)	
	Glucose	Xylose
24	41.75±4.05	20.16±2.67
48	67.73±5.04	36.46±3.83

Table 3 – Percent theoretical maximum yields of glucose and xylose (mean ± standard deviation) from wheat straw, pretreated at different time, temperatures and acid concentrations and after 48 h of enzymatic hydrolysis.

Acid concentration (%)	Reaction time (min)	Temperature			
		140 °C		160 °C	
		% Glucose	% Xylose	% Glucose	% Xylose
0.5	10	33.70±2.6	34.57±7.2	40.44±1.8	24.65±6.7
	20	45.25±1.0	37.96±7.1	46.45±2.9	21.53±6.7
	30	59.45±1.1	55.32±2.4	48.64±4.5	20.32±4.8
	45	61.24±0.9	49.55±1.0	39.42±3.3	13.21±4.7
	60	72.67±0.5	52.63±1.2	44.76±1.3	9.31±5.7
1.0	10	79.62±3.2	43.01±1.4	73.86±1.7	21.53±2.5
	20	85.39±3.6	47.97±0.9	75.83±1.6	21.45±2.6
	30	89.25±1.1	47.18±2.7	77.64±1.5	17.55±1.8
	45	62.62±9.7	37.86±1.4	68.40±1.8	14.50±1.5
	60	72.42±9.5	40.04±3.3	73.77±1.6	13.29±1.3
2.0	10	42.92±2.9	19.56±1.8	72.39±4.4	13.64±0.7[a]
	20	47.69±4.2	18.61±1.7	67.32±0.5	3.37±0.3
	30	48.76±3.2	20.68±1.9	78.32±0.2	1.81±1.4
	45	34.03±8.4	14.45±0.9	49.51±6.1[a]	1.22±0.8
	60	38.63±9.4	13.74±0.6	63.52±2.4	0.23±0.0[b]

[a,b] Significantly different within the same group of pretreatment conditions ($p<0.05$).

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IV. Characterization of Rice straw Prehydrolyzates and Their Effect on the Hydrolysis of Model Substrates, Using a Commercial *endo*-Cellulase, β-Glucosidase and Cellulase Cocktail

Kalavathy Rajan [†], Danielle Julie Carrier* [‡]

† Department of Food Science, University of Arkansas, 2650 N Young Ave, Fayetteville AR 72704 USA

‡ Department of Biological and Agricultural Engineering, 203 Engineering Hall, 1 University of Arkansas, Fayetteville AR 72701 USA

* Corresponding **Email:** carrier@uark.edu, **Phone:** +1(479) 575-2540.

Abstract

Pretreatment and enzymatic saccharification are two major upstream processes that affect the economic feasibility and sustainability of lignocellulosic biofuel production. Cellulase inhibiting degradation products, generated during dilute acid pretreatment, increase enzyme usage and therefore, it is essential to mitigate their production. In an attempt to elucidate the most deleterious degradation product to enzymatic hydrolysis, hydrolyzates were generated from rice straw and their effect on enzyme activity was determined. Ground rice straw was subjected to the following pretreatments at a combined severity factor of 1.75: T1– 160°C, pH 1.7; T2– 180°C, pH 2.25; and T3– 220°C, pH 7.0. The liquid prehydrolyzates were freeze-dried and their inhibitory effects on the activity of a commercial cellulase cocktail, *endo*-cellulase and β-glucosidase, were determined using filter paper, carboxymethyl cellulose and cellobiose, respectively. Addition of 15 g L^{-1} of T1, T2, or T3 freeze-dried prehydrolyzates resulted in decreases of 67%, 57%, and 77% of CMC-ase activity of *endo*-cellulase, respectively. In the presence of 35 g L^{-1} of T1, T2, or T3 prehydrolyzates, the filter paper activity of cellulase cocktail was reduced by 64%, 68%, and 82%, respectively. Characterization of the freeze-dried prehydrolyzates showed that T3 had significantly higher xylo-oligosaccharides and total phenolic content than T2 and T1.

Key words: Rice straw, prehydrolyzate, *endo*-cellulase, β-glucosidase, filter paper activity, cellobiase activity, xylo-oligosaccharides, phenolics, enzyme inhibition

Introduction

The estimated production of lignocellulosic fuel in the United States, in 2012, was less than 0.5 million gallons, a fraction of the revised projection of the Renewable Fuel Standards-2 of the Energy Independence and Security Act, 2007.[1] Start-up cellulosic biofuel industries are afflicted with problems ranging from feedstock availability to high production costs to scalability.[2] Cellulolytic enzymes, used in saccharification, account for 14% of the total production cost and, next to the biomass cost, is the highest input cost.[3] Carbohydrate degradation products, such as acetic acid and formic acid, and furan derivatives, such as furfural and 5-hydroxymethyl furfural, are formed during the pretreatment of lignocellulosic biomass, and have been reported to inhibit the cellulase cocktails.[4,5,6] In addition, xylo-oligosaccharides and lignin-derived compounds, released during biomass pretreatment, also inhibit commercial cellulase cocktails.[7,8] Lignin model compounds, such as tannic acid, gallic acid, vanillin, cinnamic acid, ferulic acid, *p*-coumaric acid, sinapic acid, syringaldehyde and *p*-hydroxybenzoic acid were reported to inhibit and deactivate the cellobiose activity of β-glucosidases and filter paper activities of commercial cellulase cocktails, through precipitation and competitive binding.[9,10] Similarly, the inhibitory effects of pure xylobiose and higher DP xylo-oligosaccharides have been tested against commercial cellulases on Avicel substrates. It was reported that initial glucose yields were reduced by 82% in the presence of 12.5 g L^{-1} of xylooligomers, as a result of competitive inhibition of cellulases.[11] Thus, these degradation products play a significant role in reducing the efficiency of enzymatic hydrolysis and increasing the input cost.

For the satisfactory performance of the saccharification cocktail, inhibitory compounds present in prehydrolyzates need to be mitigated, prior to enzymatic hydrolysis. Rinsing the pretreated biomass with 10X or more volumes of water have been reported to remove the

degradation products and improve the enzymatic saccharification on a laboratory scale.[8,12,13] At the demonstration scale, estimates of water consumption of the cellulosic ethanol industry are 23 to 38 L of water per liter of ethanol; detoxification with such water volumes would further decrease its sustainability.[14] Therefore, efforts were made in this study to characterize the prehydrolyzates and to determine which group of compounds affected saccharification. The long-term objective of this work is to identify which components in the crude prehydrolyzate are most inhibitory to the cellulase cocktail as a whole and to its individual enzymes, such as *endo*-cellulase and β-glucosidase.

Agricultural residues constitute the bulk of potential feedstock available in the U.S., for cellulosic fuel production.[15] Globally, rice straw alone represents 23% of all agricultural wastes and is a major feedstock source for cellulosic biofuel production in countries, such as India, where extensive land use changes would be unsustainable.[16] Because of its importance on the world scene, rice straw was used as the feedstock in this study.

Materials and methods

Chemicals

Commercial standards of glucose, arabinose (Alfa-Aesar, Ward Hill, MA), xylose, 5-hydroxymethyl furfural (HMF), furfural, *p*-coumaric acid, *trans* ferulic acid, protocatechuic acid, acetosyringone (Sigma-Aldrich, St. Louis, MO), syringaldehyde, 4-hydroxybenzoic acid, vanillin, salicylic acid, gallic acid (TCI chemicals, Montgomeryville, PA) and formic acid (Amresco, Solon, OH) were used in the characterization of the prehydrolyzates. Xylo-oligosaccharide standards (DP2 to DP6) were purchased from Megazyme International (Wicklow, Ireland). The enzymes used in this study were Cellic® CTec2, a saccharification cocktail from *Trichoderma reesei*, NS 22118, a β-glucosidase (Novozymes North America Inc.,

Franklinton, NC) and *endo*-1,4-β-D-glucanase (*endo*-cellulase) from *Aspergillus niger* (Megazyme International). The saccharification cocktail had a filter paper activity of 62 U mL^{-1}. The *endo*-cellulase had a carboxymethyl cellulase (CMC-ase) activity of 2000 U mL^{-1}. NS 22118 had a cellobiase activity of 105 U mL^{-1}. Filter paper (Grade 1 Whatman®), carboxymethyl cellulose (CMC) and cellobiose (≥ 98%) were purchased from VWR International (Houston, TX), EMD Millipore (Bedford, MA) and Alfa-Aesar (Ward Hill, MA), respectively.

Sulfuric acid (≥ 95.0%), hydrochloric acid (≥ 98.0%) and glacial acetic acid (HPLC) were purchased from EMD Chemicals (Gibbstown, NJ). Folin & Ciocalteau's (F-C) phenol reagent, 2-hydroxy–3, 5–dinitrobenzoic acid, ammonium hydroxide and potassium sodium tartrate tetrahydrate, analytical grade, were purchased from Sigma-Aldrich (Milwaukee, WI). Citric acid anhydrous, sodium citrate dihydrate, sodium acetate trihydrate and calcium chloride dihydrate were procured from Alfa-Aesar (Ward Hill, MA). Water was prepared with a Direct-Q system (Millipore, Billerica, MA) that had 18.2 MΩ resistivity.

Raw material and composition

Rice straw was obtained from the University of Arkansas Cooperative Extension Service, Little Rock, AR. The straw was a Clearfield hybrid rice variety and was originally harvested from a location corresponding to *34° 58′ 28.3152″ N* longitude and *92° 0′ 59.5224″ W* latitude (Cabot, Lonoke county, Arkansas). The biomass was stored at 4 °C in a walk-in refrigerator until use. It was ground using a Thomas Willey® Mini mill (Swedesboro, NJ) and sieved with a 20-mesh screen, such that particle size were uniformly reduced to 0.84 mm.[17] The total solids, structural carbohydrates, lignin, ash content and total extractives in the rice straw were determined per the NREL (National Renewable Energy Laboratory, Golden, CO) protocols.[18,19,20,21]

Pretreatment

Rice straw, 25 g and water, 225 mL, were loaded in a 1 L Parr 4525 reactor (Moline, IL) and agitated at 144 rpm until the end of the reaction. The three treatments were: T1 (160°C, 48 min and pH 1.7), T2 (180°C, 44 min, pH 2.25), and T3 (220°C, 52 min and pH 7.0). The conditions for pretreatment were calculated based on the combined severity factor (CSF), which is given by;

$$\text{CSF} = \log\left\{t \times \exp\left[\frac{T_H - T_R}{14.75}\right]\right\} - \text{pH} \quad (1)$$

where t is the duration of pretreatment, T_H is the pretreatment temperature and T_R is the reference temperature (100°C).[22] Concentrated sulfuric acid was used at 1.0% (w/v) and 0.1% (w/v) to adjust the pH of T1 and T2, respectively, prior to the Parr reaction. The pretreatment duration was calculated only at the desired temperature. Once the required duration had elapsed, the heating jacket was removed and the reactor was cooled using cooling coils. The liquid prehydrolyzates were separated from the pretreated biomass, using a Buchner filtration apparatus, fitted with Whatman #1 filter paper and stored frozen at −20°C in 100 mL polypropylene bottles.

Freeze-drying

The frozen prehydrolyzates were quick frozen with liquid nitrogen at −196°C, to ensure thorough freezing, and then freeze-dried in a FreeZone 18 L console freeze dry system (Labconco®, Kansas city, MO). The freeze-drying conditions were −44°C, 7.7 Pa and 72 h. Inhibitor stock was prepared by mixing the lyophilized T1, T2 and T3 prehydrolyzates with buffer, at a concentration of 100 g L^{-1}. This stock was used for further enzyme assays and for characterization studies using liquid chromatography techniques.

Characterization of prehydrolyzate

High Performance Liquid Chromatography (HPLC): The monosaccharide composition of lyophilized rice straw prehydrolyzates was determined using a Waters Alliance HPLC system (Model 2695, Waters Corporation, Milford, MA) equipped with SP-G pre-column, SP0810 analytical column (Shodex, Kawasaki, Japan) and refractive index detector (Model 2414, Waters Corporation, Milford, MA). The xylo-oligosaccharide concentration was determined by equipping the same HPLC with a Bio-Rad Aminex-HPX 42A analytical column (Bio-Rad, Hercules, CA) and a Micro-Guard de-ashing pre-column. Calibration curves for the xylo-oligosaccharides (DP 2 to DP 6) were determined using pure (>95.0%) reference compounds. The analytical columns and the detector were maintained at 85°C and 50°C, respectively. Millipore water was used as eluent at a flow rate of 0.2 mL min^{-1} and the sugars were quantified using in-house calibration curves.

Analyses of formic acid, acetic acid, HMF and furfural, were performed using the Waters Alliance HPLC system fitted with a Bio-Rad Aminex HPX-87H ion exclusion analytical column (Life Sciences Research, Hercules, CA) and photodiode array detector (Model 2996, Waters Corporation, Milford, MA). The samples were eluted with 5 mM sulfuric acid at a flow rate of 0.6 mL min^{-1} and detected at 280 nm.[23]

Total phenolics assay: The total phenolic content of the rice straw prehydrolyzates was determined using the Folin & Ciocalteau's (F-C) method, modified from a previously published protocol.[24] The F-C assay relies on the transfer of electrons from the phenolic compounds, in an alkaline medium, to phosphomolybdic/phosphotungstic acid complexes, to form blue color, which is then read spectrophotometrically, at 765 nm.[25] The 100 g L^{-1} prehydrolyzate solutions were diluted to 1.25 g L^{-1}, and a 100 µL aliquot was mixed with 200 µL of 0.2 N F-C reagent and

incubated in the dark for 5 min. A 700 µL aliquot of 7.5% sodium carbonate solution was added to the mixture and incubated in the dark at room temperature for 2 h. After the incubation period, the samples were diluted 4X with water, and their absorbance at 765 nm was determined using a spectrophotometer (Model 517601, Beckman Coulter Inc., Indianapolis, IN). Gallic acid standards (0.5 to 2.5 g L^{-1}) were used to construct a standard curve; the results were expressed in gallic acid equivalent.

LC/ESI–MS analysis: The lyophilized, rice straw prehydrolyzates were dissolved in methanol to a concentration of 100 g L^{-1} and analyzed in a Hewlett 1100 HPLC system (Hewlett Packard, Palo Alto, CA) using a Supelco C18 column (15 cm x 4.6 mm, 5µm particle size, 300 Å pore size), coupled online to a quadrapole ion trap, electron spray ionization-mass spectrometer (ESI–MS) (Bruker Esquire 2000, Bruker, Billerica, MA). The samples were eluted in a gradient system using 0.1% formic acid in water and methanol from 85:11 to 50:50 (v/v), in 35 min at a flow rate of 0.4 mL min^{-1}. The solvent system was selected based on a previously published method.[26] Mass spectrometry parameters were adjusted to optimize *m/z* signal in the range of 50 to 800. The mass spectrum was obtained in positive ion mode. The chromatogram peaks were identified based on the retention times of the reference compounds and their corresponding *m/z* values.

