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Compounds Released from Biomass Deconstruction: Understanding Their Effect on Cellulose Enzyme Hydrolysis and Their Biological Activity

Angele Djioleu
University of Arkansas, Fayetteville

Compounds Released from Biomass Deconstruction: Understanding Their Effect on Cellulose Enzyme Hydrolysis and Their Biological Activity

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by

Angèle Mezindjou Djioleu
University of Arkansas
Bachelor of Science in Biological Engineering, 2010
University of Arkansas
Master of Science in Biological Engineering, 2012

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University of Arkansas

This dissertation is approved for recommendation to the Graduate Council.

Dr. Danielle Julie Carrier
Dissertation Director

Dr. Robert Beitle
Committee Member

Dr. Edgar C. Clausen
Committee Member

Dr. Thomas A. Costello
Committee Member

ABSTRACT

The effect of compounds produced during biomass pretreatment on cellulolytic enzyme was investigated. Liquid prehydrolyzates were prepared by pretreating switchgrass using 24 combinations of temperature, time, and sulfuric acid concentration based on a full factorial design. Temperature was varied from 140°C to 180°C; time ranged from 10 to 40 min; and the sulfuric acid concentrations were 0.5% or 1% (v/v). Identified products in the prehydrolyzates included xylose, glucose, hydroxymethylfurfural (HMF), furfural, acetic acid, formic acid, and phenolic compounds at concentration ranging from 0 to 21.4 g/L. Pretreatment conditions significantly affected the concentrations of compounds detected in prehydrolyzates. When assayed in the presence of switchgrass prehydrolyzates against model substrates, activities of cellulase, β-glucosidase, and exoglucanase, were significantly reduced by at least 16%, 31.8%, and 57.8%, respectively, as compared to the control. A strong positive correlation between inhibition of β-glucosidase and concentration of glucose, acetic acid, and furans in prehydrolyzate was established. Exoglucanase inhibition correlated with the presence of phenolic compounds and acetic acid. The prehydrolyzate, prepared at 160°C, 30 min, and 1% acid, was fractionated by centrifugal partition chromatography (CPC) into six fractions; the inhibition effect of these fractions on β-glucosidase and exoglucanase was determined. The initial hydrolysis rate of cellobiose by β-glucosidase was significantly reduced by the CPC sugar-rich fraction; however, exoglucanase was deactivated by the CPC phenolic-rich fraction. Finally, biological activities of water-extracted compounds from sweetgum bark and their effect on cellulase was investigated. It was determined that 12% of solid content of the bark extract could be accounted by phenolic compounds with gallic acid identified as the most concentrated phytochemical. Sweetgum bark extract inhibited *Staphylococcus aureus* growth and copper-

induced peroxidation of human low-density lipoprotein, confirming antimicrobial and antioxidant activities of the extract. On the other hand, bark extract inhibited cellulase cocktail activity by reducing cellulose hydrolysis by 82.32% after 48 h of incubation. Overall, phenolic compounds generated from biomass fractionation are important players in cellulolytic enzyme inhibition; removal of biomass extractives prior to pretreatment could reduce inhibitory compounds in prehydrolyzate while generating phytochemicals with societal benefits.

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The Almighty God, for his infinite grace without which nothing is possible

DEDICATIONS

In memory of our love ones

Dad and Zoé

TABLE OF CONTENTS

I. Introduction

For the past decades, industrialized societies have appreciated the potential that lignocellulosic materials can offer for crafting solutions to mitigate their energy, environmental, and sustainability needs (Sun et al., 2002; Mosier et al., 2005; Kumar et al., 2009). The major constituents of lignocellulosic biomass are hemicellulose, cellulose, and lignin fraction, as shown in Fig. 1. Hemicellulose and cellulose are the polymeric carbohydrate components, whereas lignin is a polymer of phenolic compounds and is an extensive contributor to the recalcitrant nature of lignocellulosic biomass (Gottlieb and Pelczar, 1951; Mansfield et al., 1999). The biorefinery concept refers to a process by which lignocellulosic materials are converted to an array of bio-products, including fuels and chemicals that would mirror products currently derived from petroleum-based refineries (Kamm and Kamm, 2004). Lignocellulosic biomass conversion can be accomplished in a biochemical refinery platform, where polymeric carbohydrate constituents of the biomass would be hydrolyzed into fermentable sugars and converted to bio-products by microorganisms (Lynd et al., 2005). As shown in Fig. 2, the majors unit operations in a biochemical conversion platform are 1) pretreatment; 2) enzymatic saccharification; 3) fermentation; and 4) products recovery.

Biomass pretreatment is an important step in the conversion process, as it prepares the biomass for efficient saccharification and hereby influencing the overall conversion yield (Mosier et al, 2005; Kumar et al., 2009; Wyman, 1999; Agbor et al., 2011). Unfortunately, biomass pretreatment also produced degradation compounds as illustrated in Fig. 3 (Palmqvist and Hahn-Hägerdal, 2000a,b). In general, pretreatment-derived compounds can be classified into four categories, including: 1) sugars, such xylose, xylooligomers, and glucose; 2) furans, such as furfural and 5-hydroxymethylfurfural; 3) organic acids, like acetic, formic, and levulinic acid;

and 4) phenolic compounds like vanillin and *p*-coumaric (Palmqvist and Hahn-Hägerdal, 2000a,b). The amount and nature of compounds released during biomass pretreatment depend on the source of lignocellulosic material, the pretreatment technology, and the values of pretreatment parameters (Du et al., 2010).

Pretreatment-derived compounds have been shown to impede downstream operations in biomass biochemical conversion processes (Palmqvist and Hahn-Hägerdal, 2000a,b; Klinke et al., 2004; Jönsson et al., 2013). To prevent pretreatment degradation compounds to interfere with biomass saccharification, pretreated biomass is usually washed with vast volumes of water, leading to the generation of colossal amounts of water when scaled-up to biorefinery settings (Frederick et al., 2014; Rajan and Carrier, 2014a; Cantarella et al., 2004). Other biological, chemical, and physical detoxification methods have been proposed to mitigate the inhibitory effects of pretreatment derived compounds (Palmqvist and Hahn-Hägerdal, 2000a,b; Parawira and Tekere 2011; Van der Pol et al., 2014). However, prehydrolyzate detoxifications can account for up to 22% of the costs associated with biomass-to-ethanol conversion process (von Sivers et al., 1994). In addition, efficiency of biomass-to-ethanol process can be improved by including hemicellulose sugar hydrolyzate in enzymatic saccharification of cellulose, eliminating all together prehydrolyzates detoxification steps (Tengborg et al., 2001). Therefore, identification of pretreatment degradation products that impede cellulose saccharification and determination of their mechanism of inhibition would be beneficial to biomass-to- fuels/chemicals conversion processes, as this would provide insights as to how to adjust processes to mitigate their effects.