Enzyme assays

Saccharification cocktail assay: The filter paper activity of the cellulase cocktail was determined by mixing filter paper with 100 mM citrate buffer (pH 5.0), at 5% (w/v) loading, in 16 × 100 mm glass test tubes.[27] For the inhibition studies, the inhibitor stock of T1, T2 and T3 prehydrolyzates were mixed with the buffer such that their concentrations were 15, 20, 25, 30 and 35 g L^{-1}. The filter paper, buffer and inhibitor mixture was equilibrated at 50 °C, for 5 min,

in a reciprocating water bath, agitated at 100 rpm. The saccharification cocktail was subsequently added at 0.67 mg enzyme per gram of filter paper, and incubated at 50°C for 30 min. Upon completion of the reaction, the enzyme was deactivated by boiling the mixture at 100°C for 5 min. The reaction mixture was then cooled in an ice bath and centrifuged at 1286 × *g* for 10 min (IEC Spinette centrifuge, Needham, MA) to separate residual filter paper from the supernatant. The supernatant was analyzed for glucose concentration, using HPLC and the filter paper units were determined.[27]

Endo-cellulase assay: A 4% (w/v) carboxymethyl cellulose (CMC) solution, was prepared with 50 mM acetate buffer (pH 4.5) and used as the substrate.[28] For the control, CMC was mixed with the 50 mM acetate buffer (pH 4.5), at 1.2% (w/v) loading and incubated with 0.04 mg of enzyme per gram of CMC. For the inhibition assays, 50, 100, 150 and 200 µL of the inhibitor stock, corresponding to prehydrolyzate concentrations of 5, 10, 15 and 20 g L^{-1}, respectively, were mixed with the buffer. All assay samples were incubated at 40°C, for 20 min, in a reciprocating water bath, agitated at 100 rpm. At the end of the reaction, 400 µL of dinitrosalicylic acid (DNS) reagent was added, and the color was developed by boiling the mixture at 100°C for 10 min. The DNS reagent was prepared as previously reported.[28] After terminating the reaction, by cooling the samples in an ice bath, their absorbance were determined at 530 nm using a spectrophotometer (Model 517601, Beckman Coulter Inc., Indianapolis, IN), and the specific activity of the enzyme was determined.[29]

β-glucosidase assay: The cellobiase activity of NS 22118 was determined by mixing cellobiose with 100 mM citrate buffer (pH 5.0), at 1.0% (w/v) loading in 16 × 100 mm glass test tubes. For the inhibition studies, the inhibitor stock was mixed with the buffer, such that, their concentration was 15, 20, 25, 30 and 35 g L^{-1}. The mixture was equilibrated at 50 °C for 5 min.

The β-glucosidase was subsequently loaded at 3.49 mg enzyme per gram of cellobiose, and the mixture was incubated at 50°C, for 30 min, in a reciprocating water bath, agitated at 100 rpm. To terminate the reaction, the mixture was boiled at 100°C for 5 min. The mixture was then cooled in an ice bath and the glucose concentration was determined using HPLC. The cellobiose units were determined as μmol cellobiose converted per minute per gram of the enzyme.

All samples were assayed in triplicate and two blanks were prepared per assay for the sample and reagents. The control was prepared with only enzyme and substrate mixed with the buffers. The enzyme activities were determined as previously reported.[29] Analysis of variance and test of significance (Student's *t*-test) were performed using JMP Pro 9.0 (SAS, Cary, NC).

Results and discussion

Effect of combined severity on prehydrolyzate composition

The average composition of rice straw, adjusted for its moisture content is presented in Table 1. The percent glucan (35%), xylan (17%), Klason lignin (11%), acid soluble lignin (0.4%) and total ash (15%), determined as per NREL protocols, aligned with previously reported compositions.[30,31] Ground rice straw was subjected to three pretreatment conditions (T1, T2, and T3) that displayed a combined severity factor (CSF) of 1.75. It has been previously reported that a CSF of 1.75 yielded the maximum quantities of xylose and glucose upon pretreatment and enzymatic saccharification, respectively.[30] The prehydrolyzates were lyophilized and their composition determined using HPLC and colorometric analyses, are presented in Table 2. No significant differences were observed in composition of pre- and post freeze-dried hydrolyzates, except for that of acetic acid, which showed 1.8 and 1.6 fold increases, in T1 and T2, respectively.

In T1 prehydrolyzates, theoretical maximum recoveries of glucose and xylose were 5.95% and 16.46%, respectively, which was similar to previously reported values.[30] The concentrations of degradation products in T1, such as acetic acid, furfural and HMF were 1.75±0.31, 1.00±0.09 and 0.16± 0.01 g L^{-1}, respectively, which were also similar to the previously reported values.[30] As shown in Table 2, despite having constant severities, T1, T2, and T3 yielded prehydrolyzates of varied compositions. Specifically, the proportion of monosaccharides and other degradation products were significantly different in T1, T2, and T3. The mass fractions of weak acids and furan derivatives were similar for T1, T2 and T3. However, the prehydrolyzate composition of total phenolics and xylo-oligosaccharides (DP2 to DP6) were significantly higher in T3 compared to T1 and T2. It has been reported that pretreatment severity affected the kinetics of formation of degradation products.[32,33,**34**] The kinetics of xylo-oligosaccharides degradation have been previously reported to depend on the pH of the pretreatment; for example, the overall degradation rate constant of pure xylotetraose to xylose was 625.5 at pH 1.45 as opposed to 3.1 at pH 7.0, thus corroborating the presented results.[35] However, lignin depolymerization has been reported to increase as a function of temperature. During the dilute acid pretreatment of corn stover, increases in temperature at constant CSF and pH affected the resultant concentration of phenolics in the prehydrolyzates.[34] Moreover, raising the pretreatment temperature from 140°C to 190°C resulted in a 67% increase of condensed phenolics in corn stover hydrolyzates.[36] Therefore, pretreatment processing parameters can affect the composition of resulting hydrolyzates, even at constant severities. In this work, the occurrence of significantly higher concentrations of xylo-oligosaccharides and total phenolics in T3 prehydrolyzates can thus be attributed to the neutral pH and higher pretreatment temperatures, respectively.

Experimental design and enzyme inhibition

During enzyme assays, the T1, T2, T3 prehydrolyzates were lyophilized and mixed with 100 mM buffer solutions, to raise the pH to 4.8. For T1, additionally ammonium hydroxide was added, in order to increase the pH to 4.8. This procedure effectively eliminated the inhibition effect due to pH differences. In previous studies, pure reference compounds, reported to inhibit the saccharification cocktail, were individually tested to determine their inhibitory effects.[9,10] Tannic acid for example, at 0.005 mg per FPU, was reported to inhibit the filter paper activity of saccharification cocktail, by 60%.[10] Instead of reference compounds, crude prehydrolyzates were tested in this study for their inhibition effect on commercially available cellulases, *endo*-cellulase, and β-glucosidase enzyme preparations. By using authentic hydrolyzates, a true to nature evaluation of their inhibitory effect could be determined. It has been previously reported that, besides competitive inhibition, the activity of cellulases was also reduced by low accessibility of recalcitrant substrates.[37] In order to eliminate the chances of reduction in enzyme digestibility related to biomass recalcitrance, filter paper, CMC and cellobiose were used as substrates, for the inhibition studies of cellulase cocktail, *endo*-cellulase and β-glucosidase, respectively. The specific activity of the enzymes (U mg^{-1} enzyme) as a function of increasing T1, T2 and T3 prehydrolyzate concentrations are presented in Figures 1A, 1B, and 1C, respectively. The control consisted of 100% buffer solution, and as expected, exhibited the highest enzyme activity. For all three enzyme-substrate systems, decreases in enzymatic activity mirrored increases in hydrolyzate additions.

Effect of inhibitors on β-glucosidase, *endo*-cellulase and saccharification cocktail

β-glucosidase was the least inhibited by the presence of prehydrolyzates. At an inhibitor concentration of 35 g L^{-1}, the cellobiase activity of NS 22118 was reduced by 53%, 62% and

49% for T1, T2, and T3, respectively. Reductions in cellobiase activities were not significantly different between the treatment groups at any given inhibitor concentrations. It has been previously reported that β-glucosidase activity of Novozyme 188 was relatively unaffected by the derivatives of birchwood xylan.[11]

The CMC-ase activity of *endo*-cellulase decreased significantly, as a function of increases in prehydrolyzate concentration. Addition of 15 g L^{-1} of T1, T2, or T3 prehydrolyzates resulted in 67%, 57% and 77% reduction in CMC-ase activity, respectively. Addition of 20 g L^{-1} of T1, T2, or T3 prehydrolyzates to the enzyme system resulted in total loss of CMC-ase activity. The saccharification cocktail was equally susceptible to the addition of prehydrolyzates. Reduction in the filter paper activities of CTec2 was in the order of 64%, 68% and 82% in the presence of 35 mg mL^{-1} of T1, T2, or T3 prehydrolyzates, respectively. Notably, the specific activity of both enzymes were significantly lower for prehydrolyzates stemming from T3 pretreatment, compared to T1 and T2 ($P > 0.02$, $\alpha_{0.05}$), indicating that some phenolic and xylo-oligosaccharide compounds are more inhibitory to the cellulases.

Characterization of prehydrolyzates

The base peak chromatograms (BPC), which were obtained by plotting the signal of the most abundant ions, detected in each of a series of mass spectra as a function of retention time for T1, T2 and T3, are presented in Figure 2 and 3A. T1 and T2 prehydrolyzates had phenolic compounds like salicylic acid, syringic acid, vanillin, acetosyringone, *p*-coumaric and *trans*-ferulic acid. Vanillin, at 4 mg per g of protein, reportedly caused 50% reduction in the cellulolytic activity of Spezyme CP (*T. reesei*).[9] Similarly, *p*-coumaric acid, vanillin, and ferulic acid, at 1.5 mg per mg of protein, were reported to cause 10%, 20% and 30% reductions in cellobiase activity of Novozyme 188 (*A. niger*), respectively.[10] LC/ESI−MS analysis of T3

prehydrolyzate showed additional phenolic compounds, compared to T1 and T2, as shown in Figure 3A. The compound 4–hydroxybenzoic acid, that is also present in T3, was reported to be highly inhibitory to the cellobiase activity of Novozyme 188, at 1.5 mg per mg of protein.[9] Occurrence of 3,4-dihydroxybenzoic acid, 3-hydroxy-4-methoxycinnamic acid and 2-furoic acid have been reported in dilute sulfuric acid (0.7%) pretreated corn stover.[38] Compounds having identical $[M+H]^+$ ions were also detected in T3 prehydrolyzate, at retention times 11.85, 12.95 and 15.6 min, respectively. T3 also showed mass fragments corresponding to *m/z* 325 and *m/z* 163, as reported in Figure 3B. These masses can possibly be attributed to lignin-saccharide complexes, but further characterization will be required. Notably, compounds such as ferulic-*O*-glucoside and *p*-coumaric acid-*O*-glucoside detected in rice straw prehydrolyzates, were reported to be possibly inhibitory to the cellulase system.[39] Moreover, lignin-carbohydrate complexes were reported in softwood prehydrolyzates, indicating that these complexes may be more prevalent than initially anticipated.[40] Therefore, the occurrence of lignin-carbohydrate complexes in rice straw (T3) prehydrolyzates and their effect on the activity of cellulases, warrants further investigation.

Conclusion

The inhibitory effects of crude rice straw prehydrolyzates were determined using the filter paper activity of a saccharification cocktail, CMC-ase activity of *endo*-cellulase and the cellobiase activity of β-glucosidase. The β-glucosidase system was the least inhibited, and its cellobiase activity was not significantly different for all three pretreatments. However, both the cellulase cocktail and *endo*-cellulase showed significant reduction in their specific activities, in the presence of only 15 g L^{-1} of either freeze-dried prehydrolyzates. It was determined that despite constant severities, the T1, T2 and T3 prehydrolyzates were heterogeneous in nature. T3,

in particular, had significantly higher xylo-oligosaccharides and total phenolic content, which was attributed to the high temperature and neutral pH conditions of the pretreatment. Based on prehydrolyzate composition of T3, xylo-oligosaccharides, phenolics and their polymerization compounds could play an important role in inhibiting the cellulase cocktail.

Thus, characterization of crude prehydrolyzates and elucidation of their effects on individual cellulases will prove useful in identifying the key inhibitory compounds. Knowing which compounds inhibit enzymatic hydrolysis will enable the implementation of mitigation strategies that will result in an increase in process efficiency and sustainability.

Acknowledgments

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Table captions

1. Average composition (± standard deviation) of rice straw

2. Average composition (± standard deviation) of the freeze-dried rice straw prehydrolyzates expressed as percent dry weight of the biomass

Figure captions

Fig. 1. Specific activities of *endo*-Cellulase (*A.niger*) at 0.04 mg enzyme g^{-1} of CMC, cellulase cocktail (Cellic CTec2) at 0.67 mg enzyme g^{-1} of filter paper and β-glucosidase (NS 22118) at 3.49 mg enzyme g^{-1} of cellobiose, in the presence of rice straw prehydrolyzates **A.** T1 (160 °C, pH 1.7), **B.** T2 (180 °C, pH 2.25) and **C.** T3 (220 °C, pH 7.0)

Fig. 2. Base peak chromatograms (BPC) of freeze-dried rice straw prehydrolyzates, T1 (160 °C, pH 1.7) and T2 (180 °C, pH 2.25) dissolved in methanol and analyzed using LC/ESI–MS, in a positive ion mode and m/z range of 50.00 to 800.00.

Fig. 3. **A.** Base peak chromatogram (BPC) for T3 rice straw prehydrolyzate (220 °C, pH 7.0) **B.** Mass spectrum of *p*-coumaric acid-*O*-glucoside in sample T3 corresponding to BPC peak at 27.9 min.

Table 1.

Component	**(%) Dry weight**
Glucan	35.48 ± 0.99
Xylan	17.14 ± 1.81
Total ash	15.03 ± 1.32
Total lignin*	11.53 ± 0.88
Total extractives[#]	11.48 ± 1.33
Total	**90.66 ± 1.31**

*Sum of Klason lignin and acid soluble lignin

[#]Sum of water and ethanol extractives

Table 2.

Compounds	**T1**	**T2**	**T3**
Glucose	5.95 ± 0.21^{a}	0.00 ± 0.00^{c}	1.74 ± 0.65^{b}
Xylose	16.46 ± 0.75^{a}	7.07 ± 0.54^{b}	0.68 ± 0.02^{c}
Formic acid	4.34 ± 1.08	5.77 ± 1.16	6.32 ± 1.46
Acetic acid	7.32 ± 1.98	6.60 ± 2.01	8.45 ± 0.59
HMF	0.42 ± 0.04	0.20 ± 0.03	0.65 ± 0.41
Furfural	0.45 ± 0.05	0.21 ± 0.14	0.37 ± 0.29
Xylo-oligosaccharides[†]	0.16 ± 0.00	3.08 ± 0.34	4.44 ± 0.11*
Total phenolics[#]	1.34 ± 0.35^{a}	2.98 ± 0.61^{b}	4.64 ± 0.35^{c}

[†] Sum of xylobiose (DP2) to xylohexaose (DP6)

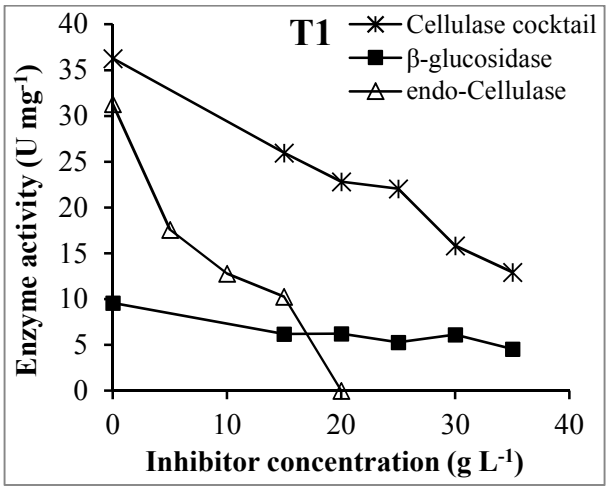
[#] Expressed as % gallic acid equivalent

*Significant difference between treatment groups (n=3), at $P > 0.05$, $\alpha_{0.05}$

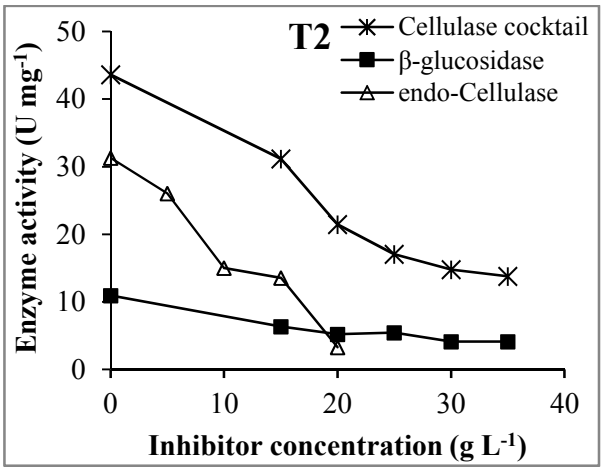
[a,b,c] Significant difference between treatment groups (n=3), at $P > 0.01$, $\alpha_{0.05}$

Fig. 1.

a)


T1
Cellulase cocktail
β-glucosidase
endo-Cellulase
Enzyme activity (U mg-1)
Inhibitor concentration (g L-1)
0
5
10
15
20
25
30
35
40
0
10
20
30
40

b)


T2
Cellulase cocktail
β-glucosidase
endo-Cellulase
Enzyme activity (U mg-1)
Inhibitor concentration (g L-1)
0
10
20
30
40
50
0
10
20
30
40

c)

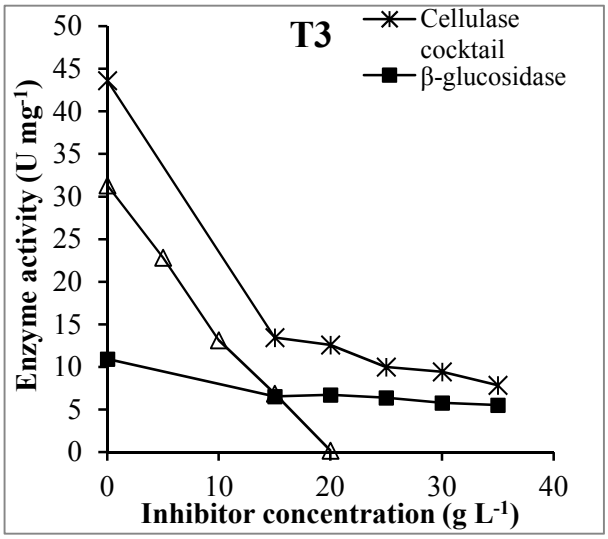

T3
Cellulase cocktail
β-glucosidase
Enzyme activity (U mg-1)
Inhibitor concentration (g L-1)
0
5
10
15
20
25
30
35
40
45
50
0
10
20
30
40

Fig.2.

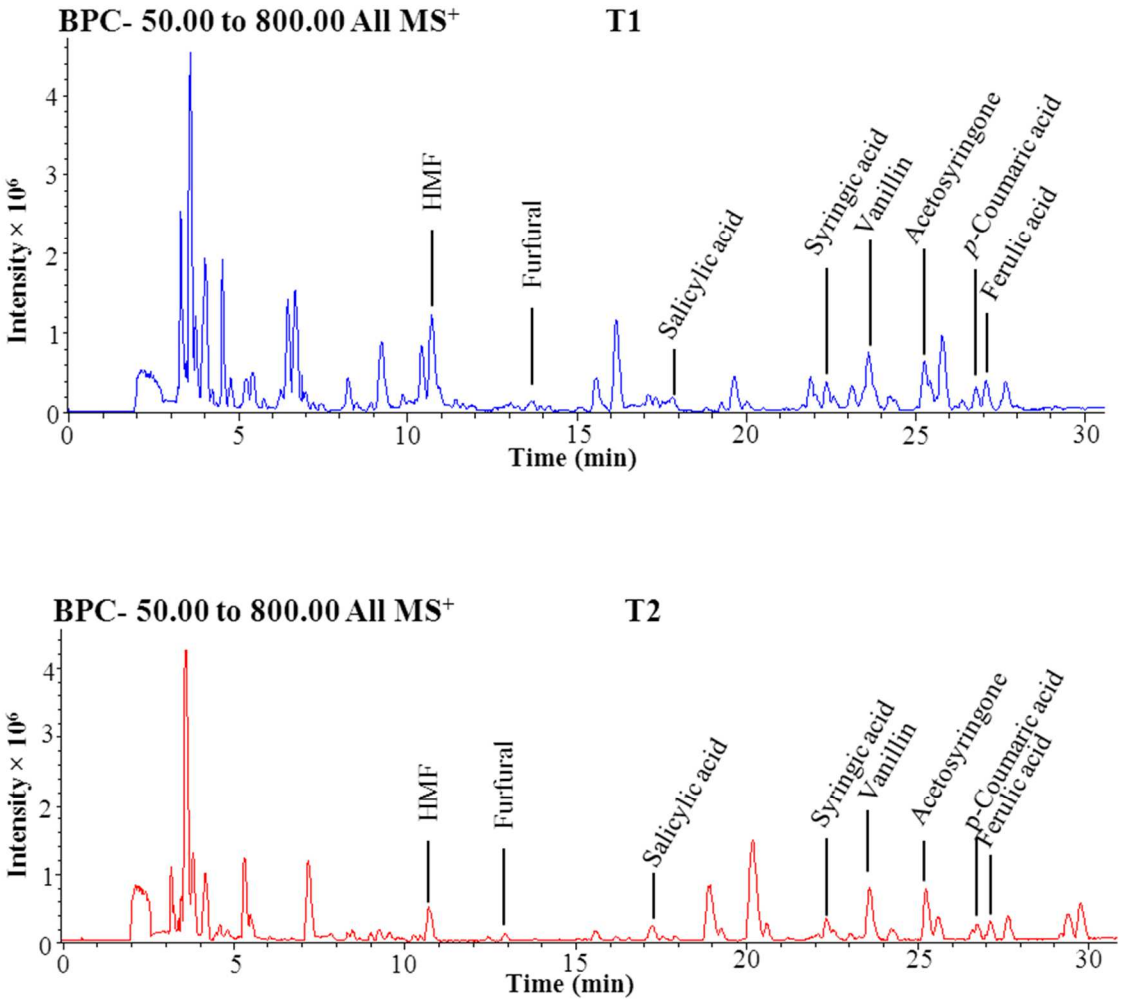

BPC- 50.00 to 800.00 All MS+
T1
Intensity × 10^6
Time (min)
HMF
Furfural
Salicylic acid
Syringic acid
Vanillin
Acetosyringone
p-Coumaric acid
Ferulic acid
BPC- 50.00 to 800.00 All MS+
T2
Intensity × 10^6
Time (min)
HMF
Furfural
Salicylic acid
Syringic acid
Vanillin
Acetosyringone
p-Coumaric acid
Ferulic acid

Fig.3a

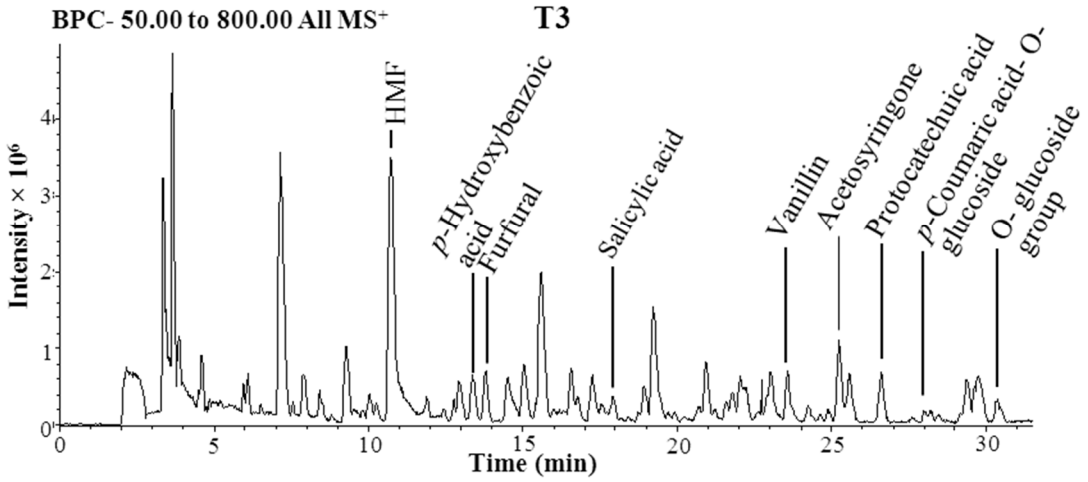
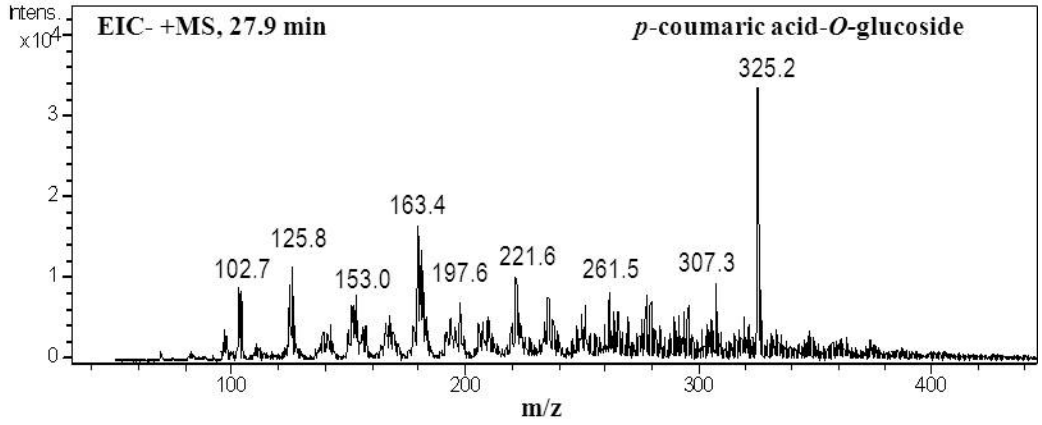

BPC- 50.00 to 800.00 All MS+
T3
Intensity × 10^6
Time (min)
HMF
p-Hydroxybenzoic acid
Furfural
Salicylic acid
Vanillin
Acetosyringone
Protocatechuic acid
p-Coumaric acid-glucoside
O-glucoside group

Fig.3b


Intens.
x10^4
EIC- +MS, 27.9 min
p-coumaric acid-O-glucoside
325.2
163.4
125.8
102.7
153.0
197.6
221.6
261.5
307.3
m/z

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V. Separation of XOS from Autohydrolyzed *Miscanthus x giganteus* Using Centrifugal Partition Chromatography

Ming-Hsu Chen[1,+], Kalavathy Rajan[2,+] , Danielle Julie Carrier[3] and Vijay Singh[1]*

1. Department of Agricultural and Biological Engineering, University of Illinois at Urbana-Champaign. 1304 W. Pennsylvania Avenue, Urbana, IL 61801, USA
2. Department of Food Science, University of Arkansas, 1180 W Cassatt St., Fayetteville, AR 72704, USA
3. Department of Biological and Agricultural Engineering, University of Arkansas, 203 Engineering Hall, Fayetteville, AR 72701, USA

+ Co First-author

*Corresponding author. Tel: 217-333-9510, Fax: 217-244-0323

E-mail address: vsingh@illinois.edu (Dr. Vijay Singh)

Abstract

Autohydrolysis of cellulosic materials for saccharification also generates xylose-oligosaccharides (XOS), due to the partial hydrolysis of xylan. Developing an efficient method for the separation and recovery of XOS from the prehydrolyzates would provide an excellent opportunity for the better utilization of the cellulosic material and for value-added co-product production. In this study, we investigated the use of centrifugal partition chromatography (CPC) for the fractionation of XOS from *Miscanthus x giganteus* (MxG). During autohydrolysis of miscanthus biomass at 180°C for 20 min, 63 % of xylan was converted into XOS and xylose. The ensuing XOS concentrate contained up to 30 % of XOS, which were distributed as 15.9 % xylobiose (DP2), 5.9 % xylotriose, (DP3), 5.6 % xylotetraose (DP4), 0.8 % xylopentaose (DP5) and 0.6 % xylohexaose (DP6). The XOS concentrate was further fractionated by CPC with a solvent system composed of 4:1:4 (v/v/v) butanol: methanol: water. Using CPC techniques, 230 mg (80 %) of DP2 to DP6 oligomers were fractionated from 1 g of XOS concentrate. The recoveries of individual XOS were 90.2 % DP2, 64.5 % DP3, 71.2 % DP4, 61.9 % DP5 and 68.9 % DP6. The purities of DP2 to DP6 fractions were 61.9 %, 63.2 %, 44.5 %, 31.5 % and 51.3 %, respectively. Presence of DP2 and DP3 in the CPC purified fractions was further validated by mass spectrometry analysis. The study provided information on fast recovery of individual XOS from crude biomass prehydrolyzate.

Key words: *Miscanthus x giganteus*, Autohydrolysis, XOS, Xylose oligomers, Centrifugal partition chromatography

Highlights

- Autohydrolysis of *Miscanthus x giganteus* at 180°C & 20 min, yielded 63.4% xylan
- Crude XOS concentrates were CPC fractionated with 4:1:4 – butanol:methanol:water
- CPC fractionation recovered 90 % DP2, 65 % DP3, 71 % DP4, 62 % DP5 and 69 % DP6
- Corresponding purities were, 62 % DP2, 63 % DP3, 45 % DP4, 32 % DP5 and 51 % DP6

Introduction

Development of cellulosic biomass for biofuels is making progress in the U.S.; according to a recent report, several commercial plants are expecting to start operation in 2014-15 (Brown and Brown, 2013). Using cellulosic biomass in fuel conversion has many benefits to the environment, energy security and rural economy (Kim and Kim, 2014). Among all bioenergy crops, *Miscanthus x giganteus* (MxG), a perennial rhizomatous grass, is a leading candidate for fuel production. MxG produces 60% more biomass than well-fertilized maize and the yield can reach 30 MT/ha/year (Somerville et al., 2010). Over 50% of the MxG cell wall consists of structural carbohydrates (cellulose and hemicellulose), which are available for biofuels and biochemicals production (Brosse et al., 2012).

The autohydrolysis involves treating biomass with hot water or steam (140 to 200°C) to open the structure of plant cell wall. At high temperatures, water acts as a weak acid and releases acetyl groups from hemicellulose, which further acidifies the reaction mixture. These hydronium ions catalyze the cleavage of glycosidic bonds within the xylan, resulting in the liberation of xylose and other sugar oligomers into the liquid phase. The sugar oligomer enriched prehydrolyzate is generally washed off and the pretreated solid residue, which is enriched in cellulose, can be further processed to ethanol (Vázquez et al., 2000).

Xylose oligomers (XOS) are indigestible carbohydrates that find application in prebiotics, since they are selectively utilized by human gut microbiota such as *Bifidobacterium* and *Lactobacillus* spp., thereby resulting in health benefits to the host (Carvalho et al., 2013). XOS have the potential of becoming value added co-products of the cellulosic ethanol industry. However, direct separation of XOS from the prehydrolyzate is a complex problem (Moure et al., 2006) because in addition to XOS, there may be other monomeric and oligomeric sugars, sugar

degradation products, acid solubilized lignin and inorganic compounds (Gullón et al., 2010). Multiple steps of separation including solvent extraction, vacuum evaporation, surface-adsorption, membrane separation, size exclusion chromatography and ion exchange chromatography have been reportedly used in sequential purification of XOS (Vázquez et al., 2000; Moure et al., 2006). XOS were purified from rice husk hydrolyzate using membrane concentration, *endo*-xylanase digestion, and anion exchange resins (Gullón et al., 2010). Activated carbon adsorption and ethanol elution was also used to recover XOS from MxG hydrolyzates (Chen et al., 2014). However, both Gullón et al. (2010) and Chen et al. (2014) recovered XOS mixtures, rather than purified oligomers that have a single degree of polymerization (DP).

Centrifugal partition chromatography (CPC) is a separation method developed from countercurrent chromatography. This liquid-liquid extraction technology uses two immiscible solvents as the stationary and mobile phase during chromatographic purification. Separation of solutes is achieved by virtue of their difference in partition coefficients. CPC has been widely used for single step preparative separations of alkaloids and flavonoids from crude plant extracts for pharmaceutical applications (Attoumbre et al., 2012; Jeon et al, 2012). Advantages of CPC include the ability to separate compounds that span a wide molecular weight range, such as XOS (Berthod et al., 1988). Birchwood xylan-derived XOS were previously fractionated by CPC into its individual oligomers with a tetrahydrofuran-based solvent system (Lau et al., 2011). The CPC method was further improved with a butanol-based solvent system (Lau et al., 2013). However, these CPC protocols were never tested with crude hydrolyzates that stemmed from biomass autohydrolysis reactions.

The objective of this study was to purify from crude MxG hydrolyzates, which were produced through autohydrolysis, XOS into their individual oligomer components. It is important to note that the starting material in this study consisted of a crude complex hydrolyzate, as opposed to a semi purified xylan material (Lau et al., 2011; Lau et al., 2013; Bunnell et al., 2015). Separation and fractionation of MxG XOS could possibly provide an opportunity to produce valuable co-products from bio-refineries, for pharmaceutical and nutraceutical applications.