Cellulose hydrolysis to glucose in a biochemical refinery platform is done by a collection of cellulolytic enzymes that work synergistically to create new accessible sites and ease product inhibition, as shown in Fig. 4 (Mansfield et al., 1999; Jørgensen et al., 2007). There are three

classes of cellulolytic enzymes: 1) β-1,4- endoglucanases (EC 3.2.1.4), 2) β -1-4-exoglucanase (cellobiohydrolase, EC 3.2.1.91), and 3) 1,4-β -glucosidases (EC 3.2.1.21). After capital cost and pretreatment, cellulolytic enzymes have the 3rd highest cost in bioethanol production (Stephen et al., 2012). It is desired to increase the biomass to enzyme ratio, either by reducing enzyme loading or increasing biomass loading, in order to make the overall biomass-to-ethanol process economically viable. However, it was reported that an increase in biomass loading during enzyme saccharification resulted in a decrease in cellulose conversion, even when biomass to enzyme ratio was kept constant (Kim et al., 2011; Ximenes et al., 2010). Such decrease in cellulose conversion can most likely be attributed to enzymatic inhibition, which can be caused by pretreatment-generated compounds that impede saccharification processes. Reports from Garcia-Aparicio et al. (2006), Kothari and Lee (2011), and Rajan and Carrier (2014b) have shown that biomass prehydrolyzates obtained, respectively, by steam explosion, dilute acid, or hot water pretreatment, inhibited cellulose hydrolysis.

Detailed investigations have been conducted to identify pretreatment degradation compounds that are inhibitory to cellulolytic enzyme. Arora et al. (2012) and Cantarella et al. (2004) reported that furans and organic acids significantly reduced cellulose hydrolysis; whereas, Ximenes et al. (2011) and Kim et al. (2013) observed that phenolic compounds deactivated cellulase enzymes. Jing et al. (2009) ranked pretreatment degradation compounds in terms of their likelihood of inhibiting cellulase enzymes as follows: lignin derivatives > furan derivatives > organic acids. However, most investigations pertaining to cellulase inhibition by pretreatment degradation compounds have been conducted with commercial standards, rather than compounds directly derived from authentic prehydrolyzates. In addition, inhibition has been mostly studied using crude cellulase enzyme cocktail and only few investigators reported on the effect of

degradation compounds on individual cellulolytic enzymes. Mhlongo et al. (2015) investigated the inhibitory effect of furans, phenolic compounds, and organic acids on endoglucanase, exoglucanase, and β-glucosidase using commercial compounds. Rajan and Carrier (2014b) reported on inhibition on individual cellulolytic enzymes by total solid derived from biomass prehydrolyzates. To the knowledge of the researcher there are no studies that examine the effect of pretreatment-derived compounds on cellulolytic enzymes. In light of these research gaps, a study, reporting on inhibitory effect of each degradation compounds group, derived from authentic prehydrolyzates, on individual cellulolytic enzymes would enrich the current knowledge on cellulose saccharification.

It is most likely that biorefinery deployment will be strongly dependent on the degree of diversification of its product portfolio (Van Heiningen, 2006; Lynd et al., 2002). Currently, main research efforts on identification of biorefinery co-products are centered on carbohydrate and lignin inspired products. Little attention is paid to extractive components that can be obtained prior to biomass pretreatment (Zhang et al., 2008; Huang et al., 2010). Extractives are biomass constituents that are hydrolysable in aqueous or organic solvents, such as ethanol, butanol, methanol, and benzene. They are secondary metabolites (phytochemicals), which are produced by plants as protection against microbial and insect attacks, and often present interesting biological activities (Rice-Evans et al., 1996; Cowan, 1999). In recent studies, extractives from potential energy feedstock, such as *Panicum virgatum* (switchgrass) and *Liquidambar styraciflua* L. (sweetgum), were reported to contain, respectively, antioxidant compounds and molecules important for the production of antiviral medications, (Uppugundla et al., 2009; Martin et al., 2010; Enrich et al., 2008; El-Readi et al., 2013; Rashed et al., 2014; Eid et al., 2015). Therefore, phytochemical extraction nested in biochemical-based biorefinery processes could result in the

production of high value phytochemical streams that could find outlets in food and drugs industries. It is important that the solvent used for such extraction be compatible with the rest of the biochemical conversion process, as it should not be toxic and prevent the extracted biomass to be subsequently processed further. Water has the advantage to not be expensive, toxic, or flammable, as opposed to organic solvents, which are usually used in phytochemicals extractions. In addition to provide added revenue, phytochemical extraction nested in biochemical-based biorefinery processes could improve processes, as phenolic compounds have been reported to precipitate proteins and enzymes (Cowan, 1999). Therefore, investigating how water extracted phytochemicals could affect cellulolytic enzyme could provide insight on how to integrate an extraction operation in a biochemical refinery platform.

Objectives

Given the gaps in biochemically-based biomass conversion literature, in terms of developing an underpinning understanding of the identification of key compounds present in pretreatment hydrolyzates that inhibit saccharification, the objectives of this project are:

1. Investigate effects of pretreatment conditions on the increase in concentration of pretreatment-generated compounds present in liquid prehydrolyzate and on the ensuing digestibility of pretreated biomass
2. Investigate the effect of whole liquid prehydrolyzates and pretreatment-generated compounds on inhibiting cellulolytic enzymes
3. Prepare water-based extracts and test them for biological activity and potential for inhibiting cellulolytic enzymes

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Figure captions

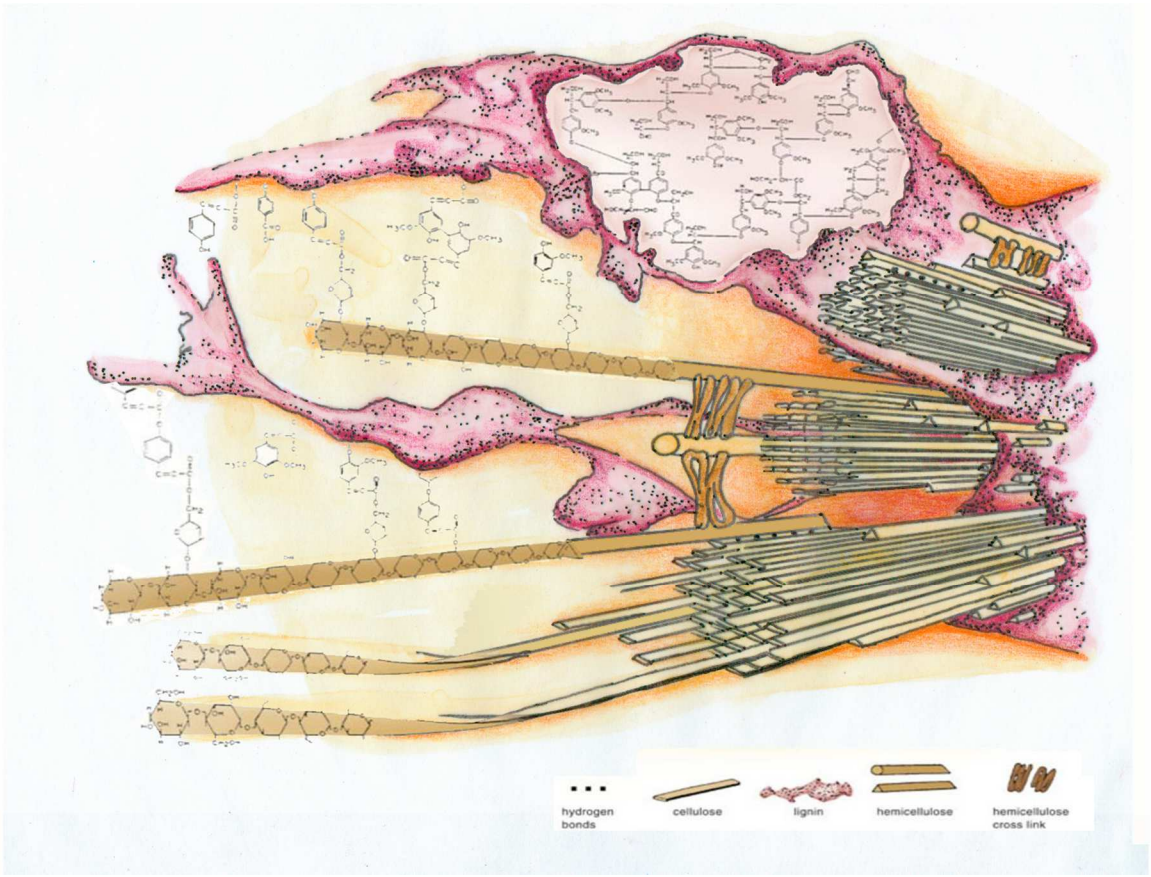
Figure 1 Structural components of lignocellulosic biomass
Drawing from Zoé Smith