Material and methods

Raw material and chemicals

Miscanthus x giganteus (MxG) was grown at the Energy Farm, University of Illinois, Urbana, IL (40.06°N, 88.19°W). The MxG was harvested in 2011 and was dried at 49°C in a convective oven, cut and ground in a hammer mill (model MHM4, Glen Mills, Clifton, NJ) to pass through a 250 μm screen. The dried and ground MxG was stored at 4°C. The moisture content was 8.2%, as determined following the NREL-LAP-001 protocol (Sluiter et al., 2008a). Xylose (DP1), arabinose, glucose and galactose were purchased from Sigma-Aldrich (St. Louis, MO). Xylobiose (DP2), xylotriose (DP3), xylotetraose (DP4), xylopentaose (DP5), and xylohexaose (DP6) were purchased from Megazyme International (Wicklow, Ireland) and their purity was reported to be greater than 95%. High performance liquid chromatography (HPLC) grade butanol and methanol were obtained from Macron Fine Chemicals (Center Valley, PA) and EMD Chemicals (Gibbstown, NJ), respectively. Water was prepared with a Direct-Q system (Millipore, Billerica, MA). Formic acid (95%) was procured from Amresco LLC (Solon, OH).

Autohydrolysis and sample preparation

The autohydrolysis was performed in a tubular reactor inserted in a fluidized bed sand bath for fast heating (Khullar et al., 2013). The 316 stainless steel tubing (SS-T12-S-065-20, Swagelok, Chicago Fluid System Technologies, Chicago, IL) reactors had an outside diameter of 19.1 mm, a wall thickness of 1.7 mm, and a length of 104.8 mm. Reactors were capped with 19.1 mm, 316 stainless steel Swagelok caps (SS-1210-C, Swagelok) on both ends. The procedure was essentially as described by Chen et al. (2014), where the loaded reactors were immersed in a fluidized sand bath (IFB-51 Industrial Fluidized Bath, Techne Inc., Burlington, NJ) preheated to 180°C and cooled in flowing room temperature tap water. Autohydrolysis was performed at a water to solid ratio of 9:1, at 180°C for 20 min. In each tubular reactor, 5 g of MxG was mixed with 45 g of water. The pretreated solids and liquids were separated by centrifugation (2600 × *g*, 5 min) in an Eppendorf 5804R centrifuge. This procedure was repeated in six reactors for four batches and corresponding hydrolyzates were combined. The resulting volume, determined to be 420 mL, was filtered through a 0.45 μm filter membrane (Millipore, Bedford, MA) and dried in a 49°C oven, removing approximately 415 mL of water. The resulting crude dried mixture was subsequently referred to as "XOS concentrate" throughout the work.

Sugar and oligomer characterization

Xylose, glucose, arabinose, galactose and XOS (from DP2 to DP6) content in the XOS concentrate was determined using a Waters Alliance HPLC system (Model 2695, Waters Corporation, Milford, MA) equipped with SP0810 (Shodex, Kawasaki, Japan) or Bio-Rad Aminex-HPX 42A (Bio-Rad, Hercules, CA) columns for monomer and oligomer quantification, respectively. HPLC was equipped with SP-G pre-column for monomer and Micro-Guard De-ashing pre-column for oligomer determinations. Purified and 0.22 μm filtered water was used as

the eluent. Column temperature was 85°C. Eluent flow rate was 0.2 mL/min, and monomers or oligomers were detected with a refractive index detector (Model 2414, Waters Corporation, Milford, MA), which was maintained at 50°C. The concentrations of xylose, glucose, arabinose, galactose and XOS (DP2 to DP6) were determined using in-house calibration curves generated from commercial standards.

The total dissolved solids and sugar content of the concentrate were determined following NREL LAP-001 (Sluiter et al., 2008a) and NREL-LAP- 014 (Sluiter et al., 2008b) protocols, respectively. Accordingly, the total yield of xylose oligomers in the XOS concentrate was determined as follows:

$$\text{Yield (\%)} = (W_h - W_i) \times 0.88 \times 100 \quad (1)$$

Where W_h was the mass fraction of xylose in the concentrate after acid hydrolysis, W_i was the initial mass fraction of xylose in the concentrate and 0.88 is the anhydro correction factor for xylose (Sluiter et al., 2008c). The total phenolic content of the concentrate was determined using the Folin & Ciocalteau's (F-C) method, based on a previously published protocol (Ainsworth and Gillespie, 2007).

Centrifugal partition chromatography

A bench scale SCPC-250 system from Armen Instruments (Dallas, TX) equipped with CherryOne Beta (C1) countercurrent chromatography control system (Chicago, IL) was used for fractionating and purifying the XOS concentrate. Samples were processed by ultrapure nitrogen at 43 psig and with an evaporative light scattering detector (ELSD) (SofTA Corp, Westminster, CO). Butanol: methanol: water solvent system in 4:1:4 (v/v/v) was used for the CPC separation (Lau et al., 2013). To prepare 2 L of the biphasic system, 889 mL of butanol was mixed with 222 mL of methanol and 889 mL of water in a separation funnel and agitated thoroughly. The upper

butanol rich organic phase and the lower aqueous phase were collected separately after 4 h and used as the mobile and stationary phases, respectively, when the CPC was operated in the ascending mode. One g of dried XOS concentrate was dissolved in 20 mL of butanol-rich phase and 8 mL of the aqueous-rich phase. The mixture was then vortexed and filtered through a 1μm PTFE syringe filter (Thermo Scientific National, Rockwood, TN) and followed by injection into the CPC system.

The stationary and mobile phases were loaded in the ascending mode at 500 RPM. The equilibrium was reached at retention volumes of 114 mL and 136 mL, respectively. The CPC run time was 344 min, at 2300 RPM and at a flow rate of 8.14 mL/min. The UV detector in the controller was equipped with a preparatory flow cell that was set at 254 nm for real-time monitoring. Fraction collection was started after 60 min and fractions of approximately 8.1 mL each, were collected every minute. To accomplish this task, two fraction collectors were connected in series: Foxy R1 (Teledyne Isco, Lincoln, NE) and Waters Fraction Collector III (Waters Corporation, Milford, MA). After 240 min of run time, the CPC was operated in the descending mode allowing for un-separated compounds to exit the column and be collected. All 284 CPC fractions were dried in a Savant SpeedVac Concentrator SPD 1010 (Thermo Scientific, Ashville, NC), at 7 Torr for 6 h and reconstituted in 0.5 mL of water. The reconstituted fractions were filtered through a 0.22 μm nylon syringe filter (Thermo Scientific National, Rockwood, TN) and analyzed using HPLC to determine the concentration of xylose monomer and oligomers (DP2 to DP6).

The CPC fractionation of MxG XOS was repeated twice. Fractions were consolidated based on the concentration of each xylose oligomer. The purity of consolidated samples was calculated as:

$$\% \text{ Purity} = \frac{Weight\ of\ xylose\ oligomer}{Total\ weight\ of\ each\ fraction} \times 100 \quad (2)$$

ESI–MS verification

Samples were dried in SpeedVac and mixed with 0.1 % formic acid solution in methanol, at 20:1 ratio and directly injected into the mass spectrometer with a syringe pump. Gas phase ions created using electrospray ionization (ESI) were analyzed with a quadrupole/time –of – flight (Q-TOF) mass analyzer (Bruker ultrOTOF-Q, Bruker Daltonics Inc., Billerica, MA). The solvent (methanol) flow rate was 3 μL/min and N_2 gas at 1 bar was used for nebulization. The temperature of the source was 180°C. Other instrument parameters were adjusted to optimize signal in the *m/z* range of 100-800 and the mass spectra were obtained in positive ion mode.

Results

Production of XOS concentrate

Miscanthus x giganteus (MxG) biomass composition, previously determined by Chen et al. (2014), was 35.9 % glucan, 19.5 % xylan, 18.5 % acid insoluble lignin, 11.3 % total extractives, 1.8 % ash and 1.1 % acid soluble lignin. Autohydrolysis of MxG at 180°C resulted in xylan being hydrolyzed into xylose monomer and oligomers. Acetyl groups present on hemicellulose chains were cleaved during heating and provided an acid source, leading to decreased pH and increased xylan cleavage. Incubation at 180°C for 20 min led to 63.4 % (w/w) xylan removal from the pretreated solids. The hydrolyzates were pooled and concentrated and the resultant XOS concentrate was determined to have a total solid content of 71.2 ± 0.3 % (w/w). In previous work, MxG autohydrolysis in a 0.6 L stainless Parr reactor, at temperatures ranging from 130°C to 150°C was reported to yield 40% (w/w) of initial xylan content (El Hage et al., 2010). In a stainless tube reactor heated at 160°C for 60 min, 65% (w/w) of xylan was

recovered as xylose and XOS (Ligero et al., 2011). Autohydrolysis conditions reported in the current work, provided comparable yields for the production of crude XOS concentrate.

The carbohydrate composition of the XOS concentrate, adjusted for moisture content, was determined to be 39.8 ± 2.1 % xylose, 4.4 ± 0.1 % glucose, 5.1 ± 0.1 % arabinose and 2.7 ± 0.3 % galactose. The total xylose oligomer content, determined as per equation (1), was 30.1 ± 1.0 %. Distribution of XOS (DP2 to DP6) in the concentrate varied from 0.6 to 15.9 % dry weight, as shown in Table 1 and it was determined that DP2 to DP6 constituted 95.5 % of the total XOS. The remaining 4.5% may be made up of higher DPs of xylose or conjugates of xylan, arabinan, galactan and/or glucan. The total phenolic content of the concentrate was 4.1 ± 0.2 % gallic acid equivalent. Presence of phenolic compounds indicated that a certain degree of lignin degradation had occurred during the autohydrolysis reaction. It was calculated that in order to produce 1 g of the XOS concentrate, autohydrolysis of 3.7 g of MxG biomass was required. Conversely, 1 g of the concentrate used for CPC fractionation contained, 159.2 mg of DP2, 59.0 mg of DP3, 56.0 mg of DP4, 8.1 mg of DP5 and 5.6 mg of DP6.

XOS separation and purification by CPC

The XOS present in the concentrate were separated using CPC, generating 284 fractions. The ELSD chromatogram corresponding to a typical separation is presented in Figure 1; fractions corresponding to the highlighted area under the curve were consolidated. The HPLC-RID chromatograms of the original concentrate and the CPC consolidated fractions are presented in Figure 2, the average yields of XOS, calculated on a dry weight basis of the concentrate, as well as the corresponding purities are presented in Table 2. Recovery of DP2 to DP6 oligomers varied between 61.9 to 90.2 %, while purity fluctuated between 31.5 to 63.2 %. The recovery of CPC fractionated XOS was the highest for DP2 at 90.2 %, based on the original composition of

XOS concentrate (Table 2). The consolidated DP2 and DP3 fractions had comparatively higher purities of 61.9% and 63.2%, respectively. The purities of CPC fractionated and consolidated MxG XOS were calculated based on their total mass fractions (Eqn. 2). In earlier reports, purities of XOS produced from purified xylan were higher, but were calculated based on area under the (chromatogram) curve, relative to other DPs and not on a mass basis (Qing et al., 2010; Lau et al., 2013)

ESI–MS verification of CPC separated fractions

Qualitative analysis of the CPC fractionated DP2 and DP3, performed using ESI–MS, was useful to verify their identity. As evidenced in Figure 3a, the major signals in consolidated DP2 fractions were *m/z* 305.1 $[M+Na]^+$ and 321.1 $[M+K]^+$ which corresponded to individual pentobiose molecule bound with sodium and potassium ions. Similarly, the major signals in consolidated DP3 fractions were *m/z* 437.1 $[M+Na]^+$ and 453.1 $[M+K]^+$ (Figure 3b) which corresponded to individual pentotriose molecule bound with sodium and potassium ions. The ESI-MS spectrums validated that the DP2 and DP3 were isolated and were the main component in each fraction.

Discussion

Production of XOS for nutraceutical applications is a topic that is garnering research efforts. Low molecular weight XOS of sizes DP2 to DP5, at 70 % purity, were reportedly fermented *in vitro* as well as *in vivo,* by prebiotic organisms belonging to the *Bifidobacterium* spp. (Hopkins et al., 1998). In one particular study, 90 % of DP3 and 84 % of DP2 were preferentially utilized by *Bifidobacterium* adolescentis (Gullón et al., 2008). Similarly certain strains of *Lactobacillus* spp. were reported to preferentially utilize xylose DP2 to DP4 when compared to other oligosaccharides, during mixed acid fermentations (Kontula et al., 1998).

There are several enzymatic and chemical hydrolysis methods reported for the production of XOS from lignocellulosic biomass. However, few reports are addressing fractionation practices. Cara et al. (2012), described the production and separation of glucose and XOS by gel filtration chromatography, but did not separate XOS into individual DP components. Jayapal et al. (2013), extracted hemicellulose from sugarcane bagasse and produced XOS using xylanases. They detected their product with reducing sugar and thin layer chromatography assays with no report on fractionation. Recently Mandelli et al. (2014), reported on the production of XOS from sugarcane bagasse, detecting and quantifying their products by capillary electrophoresis and HPAEC-PAD detection; however, this group also did not attempt to purify the xylan DPs.

Chen et al. (2014) originally used 10 % activated carbon adsorption followed by aqueous ethanol elution and obtained a theoretical maximum recovery of 47.9 % of XOS DP2 to DP9, from *Miscanthus x giganteus* biomass. However, fractionation and purification of DPs into individual components was not attempted. Lau et al. (2011) were the first to report the use of CPC in separation and purification of XOS from acid hydrolyzed birchwood xylan: best results were obtained for the separation of xylose DP2 to DP5. With the adoption of a 4:1:4 volumetric ratio of butanol, methanol and water system, Lau et al. (2013) recovered 12.5 mg/g xylan of 81.2% pure xylobiose, 9.6 mg/g xylan of 71.0% pure xylotriose, 14.2 mg/g xylan of 62.4% pure xylotetraose and 21 mg/g xylan of 51.9% pure xylopentaose from autohydrolyzed birchwood xylan. Fractionating crude MxG hydrolyzate, containing other carbohydrates and lignin derivate, increased the complexity of separation and therefore reduced the purity of CPC fractions.

Lau et al. (2013), used CPC to recover 59.1 mg/g of DP2 to DP5, from autohydrolyzed birchwood xylan, and Bunnell (2013) reported CPC recoveries of 138 mg/g of DP2 to DP6 from alkali extracted switchgrass xylan. In this study, 230 mg of xylose DP2 to DP6 were purified

from 1 g of autohydrolyzed XOS concentrate, by CPC using a similar butanol- methanol-water solvent system. This work was on purifying XOS directly from a crude autohydrolyzed concentrate, illustrating that this technique can fractionate DPs from complex matrices. To our knowledge this is a first report on XOS purification from crude biomass hydrolyzates, using CPC.

Conclusion

XOS were purified from autohydrolyzed MxG concentrate by CPC with a solvent system composed of 4:1:4 (v/v/v) butanol, methanol and water. Through the collection of samples following elution order, 80% of DP2 to DP6 were fractionated from the original concentrate. Approximately, 90.2% DP2, 64.5% DP3, 71.2% DP4, 61.9% DP5 and 68.9% of DP6 were recovered. Purities of each oligomer fraction was 61.9% DP2, 63.3% DP3, 44.5% DP4, 31.5% DP5 and 51.3% for DP6. The DP2 and DP3 fractions were validated by mass spectrometry analysis. CPC thus provides a fast and effective means of separating XOS, from biorefinery by-products, for commercial applications.

Acknowledgments

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Captions to Tables:

Table 1. Composition of autohydrolyzed XOS concentrate determined using HPLC analysis

Table 2. Yields of MxG XOS during centrifugal partition chromatography separation.

Table 1. Composition of autohydrolyzed XOS concentrate determined using HPLC analysis

Compound	% Dry wt.[a]
Xylobiose (DP2)	15.9 ± 1.5
Xylotriose (DP3)	5.9 ± 0.8
Xylotetraose (DP4)	5.6 ± 0.7
Xylopentaose (DP5)	0.8 ± 0.2
Xylohexaose (DP6)	0.6 ± 0.2
Total	28.8 ± 0.5

[a] Mean and standard deviation, *n*=3

Table 2. Yields of MxG XOS during centrifugal partition chromatography separation.

Fractionated compounds	Yield of each compound [a] (mg/g)	Recovery [b]	Purity [c]
DP2-Xylobiose	143.5±6.0	90.2%	61.9±1.9
DP3-Xylotriose	38.1±5.0	64.5%	63.2±2.3
DP4-Xylotetraose	39.8±1.0	71.2%	44.5±1.9
DP5-Xylopentaose	4.9±0.1	61.9%	31.5±1.0
DP6-Xylohexaose	3.9±0.3	68.9%	51.3±1.0

[a] Mean and standard deviation, n=2

[b] % Recovery was calculated based the theoretical maximum yield

[c] % Purity was calculated based on the total weight of each fraction

Figure captions

Fig. 1. ELSD chromatogram of centrifugal partition chromatography separated XOS from autohydrolyzed MxG concentrate.

Fig. 2. HPLC-RID chromatogram of (a) original XOS concentrate and the consolidated XOS, (b) DP2 fraction, (c) DP3 fraction, (d) DP4 fraction, (e) DP5 fraction and (f) DP6 fraction

Fig. 3. ESI mass spectrum of consolidated xylose DP2 and DP3 purified from crude XOS concentrate by CPC. (a) DP2 fraction, (b) DP3 fraction

Fig. 1

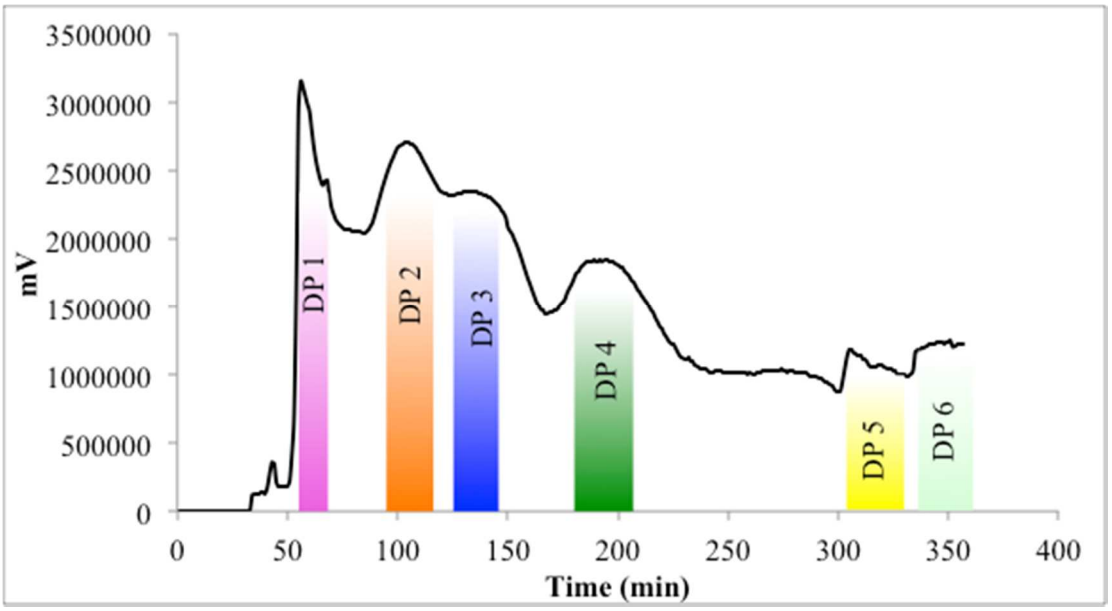

3500000
3000000
2500000
2000000
1500000
1000000
500000
0
mV
DP 1
DP 2
DP 3
DP 4
DP 5
DP 6
0
50
100
150
200
250
300
350
400
Time (min)

Fig. 2

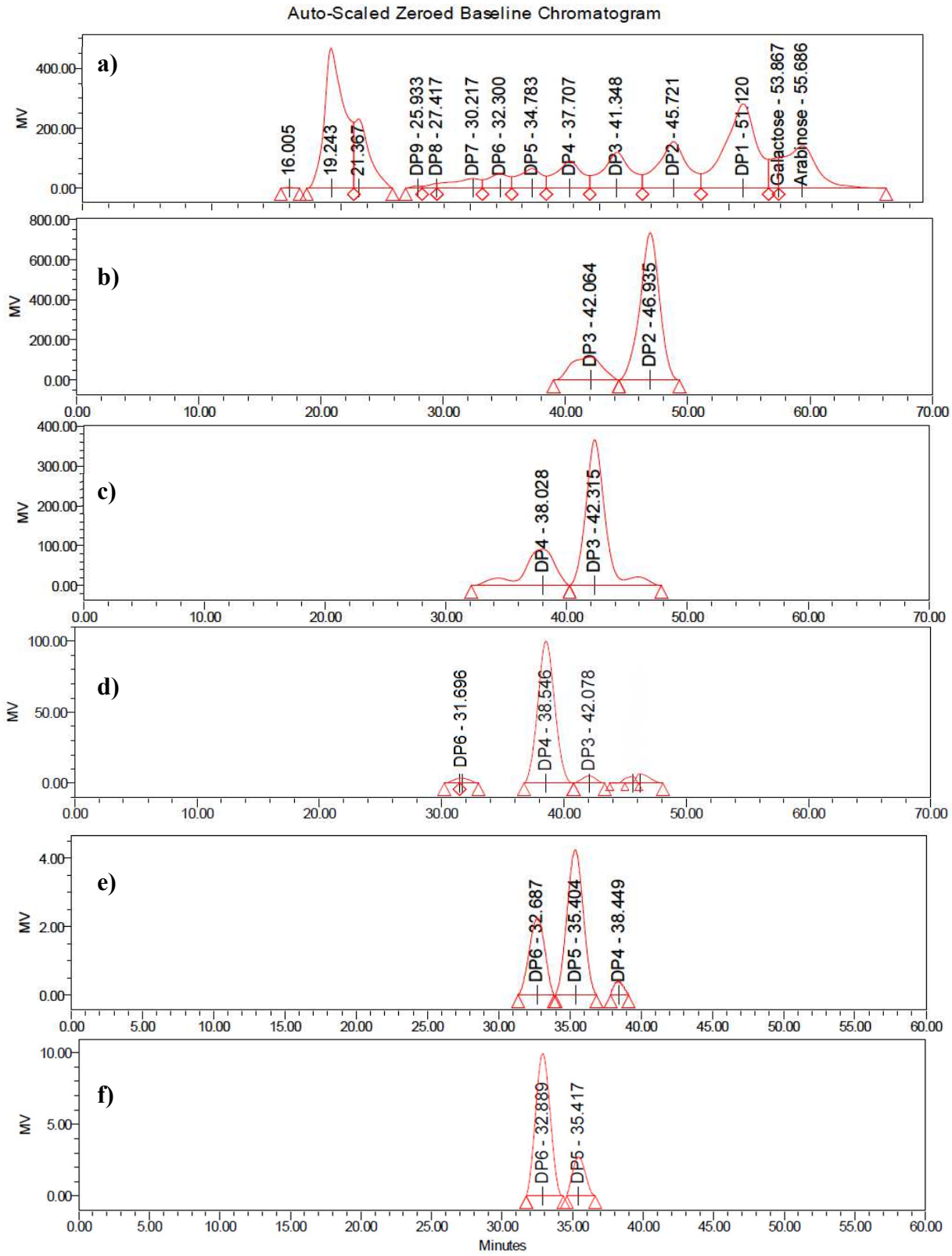

Auto-Scaled Zeroed Baseline Chromatogram
a)
16.005
19.243
21.367
DP9 - 25.933
DP8 - 27.417
DP7 - 30.217
DP6 - 32.300
DP5 - 34.783
DP4 - 37.707
DP3 - 41.348
DP2 - 45.721
DP1 - 51.120
Galactose - 53.867
Arabinose - 55.686
b)
DP3 - 42.064
DP2 - 46.935
c)
DP4 - 38.028
DP3 - 42.315
d)
DP6 - 31.696
DP4 - 38.546
DP3 - 42.078
e)
DP6 - 32.687
DP5 - 35.404
DP4 - 38.449
f)
DP6 - 32.889
DP5 - 35.417
MV
Minutes

Fig. 3a.

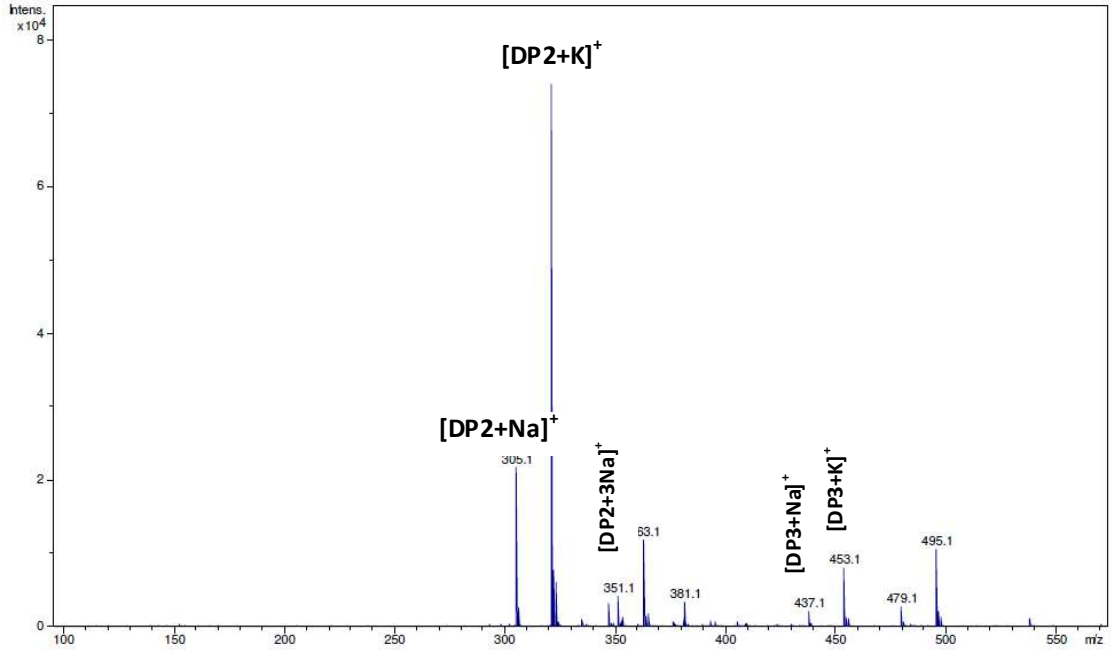
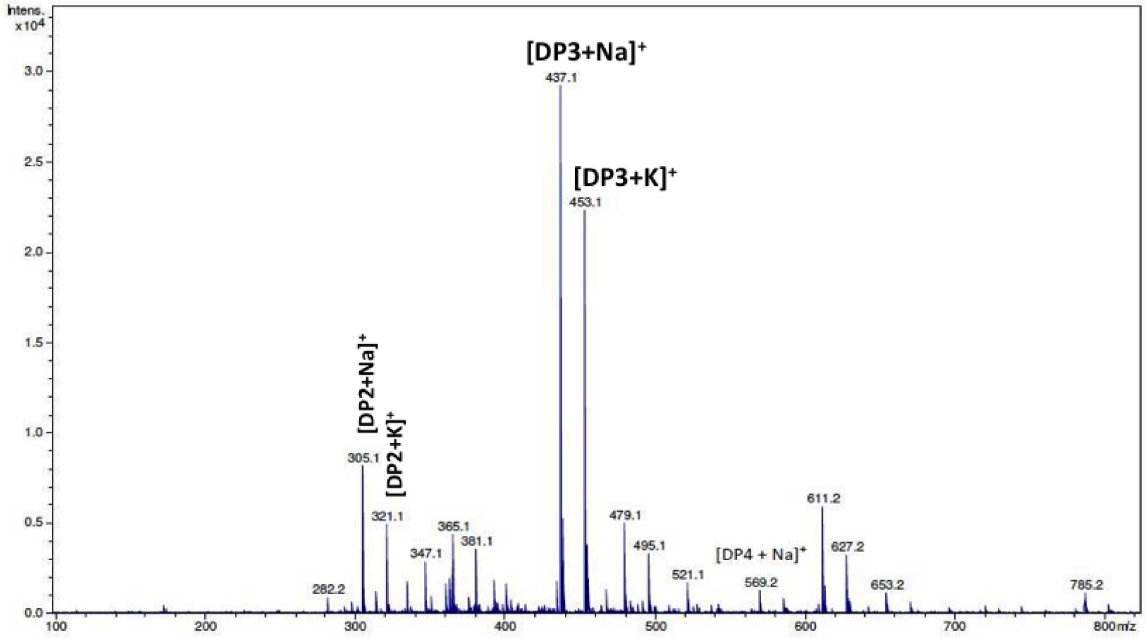

Intens.
x10^4
[DP2+K]+
[DP2+Na]+
[DP2+3Na]+
[DP3+Na]+
[DP3+K]+
305.1
351.1
381.1
437.1
453.1
479.1
495.1
m/z

Fig. 3b.


Intens.
x10^4
[DP3+Na]+
437.1
[DP3+K]+
453.1
[DP2+Na]+
[DP2+K]+
305.1
321.1
347.1
365.1
381.1
479.1
495.1
521.1
[DP4 + Na]+
569.2
611.2
627.2
653.2
785.2
282.2
m/z

A. References

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VI. Insights into *exo*-Cellulase Inhibition by the Liquid Hot Water Prehydrolyzates of Rice Straw

Kalavathy Rajan[a] and Danielle Julie Carrier[b*]

Affiliations

[a] Department of Food Science, University of Arkansas, Fayetteville, Arkansas, U.S.A.

[b] Department of Biological and Agricultural Engineering, 203 White Engineering Hall, 1 University of Arkansas, Fayetteville, AR 72701.

***Corresponding author Email:** carrier@uark.edu **Phone:** +1(479) 575-2542

Abstract

During the preconditioning of lignocellulosic biomass prior to enzymatic saccharification and fermentation for bioethanol production, the polysaccharide and lignin complex of the plant cell wall is broken down to produce several by-products. These by-products are inhibitory to the saccharification enzymes and fermentation microorganisms unless otherwise removed. In this study the prehydrolyzates from the hot water pretreatment of rice straw was fractionated using centrifugal partition chromatography (CPC) into phenolics, furans, organic acids, monomeric and oligomeric sugars. When 1 g/L of the CPC fractions rich in phenolics and acetic acid were incubated with the *exo*-cellulase enzyme from *Hypocrea jecorina*, it was determined that its specific hydrolysis rate was reduced by a factor of 0.92 and 0.87 compared to that of the control, respectively. The CPC fractions containing xylo-oligosaccharides were also inhibitory to the *exo*-cellulase but when the enzyme dosage was increased by a factor of four, the hydrolysis rates showed significant improvement. This study elucidates the impact of different inhibitors in the actual hot water hydrolyzate of rice straw on the cellulolytic enzyme efficiency.

Key words: Rice straw; hot water prehydrolyzate; exo-cellulase inhibition; centrifugal partition chromatography.