Figure 2 Schematic representation of biochemical conversion of biomass to fuels and chemicals

Figure 3 Formation of products during biomass pretreatment. XOS* = xylose oligomers, [a]HMF = hydroxymethylfurfural. Adapted from Kumar et al. (2009); Palmqvist, E., Hahn-Hägerdal, B. (2000b)

Figure 4 Schematic representation cellulose hydrolysis

Fig. 1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cellulose
lignin
hemicellulose
hemicellulose cross link

Fig. 2

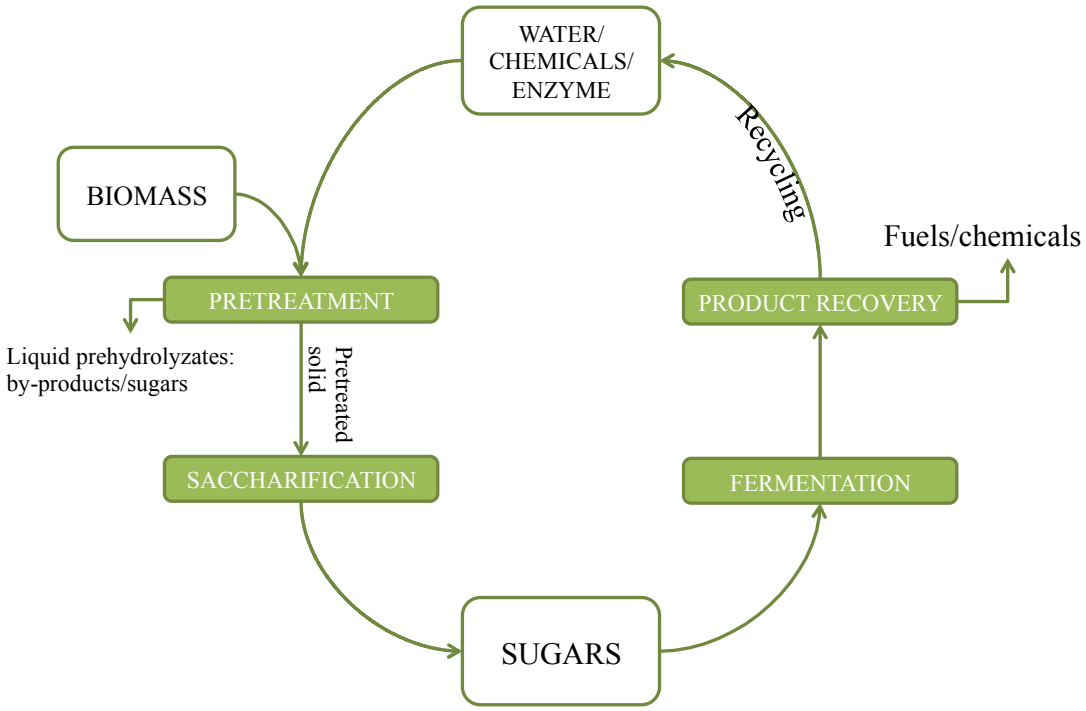
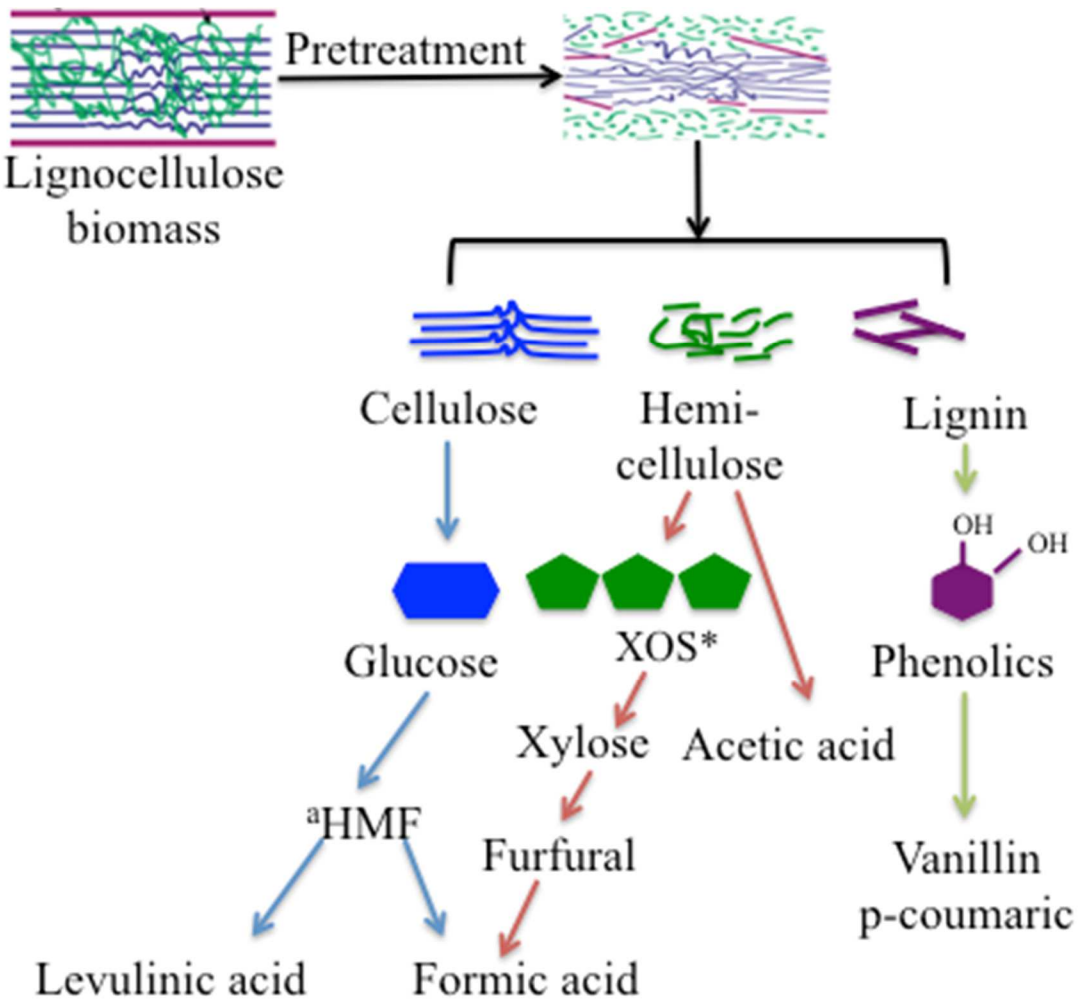