Introduction

Lignocellulosic biomass is an inexpensive and renewable source for industrial chemicals and biofuels production (Chundawat et al., 2011). Lignocellulosic material, such as rice straw, is inherently recalcitrant to bio-chemical conversion, which usually involves enzymatic hydrolysis and fermentation. In order to overcome recalcitrance, chemical, physico-chemical, mechanical and biological treatments are usually performed to precondition the biomass prior to biochemical conversions (Kumar et al., 2009). Regrettably, these pretreatments lead to the formation of several inhibitory by-products as a result of cellulose, hemicellulose and lignin degradation but it would be ill advised to omit pretreatments since the conversion yields could be compromised. Several studies have quantified by-product generation due to pretreatment. Dilute acid pretreated corn stover prehydrolyzates were reported to contain by-products: furans (23.6–26.6 g/kg), organic acids (37.0– 44.7 g/kg) and soluble monomeric phenols (3.6– 4.1 g/kg) (Chundawat et al., 2010; Du et al., 2010). Hot water pretreated maple prehydrolyzate was reported to contain 13 g/L of organic acids, 12.7 g/L of oligomeric sugars, 9.8 g/L of monomeric sugars, 4.1 g/L of furans and 1.3 g/L of phenolic compounds. Ammonia fiber exploded corn stover was reported to contain furans 0.7 g/kg, organic acids 10.7 g/kg and total phenolics 1.7 g/kg (Chundawat et al., 2010). Wheat straw prehydrolyzate recovered after alkaline wet oxidation was also reported to contain organic acids (64.6 to 114.9 g/kg), total phenolics (1.5 to 5.2 g/kg) and furans (0.0 to 1.8 g/kg) (Klinke et al., 2002). These previous studies show that water-soluble by-products are released into liquid fraction of prehydrolyzates after pretreatments such as hot water, dilute acid, wet oxidation, steam explosion and ammonia fiber explosion, complicating subsequent enzymatic hydrolysis and fermentation (Cantarella et al., 2004; Palmqvist and Hahn-Hägerdal, 2000).

Exo-cellulase, specifically cellobiohydrolase-I, accounts for approximately 60% of the extracellular enzymes secreted by *Hypocrea jecorina* (*Trichoderma reesei),* and plays an important role in cleaving cellobiose units from the reducing ends of a cellulose molecule, during the enzymatic saccharification of pretreated biomass (Tejirian and Xu, 2011). The byproducts of lignocellulose pretreatments have been reported to inhibit the saccharification efficiency of commercial *T. reesei* cellulases. *Exo*-cellulase hydrolysis of phosphoric acid swollen cellulose substrate was reduced by 50% in the presence of phenolic compounds, such as 1 mM commercial tannic acid (Tejirian and Xu, 2011). Xylo-oligosaccharides have also been reported to competitively inhibit *exo*-cellulase hydrolysis of 4-methylumbelliferyl β-D-lactoside (Zhang and Viikari, 2012). Several enzyme inhibition studies have been conducted in the presence of model inhibitory compounds (Cantarella et al., 2004; Ximenes et al., 2010) or have focused on specific group of inhibitors extracted from biomass prehydrolyzates with minimal purification (Kim et al., 2011). Here we have analyzed the inhibition of *exo*-cellulase in the presence of whole and centrifugal partition chromatography (CPC) fractionated hot water hydrolyzates of rice straw in an attempt to develop a more realistic model for cellulase inhibition by lignocellulosic byproducts.

Materials and methods

Raw material

Rice straw (Clearfield hybrid, Lonoke county, AR), obtained from the University of Arkansas Cooperative Extension Service in Little Rock, AR, was air-dried and ground to an average particle size of 0.84 mm using a Thomas Willey® Mini mill (Swedesboro, NJ). Its original composition expressed as percent dry weight of the biomass was, glucan 35.4 ± 1.0 %,

xylan 17.1 ± 1.8 %, ash 15.0 ± 1.3 %, arabinan 13.0 ± 0.1 % and total lignin 11.5 ± 0.9 % (Rajan and Carrier, 2014a).

Chemicals

Commercial standards of glucose, galactose, arabinose (Alfa-Aesar, Ward Hill, MA), xylose, 5-hydroxymethyl furfural (HMF), furfural, *p*-coumaric acid, *trans*-ferulic acid, syringaldehyde, 4-hydroxybenzoic acid, vanillin, salicylic acid, gallic acid (TCI chemicals, Montgomeryville, PA), vanillic acid, *trans*-cinnamic acid, propionic acid, butyric acid and formic acid (Amresco, Solon, OH) were used in the characterization of the prehydrolyzates. Xylobiose (DP2), xylotriose (DP3), xylotetraose (DP4), xylopentaose (DP5) and xylohexaose (DP6) of > 95% purity, were purchased from Megazyme International (Wicklow, Ireland). Cellobiohydrolase-1 (Cel7A; E.C.3.2.1.91) or *exo*-cellulase produced by *Hypocrea jecorina* (*Trichoderma reesei)* was purchased from Infinite Enzymes (State University, Jonesboro, AR). 4-Methylumbelliferyl β-D-cellobioside (MUC), a fluorescent substrate used in *exo*-cellulase assays, was purchased from Sigma-Aldrich (St. Louis, MO). Accellerase® 1500 was graciously donated by Genencor (Rochester, NY, U.S.), which had a total cellulolytic activity of 74 FPU/ mL (filter paper units). Folin-Ciocalteu (F-C) phenol reagent, 2-hydroxy–3, 5–dinitrobenzoic acid, ammonium hydroxide and potassium sodium tartrate tetrahydrate, analytical grade, were purchased from Sigma-Aldrich (Milwaukee, WI). Water was prepared with a Direct-Q system (Millipore, Billerica, MA) that had 18.2 MΩ resistivity.

Pretreatment and enzymatic saccharification

Rice straw was subjected to hot water pretreatment at 220 °C, 52 min and pH 7.0, in a 1 L Parr 4525 reactor (Moline, IL), at a 10 % solid loading (wet weight). Dilute acid pretreatment

was carried out at 160 °C, 48 min and pH 1.7, where the pH was adjusted using 1.0 % (w/w) concentrated sulfuric acid (Rajan and Carrier, 2014a). The pretreatment severity is given by

$$\text{Combined severity} = \log\{t \times \exp\left[\frac{T_H - T_R}{14.75}\right]\} - \text{pH} \quad (1)$$

where *t* was the duration of pretreatment, T_H was the pretreatment temperature and T_R was the reference temperature (100 °C). After hot water pretreatment, the liquid hydrolyzate was separated from the pretreated biomass, using a Buchner filtration apparatus, fitted with Whatman #1 filter paper and frozen at −20 °C, in 500 mL polypropylene bottles. The frozen hydrolyzate was lyophilized at −44 °C and 7.7 Pa, in a FreeZone 18 L console freeze dry system (Labconco®, Kansas city, MO) for 72 h.

The pretreated solids were washed with water at a 10 % loading and saccharified using Accellerase® 1500 enzyme, at a loading of 105 FPU/g cellulose. One gram of pretreated solids loaded with the appropriate quantity of cellulase cocktail was mixed with 5 mL of 0.1 M citrate buffer, at pH 4.8 and the volume was made up with water, such that the final mixture had 10 % solid loading. The mixture was incubated at 50 °C in a reciprocating water bath (Thermo Scientific, Nashville, TN), agitated at 100 RPM for 90 h. Samples collected every 24 h were analyzed for its glucose contents.

2.4 Determination of partition coefficients

A biphasic solvent system composed of 5:1:4 (v/v/v) butanol, methanol and water, was used for the centrifugal partition chromatography (CPC) separation (Lau et al., 2013). The partition coefficients (K) of the phenolic compounds (trans-cinnamic acid, p-coumaric acid, trans-ferulic acid, salicylic acid, vanillic acid, vanillin), furans (HMF, furfural), organic acids (formic acid, acetic acid, propionic acid, butyric acid) and sugars (glucose, arabinose, galactose

and xylose), for the given solvent system were determined using the method reported by Berthod and Carda-Broch (2004). The partition coefficient K for an ascending mode operation is given by

$$K_x = \frac{C_{Tx}}{C_{Bx}} \quad (2)$$

Where C_T was the concentration of solute *x* in the butanol-rich top phase and C_B is the concentration of solute *x* in the aqueous bottom phase.

Centrifugal Partition Chromatography (CPC) fractionation

A bench scale SCPC-250 (Armen Instrument, Saint-Avé, France) centrifugal partition chromatography (CPC) column connected to a CherryOne Beta controller (Chicago, IL) was used to fractionate the lyophilized extracts. Solvents for CPC separation was prepared as reported by Chen et al., (2015). The CPC column was initially loaded with the aqueous stationary phase at 10 mL/min for 30 min, followed by the butanol-rich mobile phase at 8 mL/min and at a rotor speed of 2,300 RPM. When the solvents reached equilibrium, the volume of the stationary phase was 114 mL. The CPC eluents were monitored in a real-time mode using a built-in UV detector set at 254 nm and by an evaporative light scattering detector (ELSD) (SofTA Corp, Westminster, CO).

Samples for the CPC fractionation were prepared by dissolving 4 g of the lyophilized rice straw extract in 20 mL of the butanol-rich top phase and 8 mL of the aqueous bottom phase, and filtered using a 5 μm PTFE syringe filter (Thermo Scientific National, Rockwood, TN). The total CPC run time was 350 min; the first 265 min was operated in ascending mode (butanol-rich phase as the solvent) and in the final 85 min, the aqueous phase was pumped through the column for the extrusion of unseparated residues. The CPC eluents were collected in two fraction collectors: (i) Foxy R1 (Teledyne Isco, Lincoln, NE) and (ii) Waters Fraction Collector III (Waters Corporation, Milford, MA). All CPC fractions were dried in a Savant SpeedVac

Concentrator SPD 1010 (Thermo Scientific, Ashville, NC), at 7 Torr for 8 h and reconstituted in water prior to the HPLC analyses. The purity of consolidated CPC fractions was calculated as:

$$\%\ \text{Purity}_x = \frac{Weight\ of\ the\ compound_x}{Total\ weight\ of\ each\ fraction} \times 100 \qquad (3)$$

High Performance Liquid Chromatography (HPLC) characterizations

Sample preparation for HPLC analysis was done by filtering the CPC fractions using 0.22 µm syringe filters (National Scientific Company, Rockwood, TN). The monomeric sugars (xylose, glucose, arabinose, galactose) and xylose oligomers (DP2 to DP6) were determined using a Waters Alliance HPLC system (Model 2695, Waters Corporation, Milford, MA) equipped with SP0810 (Shodex, Kawasaki, Japan) or Bio-Rad Aminex-HPX 42A (Bio-Rad, Hercules, CA) columns and a refractive index detector (Model 2414, Waters Corporation, Milford, MA). The furans and organic acids were quantified using a similar HPLC system fitted with a Bio-Rad Aminex (Life Sciences Research, Hercules, CA) HPX-87H ion exclusion column and a photodiode array detector (Model 2996, Waters Corporation, Milford, MA) set at wavelengths of 210 and 280 nm. Previously reported HPLC methods (Rajan and Carrier, 2014a; Rajan and Carrier, 2014b) were used for the analysis of sugars, furans and organic acids.

Phenolic compounds were analyzed using an Acquity Ultra Performance Liquid Chromatography (UPLC) system equipped with a BEH C_{18} (1.7 µm × 2.1 mm × 50 mm) analytical column and an Acquity VanGuard pre-column (Waters, Milford, MA). The samples were eluted at a flow rate of 0.4 mL/min and detected at 220, 267, 280 and 300 nm, using a photodiode array (PDA) detector. The column was heated to 50 °C. The mobile phases consisting of 0.1 % formic acid and methanol were eluted at a gradient of 88.5:11.5 to 30:70, over 3.5 min. The sugars, furans, organic acids and phenolic compounds were quantified using in-house calibration curves.

Total phenolics assay

The total phenolic content was determined using the Folin-Ciocalteu (F-C) reagent, based on a method modified from a previous publication (Ainsworth and Gillespie, 2007). The samples were diluted to an approximate concentration of 1.25 g/L, and 100 µL this aliquot was mixed with 200 µL of 0.2 N F-C reagent, followed by incubation in the dark, for 5 min. A 700 µL of aliquot 7.5% sodium carbonate solution was subsequently added to the mixture and incubated in the dark, at room temperature, for 2 h. After the incubation period, the samples were diluted by a factor of four with water, and their absorbance at 765 nm was determined using a spectrophotometer (Model 517601, Beckman Coulter Inc., Indianapolis, IN). Gallic acid standards (0.5 to 2.5 g/L) were used to build a standard curve; the results were expressed in gallic acid equivalents.

***Exo*-cellulase assay**

The *exo*-cellulase activity was determined by quantifying the amount of enzyme required to release one micromole of 4-methylumbelliferone (MUC) per minute (Boschker and Cappenberg, 1994). Substrate stock (5 µmol/mL) was prepared by first dissolving 25 mg of MUC in 1 mL of dimethyl sulfoxide and followed by adding 9 mL of 50 mmol sodium acetate buffer, at pH 5.0. Control for the *exo*-cellulase assay was prepared by mixing 25 µL of MUC, equivalent to 1 µmol/mL, with 41.7 U/mL of *exo*-cellulase and the final volume was made up to 125 µL with the sodium acetate buffer. For the enzyme inactivation kinetics 1.25 to 25 µL of the buffer was substituted with the CPC fractions, such that their concentrations were 1 to 20 g/L, respectively. The *exo*-cellulase assays were carried out in covered 96–well microtiter plates (Corning®, Radnor, PA) heated in a 50 °C water bath and agitated at 35 RPM, for up to 180 min. To stop the reaction 25 µL of the samples were transferred from the reaction plates to the reading

plates (FluoroNunc™, Fischer Scientific, Pittsburg, PA) containing 225 µL of 0.2 M sodium carbonate solution. Samplse fluorescence was analyzed using a Synergy HT (BioTek Instruments, Winooski, VT) micro-well plate reader. Calibration curves were prepared by assaying 0.2 to 1.0 µmol/mL of MUC with 41.7 U/mL *exo*-cellulase for 12 min. The *exo*-cellulase efficiency was given by

$$\text{Enzyme efficiency} = \frac{Moles\ of\ substrate\ released\ in\ presence\ of\ hydrolyzate}{Moles\ of\ substrate\ released\ by\ control} \times 100 \qquad (4)$$

All experiments were carried out in duplicates and the results were analyzed for statistical significance using MS Excel 14.0, 2011 (Microsoft Corp., Redmond, WA). Additional statistical analysis such as the Tukey HSD test was performed using JMP Pro 11 (SAS, Cary, NC).

Results and discussion

Characterization of cellulolytic inhibitors

Rice straw was subjected to dilute acid (160 °C, 48 min, pH 1.7) and hot water (220 °C, 52 min) pretreatments such that their combined severity was 1.75, which was reported to provide the maximum glucose recovery upon enzymatic saccharification of the pretreated solids (Hsu et al., 2010). The average glucan yields after 90 h were 81 ± 6 % for dilute acid and 75 ± 5 % for hot water pretreatments (Fig.1). As expected, there were no significant differences in glucan yields between the two pretreatment conditions, owing to the similar pretreatment severities. However, their prehydrolyzate compositions differed, as previously reported by Rajan and Carrier (2014a). When the lyophilized prehydrolyzates were tested against the specific saccharification activities of cellulolytic enzymes, hot water prehydrolyzate at 20 g/L exhibited the highest inhibitions of the cellulase cocktail (71%), *endo*-cellulase (99%) and β-glucosidase

(38%) (Rajan and Carrier, 2014a). In this study, 20 g/L of the lyophilized hot water and dilute acid hydrolyzates were tested against *exo*-cellulase enzyme and it was determined that its activity was reduced by 86% and 69%, respectively, compared to the control. The composition of lyophilized hot water extracts, determined using HPLC analysis and F-C reagent assay, is provided in Table 1; compounds in the hot water hydrolyzates namely, furans, organic acids, monomeric and oligomeric sugars and phenolics have been previously reported to be inhibitory to the cellulolytic enzymes (Duarte et al., 2012; Ximenes et al., 2010). These compounds are formed as a result of degradation of structural carbohydrates and lignin during biomass pretreatments (van der Pol et al., 2014). The hot water hydrolyzates were also determined to contain significantly higher quantities of xylo-oligosaccharides and total phenolics than that of dilute acid hydrolyzate (Rajan and Carrier, 2014a). For the subsequent studies, characterization of the hot water hydrolyzate was carried out, since it exhibited a higher degree of inhibition of the cellulolytic enzyme, and also had a wide spectrum of known cellulolytic inhibitors.

CPC fractionation of rice straw prehydrolyzates

CPC has been used in large-scale purifications of a wide range of naturally occurring chemical compounds such as phenolic derivative, terpenes, saponins, alkaloids and antibiotics (Yoon et al., 2010). Recently, CPC was also employed to purify xylose and xylo-oligosaccharides from the hot water hydrolyzates of birchwood (Lau et al., 2013), switchgrass (Bunnell et al., 2015) and miscanthus (Chen et al., 2015). Since xylose and xylo-oligosaccharides constituted a sizeable portion of the characterized hot water hydrolyzates of rice straw, a solvent system suitable for fractionating these compounds was sought. A biphasic 5:1:4 (v/v/v) butanol, methanol and water solvent system reported by Lau et al., (2013) was employed for the subsequent CPC separation.

For the chosen biphasic system, partition coefficients (K) of the various compounds identified in the hot water hydrolyzates, were determined and provided in Table 2. The K value depends on the solubility of a compound in a biphasic solvent system, once it attains equilibrium (Berthod and Carda-Broch, 2004). It also determines the order of elution of a target compound during the CPC fractionation; compounds having K >1 elute first, followed by compounds having K=1 and then K<1 (Ito, 2005). It was observed that in the butanol-methanol-water (5:1:4) solvent system, the phenolic compounds and furfural had a K-value greater than 1, HMF and acetic acid had a K-value equal to 1 and formic acid and all the sugars had a K-value less than 1 (Table 2). Lau et al., (2013) had previously reported that the partition coefficients for xylo-oligosaccharides using an identical biphasic solvent system were 0.044 for DP2, 0.015 for DP3 and 0.008 for DP4.

A typical ELSD chromatogram of the CPC fractionation of the rice straw hot water hydrolyzates is presented in Fig. 2. Individual CPC fractions were analyzed by HPLC and pooled together based on a target compound exhibiting the highest mass fraction. The results of consolidation are presented in Fig. 3, which in a sense is a heat map of compounds determined in the consolidated CPC fractions. Possessing a high K value, the targeted phenolic compounds had a very low polarity index for the chosen solvent system (Ingkaninan et al., 1999); therefore they eluted together (fraction 1) during the first 20 min of the run, without proper separation (Fig. 2). HMF (10.1 ± 1.8 %), furfural (3.9 ± 0.3 %), propionic acid (15.4 ± 1.6 %) and butyric acid (5.6 ± 0.7 %) eluted following the phenolics and were consolidated into fraction 2. A fairly pure form of acetic acid (89.7 ± 2.6 %) mixed with formic acid (9.37 ± 0.7 %) was obtained in fraction 3. Fraction 4 was a mixture of acetic acid (57 %) and formic acid (37 %), fraction 5 was a mixture of xylose (23.5 ± 4.6 %), arabinose (22.73 ± 4.0 %) and xylobiose (5.5 ± 0.1 %) and fraction 6

was a mixture of xylobiose (31.1 ± 1.5 %) and xylotriose (8.5 ± 0.7 %). Fraction 7 and 8 contained consolidated xylotriose (67.5 ± 2.8 %) and xylotetraose (65.3 ± 2.7 %), respectively. Fraction 9 contained a mixture of xylohexaose (22.8 ± 0.6 %), xylopentaose (9.6 ± 2.9 %) and other unknown higher polymers. Higher polymers of xylose and other sugars had a very high polarity index for the chosen solvent system and hence did not separate properly and were eluted towards the end of the CPC run (Fig. 2).

Effect of CPC fractions on *exo*-cellulase efficiency

Using CPC, the hot water hydrolyzates of rice straw were fractionated and consolidated into 9 different fractions having relatively pure groups of phenols, furans, organic acids and monomeric and oligomeric sugars. Fractions 1 to 9 were incubated individually with the *exo*-cellulase-MUC mixture, at concentrations ranging from 0 to 8 g/L, for 60 min. The efficiency of *exo*-cellulase plotted against the concentration of CPC fractions is presented in Fig. 4. It was determined that, even at 1 g/L, the phenol-rich fraction 1 was highly inhibitory to the *exo*-cellulase system, reducing the enzyme efficiency by 75 %. The phenolic compounds identified in fraction 1, using UPLC, analysis include salicylic acid (3.8 ± 0.6 %), *p*-coumaric acid (2.7 ± 0.3 %), vanillic acid (2.6 ± 0.3 %), *trans*-ferulic acid (2.1 ± 0.6 %), vanillin (1.4 ± 0.2 %), syringaldehyde (1.0 ± 0.1 %), *trans*-cinnamic acid (0.9 ± 0.0 %) and 4-hydroxybenzoic acid (0.2 ± 0.0 %). Other reports address the inhibitory effect of phenols on saccharification enzyme systems, where surrogate solutions based on prehydrolyzate composition were tested. Surrogate solutions of 2 g/L syringaldehyde were reported to inhibit the carboxymethyl cellulase (CMCase) activity of *T. reesei* cellulases by only 5% (Ximenes et al., 2011). And no inhibition of *exo*-cellulase activity of *T. reesei* cellulases was reported in the presence of surrogate solutions of 2 g/L *trans*-cinnamic acid, *trans*-ferulic acid, *p*-coumaric acid, vanillin,

syringaldehyde, and 4-hydroxybenzoic acid (Ximenes et al., 2011). Phenolic compounds, at 1 mg/mg of protein, extracted from the liquid hot water pretreated maple wood prehydrolyzate, was also reported to inhibit the *T. reesei exo*-cellulase by only 5% (Kim et al., 2011). Interestingly, a severe reduction of *exo*-cellulase efficiency was observed in this study when using authentic fractions and not synthetic solutions. When using 10 g/L of surrogate solution prepared from vanillin, syringaldehyde, *trans*-cinnamic acid and 4-hydroxybenzoic acids, Ximenes et al., (2011) reported between 19 and 90 % inhibition of *T. reesei* cellulase cocktail. In this work, 2 g/L of fraction 1 resulted in 80 % inhibition of purified *exo*-cellulase, indicating that authentic preparations could be more potent than surrogate solutions.

Incubation of *exo*-cellulase in the presence of 2 g/L of fraction 2 resulted in 75% loss of enzyme efficiency. Furfural, at 2 g/L, has been reported to inhibit the Accellerase® 1500 saccharification, by 10% (Arora et al., 2012). HMF, at 2 g/L, was reported to inhibit the filter paper activity of *T. reesei* cellulases by 7% (Jing et al., 2009). Fermentation by-products such as propionic acid (15 g/L) and isobutyric acid (9 g/L) have been reported to be inhibitory to CMCase activity of recombinant *Bacillus spp*. (Yoon et al., 1994). Occurrence of propionic acid and butyric acid is rarely reported in lignocellulosic hydrolyzates, and their potential inhibitory action on specific cellulose cocktail enzymes has not been previously described.

Fractions 3 and 4, at concentrations of 8 g/L, resulted in the loss of 85% of *exo*-cellulase enzyme efficiency. Acetic acid fractionated from hydrolyzates of steam-pretreated willow was reported to inhibit *T. reesei* cellulase hydrolysis by 10 %, at 8 g/L (Palmqvist et al., 1996). Formic acid, at 10 g/L, was reported to inhibit the Accellerase® 1500 saccharification of Avicel, by 81% (Arora et al., 2012). The inhibition of cellulases observed in these above-mentioned studies may be in part due to the inhibition of *exo*-cellulases.

The oligomer-rich fractions (6, 7, 8) were not as inhibitory to the *exo*-cellulase system, as that of fraction 5, which was rich in xylose and arabinose. Fraction 5, at 2 g/L, caused 50 % reduction in *exo*-cellulase efficiency, which is in contrast to Zhang and Viikari (2012), where *T. aurantiacus exo*-cellulase was not inhibited by 2 g/L of xylose. Xylose DP2 and DP3 were reported to be competitive inhibitors of *exo*-cellulase. In the presence of 2 g/L of birchwood-derived xylo-oligosaccharides, the *exo*-cellulase saccharification of Avicel was inhibited by 25 % (Zhang and Viikari, 2012). In this study, 2 g/L of xylo-oligosaccharides from fractions 6, 7 and 8, were also determined to reduce *exo*-cellulase efficiency by 25 to 35 % (Fig. 4). Fraction 9, which contained DP5, DP6 and other unknown oligosaccharides, was found to be highly inhibitory to the *exo*-cellulase; addition of 2 g/L led to 71% loss of enzyme efficiency. Other oligosaccharides such as manno-oligosaccharides from softwood hydrolyzates were reported to cause 50 % reduction in *exo*-cellulase at a concentration of 10 g/L (Xin et al., 2014). Further characterization of fraction 9 would shed light on exactly which compounds are responsible for the *exo*-cellulase inhibition.

Effect of CPC fractions on MUC conversion rates

Fractions 1, 2, 3, 8 and 9 were further evaluated for their inhibitory effect on *exo*-cellulase activity by incubating with 8 g/L of the CPC fractions. The *exo*-cellulase loading was increased by a factor of 4 to ensure 100 % substrate conversion in the positive control within the time frame of the experiment. Figure 5a depicts the concentration of MUC released (μmol/mL), when hydrolyzed by *exo*-cellulase as a function of time, while Figure 5b illustrates the linear substrate conversion rates of *exo*-cellulase, 15 min after incubation. The positive control presented in Figure 5a was composed of only the enzyme and substrate, whereas the “original hydrolyzate” contained 8 g/L of crude hot water rice straw hydrolyzate prior to CPC

fractionation. The MUC hydrolysis rates after 15 min of incubation with *exo*-cellulase was 0.056 μmol/mL/min for the control and 0.009 μmol/mL/min in the presence of the original hydrolyzate. In the presence of CPC fractions 1, 2, 3, 8 and 9 the MUC hydrolysis rate by the *exo*-cellulase was 0.006, 0.007, 0.008, 0.017 and 0.019 μmol/mL/min, respectively (Fig. 5b). Thus adding the original hydrolyzate reduced the *exo*-cellulase activity by 84 %, which was higher than in the presence of xylo-oligosaccharides, but lower than with other CPC fractions (Fig. 5b). Fractions 1, 2 and 3 were highly inhibitory to the *exo*-cellulase, whose activity did not improve beyond 16 % of that of the control (Fig. 5b). Conversely, fractions 8 and 9 reduced the *exo*-cellulase activity by 66 % and 70 %, respectively (Fig. 5b) and after 120 min in the presence of fraction 9, the MUC hydrolysis rate reached the same level as that of the control (Fig. 5a).

The substrate conversion rate of *exo*-cellulase was significantly lowered by 89%, in the presence of the phenolics rich CPC fraction 1 ($p< 0.001$, $\alpha_{0.05}$). Lignin-derived phenols have been reported to deactivate and inhibit saccharification cocktails and β-glucosidase. Phenols at 0.5 mg/mg protein, have been reported to deactivate up to 60 % of the cellulase cocktail within 1 h of incubation via non-competitive binding (Kim et al., 2011). In this work, the *exo*-cellulase may have been deactivated and precipitated by the phenolics contained in fraction 1, since insoluble residues were observed in the reaction wells of these samples. It has been reported that deactivation of cellulolytic enzymes can be prevented by precipitating the lignin-derived soluble phenols using bovine serum albumin (Yang and Wyman, 2009).

Furans and organic acids have also been reported to reduce the hydrolysis rates of saccharification enzymes. In this study, acetic acid at 7 g/L in fraction 3, showed a high inhibition of MUC hydrolysis by *exo*-cellulase (Fig. 5b). This is in contrast to other studies, where acetic acid, at 2 g/L, was not inhibitory to steam exploded spruce wood saccharification

by *T. reesei* cellulases (Cantarella et al., 2004). This group also reported 15 % reduction in the rate of formation of reducing sugars when placed in the presence of 2 g/L of HMF and furfural during the enzymatic saccharification of the steam exploded spruce wood. However in this study, MUC hydrolysis rates did not increase over time in the presence of furans and organic acids.

Mixed xylo-oligosaccharides at 8.3 g/L produced from hot water hydrolyzed birchwood xylan decreased the initial rate of Avicel hydrolysis by *T. reesei* cellulases down to 79%, (Qing et al., 2010). This group also reported that, even after 100 h of incubation, the hydrolysis rates only reached 50 % of those of the control; it was speculated that rapid hydrolysis of xylo-oligosaccharides by commercial *T. reesei* cellulases to xylobiose and xylose may have been responsible for the increase in cellulose hydrolysis rates (Qing et al., 2010). Although *exo*-cellulase used in this study was not tested for xylanase activity, increases in dosage of *exo*-cellulase could be effective for improving cellulose hydrolysis rates, which would otherwise be inhibited by xylo-oligosaccharides.

Conclusion

Fractionation of hot water hydrolyzates of rice straw using centrifugal partition chromatography (CPC) provided an effective methodology for comparing the inhibition of *exo*-cellulase by the different classes of chemical compounds originally present in the prehydrolyzate. Phenolic compounds were determined to be highly inhibitory to the *exo*-cellulase efficiency followed by furans and organic acid mixtures, particularly acetic acid. While the xylo-oligosaccharides were also inhibitory to the initial hydrolysis rate of *exo*-cellulase, the enzyme efficiency increased over time, at additional enzyme loadings and eventually reached 60 to 100 % of its original efficacy. Thus, this study provided an insight into which class of by-products in a pretreatment hydrolyzate was highly inhibitory to the cellulolytic enzyme systems.

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Figure captions

Fig. 1 Glucan yields after enzymatic saccharification of hot water (220 °C, 52 min) and dilute acid (160 °C, 48 min, 1% sulfuric acid) pretreated rice straw, using Accellerase® 1500 enzyme, at 105 FPU/g glucan loading.

Fig. 2 A typical ELSD (evaporative light scattering detector) chromatogram of CPC (centrifugal partition chromatography) fractionation of rice straw hot water hydrolyzates.

Fig. 3 A heat-map of the centrifugal partition chromatography (CPC) fractionated hot water hydrolyzates of rice straw, based on the percent mass fraction of a test compound in each CPC fraction.

Fig. 4 Efficiency of *exo*-cellulase (*Hypocrea jecorina*) in the presence of CPC (centrifugal partition chromatography) fractionated hot water hydrolyzates of rice straw at a concentration ranges of 0- 8 g/L, incubated at 50 °C, pH 5.0, for 60 min.

Fig. 5 **(A)** Amount of MUC (4-Methylumbelliferyl β-D-cellobioside) hydrolyzed by *Hypocrea jecorina exo*-cellulase in the presence of 8 g/L of CPC (centrifugal partition chromatography) fractionated hot water hydrolyzates of rice straw, incubated at 50 °C, pH 5.0, for up to 120 min. **(B)** Linear substrate hydrolysis rates (μmol/mL/min) of *exo*-cellulase in the presence of CPC fractions, 15 min after incubation. Tukey HSD test was performed for $n=2$. Levels not connected by same letters are significantly different.

Table 1. Composition of the lyophilized hot water (220 °C, 52 min) extract of rice straw analyzed using HPLC.

Compounds	% Freeze dried extract
Formic acid	11.45 ± 0.25
Acetic acid	13.24 ± 2.5
Propionic acid	7.55 ± 0.59
Butyric acid	5.07 ± 0.52
Furfural	1.83 ± 0.59
HMF	0.41 ± 0.09
Glucan	2.56 ± 0.03
Galactose	0.94 ± 0.00
Arabinose	0.59 ± 0.00
Xylose	2.61 ± 0.30
Xylobiose	3.48 ± 0.85
Xylotriose	3.72 ± 1.99
Xylotetraose	4.62 ± 1.57
Xylopentaose	1.59 ± 0.20
Xylohexaose	0.84 ± 0.20
Other xylan	1.77 ± 0.13
Other arabinan	0.32 ± 0.05
Other galactan	0.75 ± 0.19
Total Phenolics[#]	6.14 ± 0.21
Total	**69.48 ± 3.83**

[#] Expressed as % gallic acid equivalent, determined using Folin-Ciocalteu reagent

Table 2. Partition coefficients of reference compounds for a biphasic 5:1:4 (v/v/v) butanol, methanol and water solvent system.