WATER/
CHEMICALS/
ENZYME
BIOMASS
PRETREATMENT
Liquid prehydrolyzates:
by-products/sugars
Pretreated
solid
SACCHARIFICATION
SUGARS
FERMENTATION
PRODUCT RECOVERY
Recycling
Fuels/chemicals

Fig. 3


Pretreatment
Lignocellulose biomass
Cellulose
Hemi-cellulose
Lignin
OH
OH
Glucose
XOS*
Phenolics
Xylose
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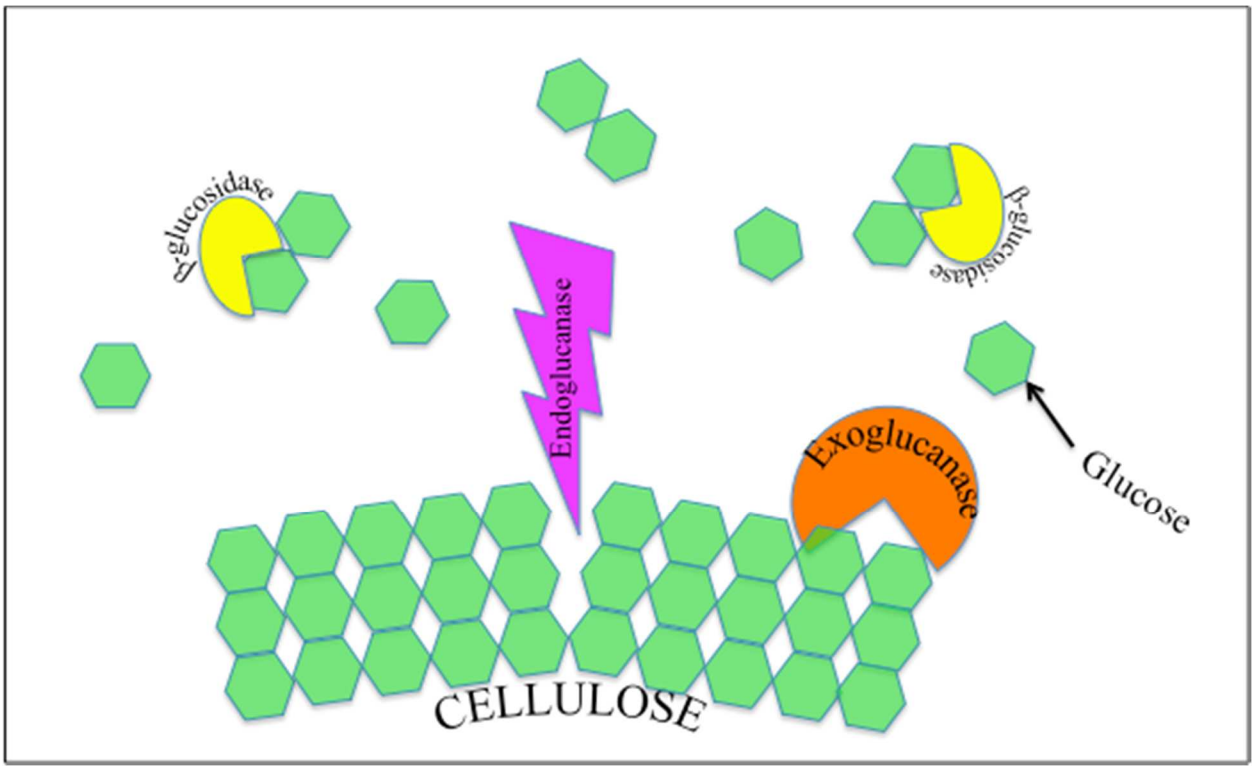
Furfural
Vanillin
p-coumaric
Levulinic acid
Formic acid

Fig. 4


β-glucosidase
Endoglucanase
β-glucosidase
Exoglucanase
Glucose
CELLULOSE

II. Effects of dilute acid pretreatment conditions on xylose and glucose concentration in prehydrolyzates, glucan content, and digestibility of pretreated switchgrass using a full factorial design

Abstract

Pretreatment is an important unit operation in the biochemical conversion of biomass because it governs the overall conversion efficiency. The effect of dilute acid pretreatment parameters on prehydrolyzate xylose and glucose concentrations, as well as on glucan content and digestibility of pretreated switchgrass, was determined using a full factorial design. Temperature was varied from 140°C to 180°C; time ranged from 10 to 40 min; and the sulfuric acid concentrations were 0.5% or 1% (v/v). Results showed that the pretreatment temperature was the most significant ($P < 0.05$) influential factor on switchgrass saccharification. The prehydrolyzate xylose levels were significantly impacted by pretreatment temperature and time, while no significant ($P > 0.05$) effect of acid concentration was observed on prehydrolyzate xylose concentrations. The resulting xylose concentrations decreased with increasing temperature and time from 21.71 g/L (140°C, 20 min, 0.5% acid) to nearly zero (180°C, 10 min, 1% acid). Prehydrolyzate glucose concentrations were dependent on all three pretreatment factors. Increasing the temperature during 0.5% (v/v) acid pretreatment yielded a maximum of 10.5 g/L glucose. Conversely, increasing the reaction temperature during 1.0% (v/v) acid pretreatment at times greater than 30 min resulted in glucose degradation. Glucan content and digestibility of pretreated switchgrass responded differently to the pretreatment parameters. Glucan content decreased with an increase in temperature, whereas digestibility rose. The highest glucan content of 64.3 % was obtained at 140°C, 30 min, and a 0.5% acid concentration (v/v), while pretreatment conditions of 180°C, 10 min and a 0.5% acid (v/v) yielded the highest

recorded digestibility of 95.8%. Interaction between temperature and acid concentration had a significant impact on glucan content, whereas digestibility was most influenced by the interaction of temperature and time. Definition of pretreatment conditions that simultaneously maximize xylose and glucose yield from switchgrass fractionation were not identified because xylose concentrations and glucan content responded differently to processing conditions, as compared to glucose level and digestibility. Overall, this study provides valuable insights to make technoeconomic decisions when designing a conversion process for a biochemical refinery platform using switchgrass as the feedstock.

1. Introduction

For the past few decades, industrialized societies have appreciated the potential that lignocellulosic materials can offer for crafting solutions to mitigate their energy, environmental, and sustainability needs (Kumar et al., 2009). Lignocellulosic materials, such as agricultural residues, forestry products, municipal solid waste and energy crops represent the most abundant form of organic carbon on earth (Kamm and Kamm, 2004). Lignocellulosic materials generally consist of three polymeric fractions: hemicellulose, cellulose, and lignin. Hemicellulose is a polymer of five-carbon sugars, such as xylose and arabinose, whereas cellulose is a long chain of hexose sugars linked together by β-(1,4)-glycosidic bonds (Kumar et al., 2009). Lignin, which is the polymer responsible for the recalcitrant nature of lignocellulosic biomass, consists of polyphenol compounds (Gottlieb and Pelczar, 1951; Mansfield et al., 1999). Through biochemical processes, hemicellulose and cellulose can be hydrolyzed into monomeric sugars, which can be further converted to fuels and chemicals via fermentation processes (Mosier et al., 2005; Wyman, 1999). However, it is very difficult to obtain carbohydrate monomers from lignocellulosic carbohydrate polymers, as they are part of an intricate and stable structure. Therefore, lignocellulosic materials first need to be pretreated, as this unit operation disturbs its stable structure, and facilitates cellulase enzymatic activity, which is critical for the hydrolysis of carbohydrate polymers into their monomeric moieties (Mosier et al., 2005; Wyman, 1999; Agbor et al., 2011).