Compound	K-value*
trans-Cinnamic acid	18.14 ± 2.54
p-Coumaric acid	13.11 ± 3.67
4-Hydroxybenzoic acid	9.84 ± 2.49
trans-Ferulic acid	9.40 ± 0.21
Vanillic acid	6.73 ± 1.75
Vanillin	6.47 ± 1.21
Furfural	4.26 ± 0.11
5-Hydroxymethyl furfural	1.17 ± 0.01
Acetic acid	1.07 ± 0.1
Formic acid	0.50 ± 0.1
Glucose	0.41 ± 0.03
Xylose	0.23 ± 0.03
Arabinose	0.19 ± 0.01
Galactose	0.11 ± 0.08

*K values were calculated as solute affinity to the aqueous-rich bottom phase

Fig. 1

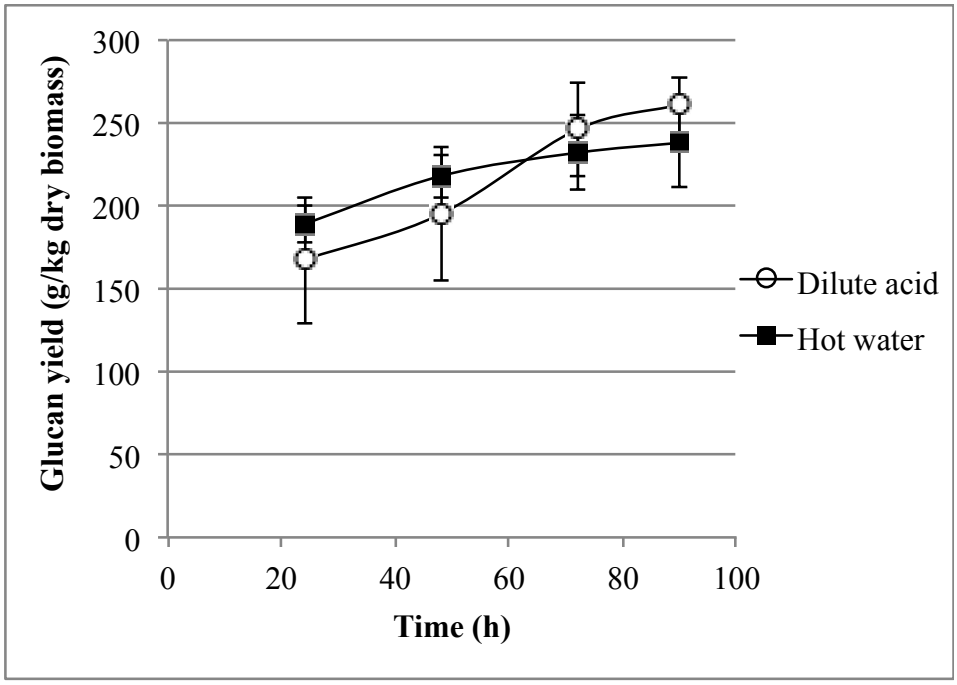

Glucan yield (g/kg dry biomass)
300
250
200
150
100
50
0
0
20
40
60
80
100
Time (h)
Dilute acid
Hot water

Fig. 2

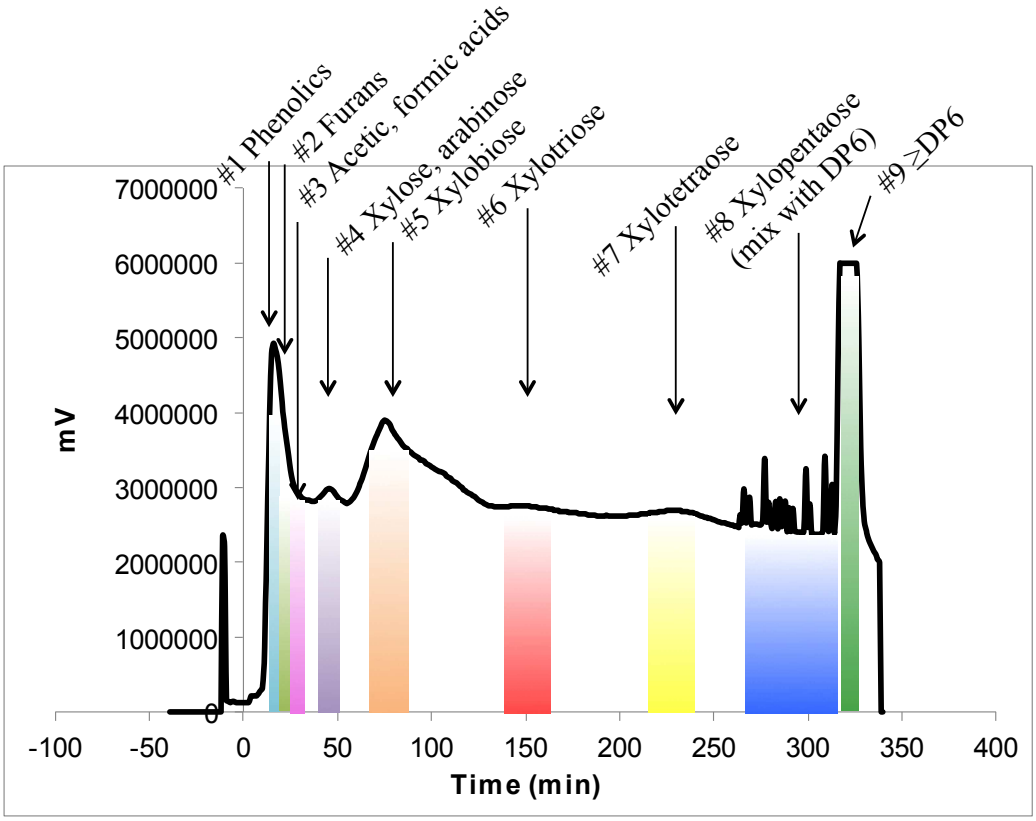

#1 Phenolics
#2 Furans
#3 Acetic, formic acids
#4 Xylose, arabinose
#5 Xylobiose
#6 Xylotriose
#7 Xylotetraose
#8 Xylopentaose
(mix with DP6)
#9 ≥DP6
7000000
6000000
5000000
4000000
3000000
2000000
1000000
0
mV
-100
-50
0
50
100
150
200
250
300
350
400
Time (min)

Fig. 3

CPC fraction #	Compounds (% fraction weight)																				
	Phenolics								Organic acids and furans						Sugars						
	Vanillic acid	Cinnamic acid	4-HBA	Vanillin	Syringaldehyde	p-Coumaric acid	Ferulic acid	Salicylic acid	HMF	Furfural	Propionic acid	Butyric acid	Acetic acid	Formic acid	Xylose	Arabinose	DP2	DP3	DP4	DP5	DP6
1	2.55	0.87	0.02	0.69	0.33	1.31	0.64	0.00													
2	0.77	0.12	0.17	1.35	1.04	2.69	2.13	3.81													
3	0.22	0.03	0.02	0.07	0.24	0.24	0.42	0.76	0.00	0.00	0.00	0.00	0.00	0.00							
4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	10.16	2.36	0.94	0.79	0.46	0.00							
5									5.46	3.94	15.38	5.57	27.05	8.22							
6									4.48	0.00	1.04	0.97	89.72	9.37	0.00	0.00	0.00				
7									2.06	0.00	1.38	0.99	56.83	37.44	7.85	6.06	2.72				
8									0.00	0.00	0.11	0.00	5.88	4.22	23.48	22.73	5.54				
9									0.23	0.00	0.00	0.00	0.00	0.00	19.87	42.54	7.39				
10															13.08	12.10	8.85				
11															8.06	0.00	12.25	0.00			
12															7.71	0.00	16.04	4.49			
13															5.05	0.00	25.76	5.65			
14															1.71	0.00	31.11	8.47			
15															1.37		28.78	9.56			
16															0.00		28.78	25.92	0.00		
17																	9.02	48.65	2.89		
18																	3.42	67.54	3.61		
19																	2.72	54.81	16.37		
20																	0.00	45.37	37.32		
21																		22.38	28.41	0.00	
22																		9.65	54.42	0.95	
23																		10.88	65.25	9.54	
24																		6.98	46.35	8.36	0.00
25																		7.39	47.92	17.26	2.40
26																		0.00	16.37	12.53	7.05
27																			5.78	9.58	22.78
28																			0.00	2.88	6.45
29																				0.95	5.55
30																				0.39	2.10

Fig. 4

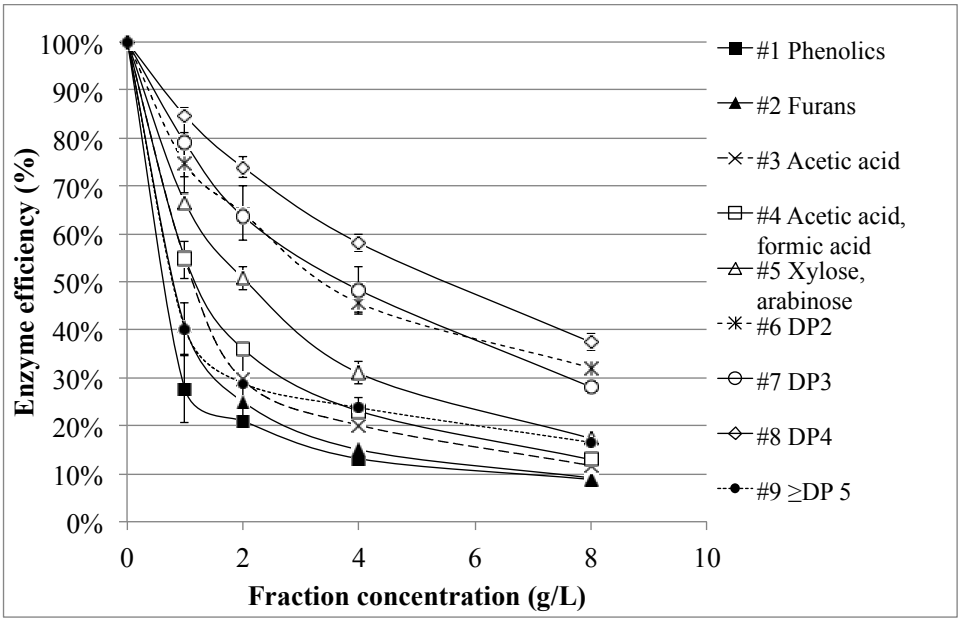

100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Enzyme efficiency (%)
0
2
4
6
8
10
Fraction concentration (g/L)
#1 Phenolics
#2 Furans
#3 Acetic acid
#4 Acetic acid, formic acid
#5 Xylose, arabinose
#6 DP2
#7 DP3
#8 DP4
#9 ≥DP 5

Fig. 5a

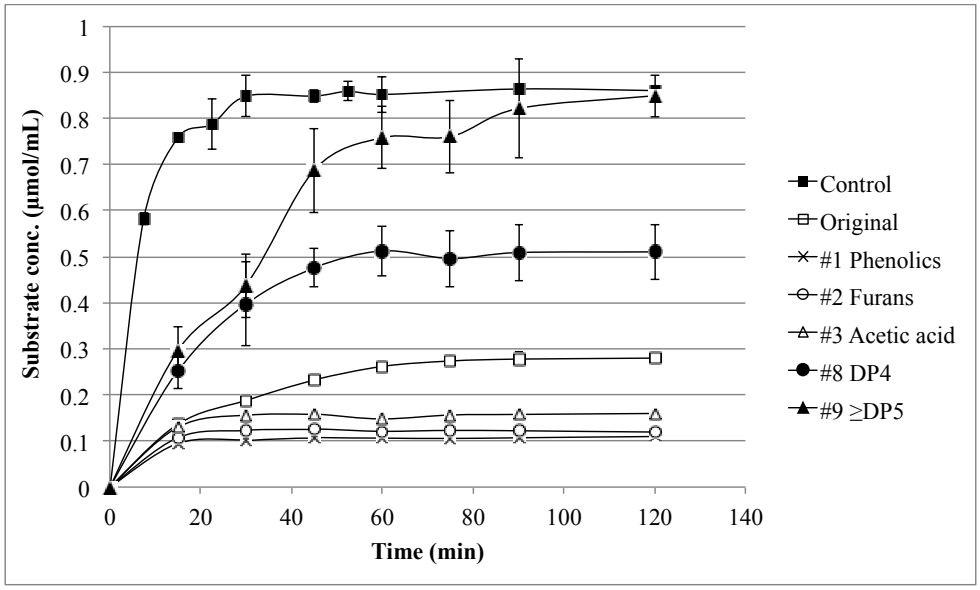
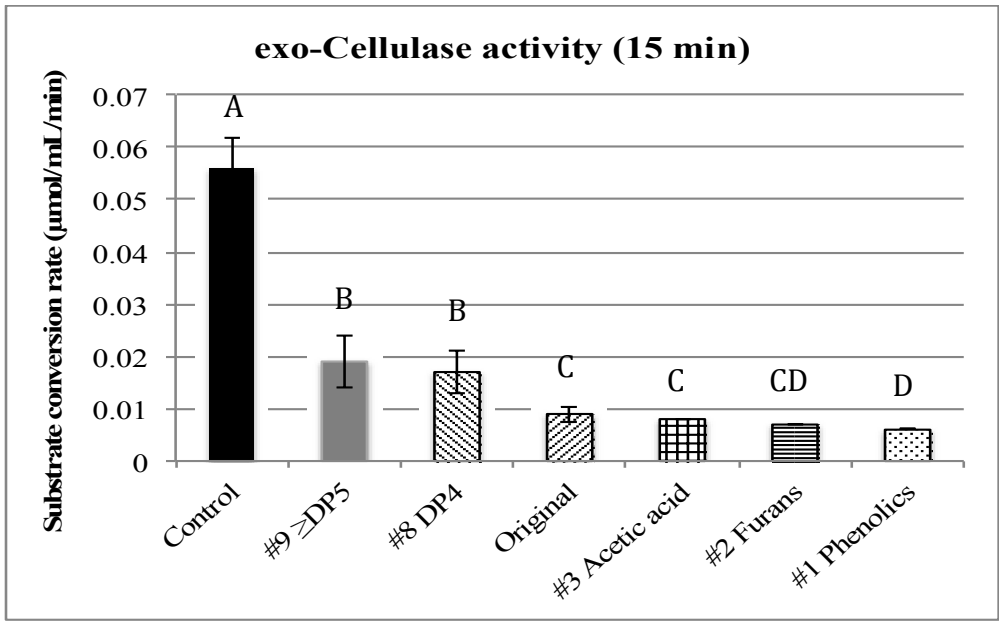

Substrate conc. (µmol/mL)
Time (min)
Control
Original
#1 Phenolics
#2 Furans
#3 Acetic acid
#8 DP4
#9 ≥DP5

Fig. 5b


exo-Cellulase activity (15 min)
Substrate conversion rate (µmol/mL/min)
A
B
B
C
C
CD
D
Control
#9 ≥DP5
#8 DP4
Original
#3 Acetic acid
#2 Furans
#1 Phenolics

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VII. Conclusion

Pretreatment hydrolyzates of rice straw were characterized and analyzed for the presence of compounds inhibiting cellulolytic enzymes. It was determined that the hot water hydrolyzate composed of 13.2 % acetic acid, 11.5 % formic acid, 7.6% propionic acid, 5.1 % butyric acid, 1.0 % furans, 6.7 % monomeric sugars, 17.1 % oligomeric sugars and 6.1 % phenolics, was highly inhibitory to the activities of *exo*–cellulase, *endo*–cellulase and β-glucosidase enzymes. The hot water hydrolyzate was subsequently fractionated using centrifugal partition chromatography that employed a biphasic solvent system composed of 5:1:4 (v/v/v) butanol, methanol and water. The CPC fractions were consolidated into different groups rich in phenolics, furans, acetic acid, xylose, xylobiose, xylotriose, xylotetraose and higher xylan-derived oligomers. When the CPC fractions were incubated with *exo*-cellulase at a concentration of 1 g/L, it was revealed that the fractions rich in phenolics, furans, higher xylan-derived oligomers and acetic acid inhibited the enzyme efficiency by 75 %, 60 %, 60 % and 45 %, respectively. The initial hydrolysis rate of *exo*-cellulase was significantly lowered by 89 % ($p < 0.001$, $\alpha_{0.05}$), when incubated with 8 g/L of CPC fraction rich in phenolics. Conversely, at a higher enzyme loading, the hydrolysis rate of exo-cellulase was shown to improve despite the addition of oligomeric sugars. Thus it was concluded that in order to improve the efficiency of the cellulases, it is important to mitigate acetic acid and phenolic compounds, because these compounds even at 1 g/L were highly inhibitory to the enzymatic saccharification. In the future, lignocellulosic pretreatment methods may be advanced such that the lignin degradation is minimized.