Several pretreatment techniques have been developed to reduce the recalcitrance of cellulosic fractions to enzyme saccharification. Pretreatment techniques can be classified as physical, chemical, or a combination of both (Mosier et al., 2005; Kumar et al., 2009). Physical pretreatments open the plant cell walls and minimize cellulose crystallinity, which is reported to

be a significant factor that confers the resistance of cellulose to saccharification (Mansfield et al., 1999). Physicochemical pretreatments, such as steam explosion, rely on the sudden change in pressure to cause an explosive decompression of the biomass fiber. Physicochemical pretreatments partially remove hemicellulose and lignin, and increase accessible surface area. Chemical pretreatments use a chemical catalyst to either hydrolyze the hemicellulosic or lignin fractions of the biomass, exposing cellulose to enzymatic attack. Chemical catalysts that are most frequently used are dilute acid, lime (calcium hydroxide) and ammonia (Mosier et al., 2005; Kumar et al., 2009).

Among the leading pretreatment techniques, dilute acid pretreatment (DAP) has gained popularity due to its efficacy and its resulting high monomeric sugar yields on a wide range of lignocellulosic materials (Mosier et al., 2005; Agbor et al., 2011). DAP is usually conducted at moderate temperatures (120°C-200°C) for time periods ranging from 1-60 min, and using acid concentrations in the range of 0.5-2%. In general, DAP results in the hydrolysis of the hemicellulosic fraction, thereby exposing the cellulose fraction to enzymes, enhancing saccharification (Mosier et al., 2005; Kumar et al., 2009). Although hydrolysis of hemicellulose during DAP facilitates access to the cellulose fraction of the biomass, it also exposes the resulting xylose to the presence of acid, which can lead to its degradation, especially at elevated temperature (Kumar and Wyman, 2008; Esteghlalian et al., 1997; Lau et al., 2015). In addition, premature hydrolysis of the amorphous surface of the cellulose fraction to glucose can also occur during DAP, equaling exposing glucose to degradation processes (Shi et al., 2011; Mosier et al., 2005; Agbor et al., 2011; Xiang et al., 2004). Degradation of xylose and glucose during DAP is highly undesirable because it reduces the yield of fermentable sugars and produces compounds

that are inhibitory to enzyme saccharification and fermentation organisms (Cantarella et al., 2004; Palmqvist and Hahn-Hägerdal, 2000).

Switchgrass (*Panicum virgatum*) is a C4 perennial, warm-season grass, which grows naturally in North America and Canada (Parrish and Finke, 2005). As a forage crop, switchgrass has documented uses as an animal feed, as well as for soil conservation and ornamental purposes. In the past 30 years, the US Department of Energy has recognized the potential of switchgrass to become an important herbaceous energy crop that could be used for fuels and chemical production; its potential is mainly due to its high productivity across a wide geographic range, low resource requirement, ability to grow on marginal quality land, and resistance to severe weather (Sanderson et al., 1996; McLaughlin and Kzos, 2005; Parrish and Finke, 2005).

Numerous studies have been reported on the hydrolysis of hemicellulose and cellulose from switchgrass using DAP and enzymatic saccharification. The Biomass Refining Consortium for Applied Fundamentals and Innovation (CAFI) reported that xylose and glucose yields resulting from DAP and enzyme sacchirification of switchgrass were competitively similar to sugar yields stemming from switchgrass pretreated with other leading pretreatment techniques (Wyman et al., 2011). Kinetic models of xylose formation and degradation during DAP of switchgrass have been developed at different temperatures and acid concentrations. While Morinelly et al. (2009) and Yat et al. (2008) concluded that xylose concentration is highly dependent on DAP temperature and acid concentration, Esteghlalian et al. (1997) only observed an acid effect at elevated temperature. Using switchgrass as a feedstock, process temperature, acid concentration, and time have been reported to also have an impact on the combined xylose and glucose yields from DAP and enzymatic hydrolysis (Jensen et al., 2010; Shi et al., 2011).

Although there is a large body of work that highlights the impact of individual DAP

parameters on sugar recovery from switchgrass, there is a gap in knowledge with respect to the interaction between DAP factors on sugar yields. Additionally, comparatively little attention has been given to the impact of DAP conditions on glucose formation and ensuing degradation products during DAP of switchgrass. This could be justified by the fact that cellulose hydrolysis is not the major reaction that takes place during DAP. However, it is important to acknowledge that some cellulose hydrolysis does occur during DAP, especially at conditions necessary to produce digestible biomass and high combined xylose and glucose concentrations (Jensen et al., 2010; Lloyd and Wyman, 2005; Shi et al., 2011).

This study examined the impact of DAP parameters on resulting concentrations of xylose and glucose in pretreatment hydrolyzates, as well as the digestibility of the ensuing pretreated switchgrass. A 3x4x2 full factorial designed was used; experimental factors were temperature, time, and acid concentration.

2. Materials and Method

2.1. Raw biomass

Switchgrass (*Panicum virgatum*), specifically the Alamo cultivar, was used for this study. The biomass was harvested on July 4th 2009, one year after being planted at the University of Arkansas Agricultural Research and Extension Center in Fayetteville, AR (36.0625° N, 94.1572° W). The collected biomass was air-dried and ground to pass through a 20-mesh screen with a Thomas Willey® mini mill (Swedesboro, NJ). Ground biomass was stored at 4°C in sealed containers until used for compositional analysis and dilute acid pretreatment (DAP).

2.2. Compositional analysis

The mass of extractives, structural carbohydrates, and acid insoluble lignin (AIL) contained in raw biomass was established by protocols from the National Renewable and Energy

Laboratory (NREL) (Golden, CO) (Sluiter et al., 2008 a,b). All determinations were done in duplicate to yield average results and standard deviations presented in Table 1. Glucan content in dilute acid pretreated switchgrass was determined using the same protocol (Sluiter et al., 2008b).

2.3. Dilute acid pretreatment (DAP)

Raw switchgrass was pretreated with dilute H_2SO_4 in a 1-L Parr 4525 reactor (Moline, IL). Twenty-four different combinations of pretreatment temperatures, time, and acid concentration were used, as listed in Table 2. Values for pretreatment temperature, time, and acid concentration were chosen based on pretreatment conditions commonly used in literature for switchgrass (Jensen et al., 2010; Lloyd and Wyman, 2005; Shi et al., 2011; Estghlalian et al., 1997; Saha et al., 2005). Biomass and dilute acid were loaded in the reactor at a 1:10 ratio and agitation in the reactor was set at 144 RPM. Biomass and the acid mixture were heated to the desired temperature for 10-15 min. The recording of pretreatment time started after desired temperature was reached. Reaction was stopped by circulation of cold water through a cooling coil until the reactor reached a safe handling temperature. The ensuing slurry was then separated by vacuum filtration into a solid and liquid portion through a perforated Büchner funnel lined with a Whatman N° 1 filter paper. The liquid hydrolyzate (prehydrolyzate) was stored at -20°C until used for analysis. The solid hydrolyzate (pretreated switchgrass) was washed at room temperature with Millipore filtered water. Washed pretreated switchgrass was then separated by vacuum filtration through a perforated Büchner funnel lined with a Whatman N° 1 filter paper and stored at -20°C until needed for compositional analysis and enzyme saccharification. All pretreatment experiments were duplicated.

2.4. Enzyme saccharification of pretreated switchgrass

Glucan contained in pretreated switchgrass was hydrolyzed with Accelerase 1500®, an

industrial enzyme cocktail generously donated by DuPont Industrial Biosciences (Cedar Rapids, IA). Total cellulase activity of the enzyme cocktail was determined to be 25 FPU/mL based on the IUPAC protocol (Ghose, 1987). Enzyme saccharification assay was adapted from the NREL protocol described by Selig et al. (2008), except that assay was not supplemented by addition of antibiotic, β-glucosidase, or xylanase. Saccharification was conducted for 48 h in an agitated water bath (Thermo Scientific, Nashville, TN, U.S.) set at 50°C and 100 RPM. The resulting slurry was centrifuged at 1286 *g* for 2 min (IEC Spinette centrifuge, Needham, MA); the liquid fraction (enzymatic hydrolyzate) was collected and stored at -20°C until further analysis. Assays were performed in duplicate.

2.5. Characterization of liquid hydrolyzate

Monosaccharides in liquid hydrolyzate from compositional analysis and DAP were analyzed with a Waters 2695 Separations module (Milford, MA) equipped with a Shodex precolumn (SP-G, 8 μm, 6 x 50 mm) and Shodex column (SP0810, 8 μm x 300 mm). Millipore filtered water (0.2 mL/min) was the mobile phase; the column was heated to 85˚C with an external heater. Sugars were detected with a Waters 2414 Refractive Index Detector (Milford, MA). Hydrolyzates were neutralized with either calcium carbonate or ammonium hydroxide and filtered through a 0.2 μm pore filter prior to analysis.

Furfural and HMF in the prehydrolyzate were analyzed with a Waters 2695 Separations module equipped with a Bio-Rad Aminex HPX-87H Ion Exclusion 7.8 mm X 30 mm column, heated to 55˚C. The mobile phase was 0.005 M H_2SO_4 flowing at 0.6 mL/min. Compounds were detected with a UV index using the Waters 2996 Photodiode Array detector set at 210 nm. Glucose in enzymatic hydrolyzates was detected with a glucose analyzer, YSI 2900 (YSI Inc., Yellow spring, OH) as described by Mohanram et al. (2015).

2.6. Statistical Analysis

JMP Pro 11 from SAS Institute was used to develop a 3x4x2 full factorial design. The factors of interest were temperature (X_1), time (X_2) and acid concentration (X_3). The levels for each factors were: X_1 = 140 °C, 160 °C, 180 °C; X_2 = 10, 20, 30, 40 min; X_3 = 0.5% and 1%. The response variables investigated were the concentration of xylose, glucose in the prehydrolyzate, glucan content, and digestibility as a percentage of the pretreated switchgrass. Experimentally obtained values for the response variables were subjected to a linear regression analysis to determine main and interaction effects of factors using the Least Square method. Models were validated with an analysis of variance. The general form of the linear models obtained is given in Equation (1). Significance for any statistical results was established for *p*-value < 0.05.

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 \left(\frac{x_3 - 0.75}{0.25}\right) + \beta_4 (x_1 - 160) * (x_2 - 25) + \beta_5 (x_1 - 160) * \left(\frac{x_3 - 0.75}{0.25}\right) + \beta_6 (x_2 - 25) * \left(\frac{x_3 - 0.75}{0.25}\right) + \beta_7 * (x_1 - 160) * (x_2 - 25) * \left(\frac{x_3 - 0.75}{0.25}\right) \quad (1)$$

y = response variable

$\beta_{1\text{-}7}$ = regression coefficient

x_1 = pretreatment temperature

x_2 = pretreatment time

x_3 = sulfuric acid concentration of pretreatment

3. Results and Discussion

3.1. Effects of dilute acid pretreatment conditions on chemical composition of prehydrolyzate

The analysis of dilute acid prehydrolyzate produced from switchgrass showed that the composition of the prehydrolyzate determined in this work was similar to typical dilute acid

prehydrolyzates previously reported in the literature (Djioleu et al., 2014; Jensen et al., 2010; Shi et al., 2011). As expected, xylose and glucose were the main fermentable sugars present in the prehydrolyzate. Furans, such as HMF and FF, were also detected. Xylose resulted from the hydrolysis of the biomass xylan fraction, while glucose was the product of hydrolysis of biomass non-structural carbohydrate or cellulose fractions, as shown in Table 1. Both xylose and glucose could further degrade into FF and HMF, respectively (Palmqvist and Hahn-Hägerdal, 2000).

The concentrations of the compounds in the prehydrolyzate were dependent on the DAP conditions. Figure 1 shows how the concentrations of xylose, glucose, FF and HMF varied with pretreatment temperature and time when the acid concentration was maintained at 0.5% (v/v). Prehydrolyzate concentrations of, glucose, furfural, and HMF increased as the pretreatment temperature increased, whereas xylose concentration declined with increasing pretreatment temperature. At a 0.5% (v/v) acid concentration, the maximum xylose concentration was 21.7 g/L and was obtained when the switchgrass was pretreated at 140°C for 20 min. When using a 0.5% (v/v) acid concentration, a pretreatment time of 40 min yielded the lowest xylose concentrations for all examined pretreatment temperatures; the xylose concentrations dropped from 13.9 g/L to nearly zero when the pretreatment temperature was increased from 140°C to 180°C.

The decrease of xylose in the prehydrolyzate was attributed to its degradation to furfural; in this study, furfural concentrations were observed to increase when pretreatment temperatures were raised. This observation mirrored findings reported in other investigations of DAP on biomass (Kamireddy et al., 2014; Noureddini and Byun, 2010). Surprisingly, the highest furfural concentration, 7.14 g/L, was obtained at a temperature of 180°C and a pretreatment time of 30 min, which was not at the condition where the lowest xylose concentration was recorded, 5.17

g/L. This observation indicated that furfural vanished during pretreatment, especially when pretreatment times and temperatures were longer than 30 min and above 160°C. Disappearance of furfural in the prehydrolyzate could be attributed to its degradation to formic acid (Palmqvist and Hahn-Hägerdal, 2000). Formic acid was detected in the prehydrolyzate; however, its concentration was not tracked in this study. A pretreatment time of 10 min yielded the lowest furfural concentration at all temperatures. Contrary to what was observed at a 40 min pretreatment time, low furfural concentrations at 10 min were mainly due to slow furfural formation, instead of fast degradation because of reported low sugar degradation at such mild conditions (Noureddini and Byun, 2010; Esteghlalian et al., 1997; Morinelly et al., 2009).

The presence of glucose in the prehydrolyzate indicated that hydrolysis of the cellulose fraction occurred during pretreatment. Hydrolysis of non-structural cellulose, the amorphous portion of the structural cellulose, has been reported to occur under acidic conditions, especially when severe temperatures are used (Mansfield et al., 1999; Kumar et al., 2009). Overall, the accumulation of glucose in the prehydrolyzate followed identical trends no matter how long the biomass was pretreated. However, at a pretreatment temperature of 180°C and time of 10 min, the glucose concentration was 1.52 g/L, which was significantly lower than 10 g/L, the average concentration obtained at 20, 30 and 40 min. It was important to note that increasing temperature affected glucose concentration differently. For example, at a pretreatment time of 40 min, an increase in temperature from 140°C to 160°C resulted in an increase in glucose concentration by 39.6%, while an increase in temperature from 160°C to 180°C increased glucose prehydrolyzate concentrations by 260.5%.

HMF is the direct degradation product of glucose (Palmqvist and Hahn-Hägerdal, 2000; Xiang et al., 2004). The fact that both glucose and HMF simultaneously increased with

temperature indicated that glucose was degraded as soon as it was released into the prehydrolyzate. This is a reason why it is important to prevent premature cellulose hydrolysis during DAP. The lowest glucose concentration of 1.52 g/L was obtained at 180°C, 10 min and the highest concentration of 11.24 g/L occurred at 180°C, 30 min.

The composition of switchgrass prehydrolyzates, with respect to pretreatment temperature and time at 1% (v/v) acid concentration, is presented in Fig. 2. The concentration of xylose varied with temperature and time in a similar manner as for the 0.5% (v/v) acid concentration experiments. Xylose degradation was more pronounced as temperature increased, resulting in its complete disappearance at 180°C, for all pretreatment times. In addition, when the pretreatment time was 40 min, a 20°C increase in temperature from 140°C to 160°C resulted in xylose concentration dropping by 75.2%, whereas a similar increase in temperature at a 0.5% (v/v) acid concentration resulted in only a 50% decrease.

The effect of pretreatment temperature and time on glucose and FF at a 1% (v/v) acid was different than that observed with 0.5% (v/v) acid. Glucose concentrations increased with temperature when the biomass was pretreated for less than 30 min; however, for pretreatments longer than 30 min, the glucose concentrations rose with temperature up to 160°C, but declined with further increases in temperature. The results in this work demonstrated that increasing acid concentrations intensified glucose disappearance. Interestingly, glucose disappearance could not be entirely attributed to its degradation to HMF, where HMF concentrations in prehydrolyzates obtained with a 1% (v/v) acid concentration were similar to those produced in 0.5% (v/v) acid. A simultaneous degradation of HMF would definitely explain this discrepancy. Glucose decreases in prehydrolyzates could also be due to the recombination of glucose with lignin, a phenomenon observed by Xiang et al. (2004) under acidic conditions and elevated temperature.

The amount of glucan that remained in the pretreated switchgrass ensuing from the 24 different combinations of DAP conditions was determined as a percentage of the pretreated biomass on a dry basis. Glucan contained in the 24 pretreated switchgrass was hydrolyzed with cellulose saccharifying enzymes. The glucose recovered from the enzymatic hydrolyzate was used to calculate the digestibility of the pretreated biomass as a percentage of the amount of glucan content in the pretreated biomass recovered as glucose in the enzymatic hydrolyzate. Glucan content and digestibility of the 24 pretreated switchgrass are presented in Fig. 3 and 4, respectively. Results in Fig. 3 show that for both acid concentrations, the glucan content in the pretreated biomass declined as the pretreatment temperature rose. When pretreatment was performed with 1% (v/v) acid, the pretreatment time did not affect the resulting glucan content. Cellulose degradation of biomass during DAP was more prominent at higher acid concentrations, especially at temperatures above 160°C. This could possibly explain why the glucan content of the pretreatment biomass decreased from 55% to near zero when the temperature was increased from 160°C to 180°C in 1% (v/v) acid. In sum, the decline in the glucan content of biomass pretreated at higher temperatures was correlated with increases in glucose concentrations in the prehydrolyzates.

Results in Fig. 4 illustrate how digestibility of pretreated biomass was affected by DAP conditions. Unfortunately, biomass pretreated at 180°C with 1% (v/v) acid was severely damaged; glucan content was very low (< 13%) and, as such, ensuing pretreated biomass at those conditions were not submitted to enzymatic hydrolysis. In general, switchgrass susceptibility to enzyme saccharification increased with pretreatment temperature. However, with a 1% acid concentration, biomass digestibility slightly decreased as the temperature increased when the pretreatment time was 40 min. These results indicate that prolonged pretreatment of switchgrass

at high temperature and high acid concentration could impede biomass digestibility. Such a decline in digestibility could be attributed to lignin re-deposition on the biomass. Selig et al. (2007) reported that lignin droplets form and re-deposit on biomass in acidic media at elevated temperatures, negatively affecting biomass saccharification by preventing adsorption of cellulase enzyme (Mansfield et al., 1999). Such phenomenon would also corroborate the unexpected decline in the glucose concentration reported earlier, particularly if lignin droplets could be characterized as glucose and lignin combinations. It is also important to appreciate that, although using harsher pretreatment temperatures significantly improved biomass digestibility, this unfortunately significantly reduced the amount of glucan that was available for saccharification. Consequently, higher digestibility may not necessarily correlate with higher glucose yields. Therefore, a better way to measure the impact of DAP conditions on pretreatment effectiveness might be to evaluate the impact on total sugar recovery from pretreatment and enzymatic hydrolysis (Lloyd and Wyman, 2005; Jensen et al., 2010). This strategy would ensure that maximum total fermentable sugars (xylose + glucose) are recovered, because analysis also showed that it was not possible to delineate DAP conditions that simultaneously optimized xylose and glucose yields. This is due to the fact that xylose concentrations and glucan content responded differently to processing conditions, as compared to glucose composition and digestibility.

3.2. Statistical impact of DAP conditions on xylose, glucose, glucan content and digestibility

The results presented in Fig. 1-4 showed that the composition of the dilute acid prehydrolyzate of switchgrass, as well as the glucan content and the digestibility of the pretreated biomass, depended on the DAP conditions. However, the significance of the effects of each individual DAP parameter or their interactions on the response variables was not clearly

established. A full factorial model, an experimental strategy where all factors of the experiments are simultaneously varied with all possible combinations of factors levels tested, allows for the determination of individual and interaction effects of factors on a response variable (Montgomery, 1984). Such a model was employed to estimate the influence of pretreatment temperature, time and acid concentration on xylose and glucose concentration in the prehydrolyzate, glucan content and digestibility of the pretreated biomass. Using the experimental data in Table 2, a linear regression was developed for each response variable. This regression analysis for digestibility did not include data obtained at 180°C because digestibility data on biomass pretreated at 180°C with 1% (v/v) acid were not available. Two-way and three-way interactions effects were also evaluated.

The general form of the linear center polynomial obtained from the regression analysis is shown in Equation 1. In a center polynomial, the parameter estimates for the model are centered and in the scale of the actual factor setting, as opposed to being orthogonally coded. The center polynomial was used to prevent interaction effects in overwhelming the main effects by ensuring that the test of main effects was independent of the test of interaction effects (JMP, Statistical Discovery from SAS). Regression models for xylose, glucose, glucan content and digestibility were validated with ANOVA, and the results are shown in Tables 3-6, respectively. All models were found significant, as their *p* -values were below 0.05. The goodness-of-fit of the models to the experimental data were measured with R-squared (R^2). R-squared values for the developed models were 94%, 73%, 86% and 86% for xylose, glucose, glucan content and digestibility, respectively. These values for R^2 indicate that the developed models could not explained at most 14% of the variation in the data, except for glucose where the fairly average R^2 could be justified by the difficulty in measuring its concentration in a dilute acid prehydrolyzate due to low

concentration. The main and interaction effects of DAP factors on xylose, glucose, glucan content and digestibility were estimated as the regression coefficients for the models developed for each response variables. Regression coefficients were validated with an ANOVA analysis as well; the values and *p*-values for the significant coefficients are presented in Table 7.

The results in Table 7 show that pretreatment temperature had the highest significant influence on all the DAP outputs. Pretreatment time was also a significant factor for xylose concentration in the prehydrolyzate. No interaction effect was detected for xylose. Surprisingly, acid concentration did not have a significant effect ($p > 0.05$) on xylose level in the prehydrolyzate. Xylose has been reported to undergo hydrolytic cleavage in acidic conditions (Kumar and Wyman, 2008; Yat et al, 2008); however, other investigators have observed that increases in acid did not promote xylose cleavage (Lau et al., 2015; Esteghlalian et al. 1997; Morinelly at al. 2009). Specifically, Esteghlalian et al. (1997) noticed that acid concentration decreased xylose concentration of switchgrass dilute acid prehydrolyzates only at pretreatment temperature greater than 180°C. Moreover, Lau et al. (2015) reported that the exponent coefficient (n= 0.002) of the xylose hydrolysis rate constant (k), with a general expression shown in Equation 2, was significantly low ($p > 0.05$), to the point that acid concentration did not have a significant effect on the hydrolysis rate of xylose derived from switchgrass hemicellulose oligomers. Morinelly et al. (2009) made a similar observation when switchgrass was pretreated with acid concentration ranging from 0.25-0.75 % at temperatures of 150-175 °C, attributing this lack of acid effect on xylose degradation to the neutralizing capacity of switchgrass generated by its ash and mineral content. It is important to appreciate that the lack of significant impact that acid had on xylose concentration in dilute acid prehydrolyzate is only for switchgrass. Other investigators have reported the inverse trend when determining the effect of DAP conditions on

xylose concentration from sunflower hulls (Kamireddy et al., 2014), rapeseed straw (Castro et al., 2011), and corncob (Cai et al., 2012).

$$K = A_0 C_a^n e^{-E/RT} \qquad (2)$$

where K = hydrolysis rate constant; C_a = acid concentration; R = gas constant; T = pretreatment temperature; E = activation energy; A_0 and n are kinetic parameters.

The results in Table 7 also show that, while pretreatment temperature and acid concentration were the main significant factors influencing glucan content in pretreated switchgrass, pretreatment temperature was the only main factor with a significant effect on switchgrass digestibility. Glucan content was significantly affected by the interaction between pretreatment temperature and acid concentration, whereas digestibility was significantly affected by the interaction between pretreatment temperature and time. Although glucose concentrations in the prehydrolyzates were not affected by pretreatment time, the overall yield from DAP and enzymatic saccharification could be affected, and this would be due to the fact that the two-way interaction between pretreatment time and temperature was a significant factor in maximizing biomass digestibility.

The effect of two-way interactions between experiment factors on a response variable can be displayed with a surface plot. A surface plot is a three-dimension graph illustrating how a variable change with respect to two other independent variables (Montgomery, 1984). Figure 5 presents the surface plots for the predicted xylose and glucose concentrations of switchgrass dilute acid prehydrolyzates, glucan content and digestibility of pretreated biomass. Projections of the surface plots were also displayed below the surface as contour plots (Fig. 5). With the exception of xylose, all of the surface plots were curved, indicating that factors interaction had a significant influence on the response of glucose, glucan content and digestibility (Montgomery,

1984). Increasing pretreatment temperature glucose concentration in prehydrolyzate, glucan content and digestibility of pretreated biomass. However, increasing acid concentration would cause glucose and glucan content to rise at low temperature but drop at high temperature. Similarly, digestibility would get better with prolong pretreatment at low temperature but diminish with time at high temperature.

4. Conclusion

The conversion of lignocellulosic biomass to fermentable sugars is laborious and cost demanding. Biomass pretreatment is an important unit operation in this process; it will determine the overall conversion efficiency. Therefore, it is very important to choose productive pretreatment techniques and conditions. Results in this work illustrated how DAP factors and their interactions impacted the conversion of switchgrass into fermentable sugars. Such an analysis could provide useful insights to make technoeconomic decisions when designing a conversion process for a biochemical refinery platform using switchgrass as the feedstock.

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Tables

Table 1: Composition of raw switchgrass (% dry weight)[a]

Ethanol extractive	4.79 ± 0.08
Glucan	35.69 ± 1.21
Xylan	24.17 ± 1.64
Arabinan	6.42 ± 0.69
AIL[b]	21.52 ± 2.01

[a]data are average and standard deviation of two replications
[b]Acid insoluble lignin

Table 2: experimental response variables for dilute acid pretreatment of switchgrass

Temp (°C)	Time (min)	Acid conc (%V)	Xylose (g/L)	Glucose (g/L)	Furfural (g/L)	HMF (g/L)	GPB (%)	DPB (%)
140	10	0.5	17.72	2.02	0.80	0.06	62.91	38.97
140	20	0.5	21.71	1.93	0.62	0.05	57.47	48.88
140	30	0.5	19.14	2.02	0.79	0.07	64.28	48.15
140	40	0.5	13.89	2.07	1.12	0.08	62.22	60.74
160	10	0.5	13.44	3.33	1.63	0.13	55.65	85.54
160	20	0.5	12.50	4.34	2.69	0.22	55.71	74.12
160	30	0.5	11.77	3.52	2.72	0.14	50.14	81.29
160	40	0.5	6.89	2.89	2.85	0.21	52.48	79.39
180	10	0.5	5.98	1.52	4.17	0.20	44.37	95.80
180	20	0.5	2.45	10.75	7.15	0.93	46.83	68.77
180	30	0.5	1.98	11.35	6.52	1.35	25.05	78.41
180	40	0.5	0.00	10.43	5.17	1.21	34.41	95.66
140	10	1	17.75	1.72	0.81	0.05	63.94	57.10
140	20	1	22.76	1.99	1.39	0.06	54.75	49.00
140	30	1	21.43	2.64	1.53	0.08	63.67	59.33
140	40	1	19.73	2.85	2.26	0.10	62.12	64.07
160	10	1	9.91	4.57	2.75	0.16	51.95	75.74
160	20	1	12.00	3.73	4.14	0.19	54.66	79.25
160	30	1	4.80	6.11	5.35	0.26	50.20	86.12
160	40	1	4.89	4.05	5.03	0.22	55.55	58.02
180	10	1	0.00	7.62	3.42	0.70	13.62	N/A
180	20	1	0.00	6.99	4.16	1.03	9.58	N/A
180	30	1	0.00	3.40	2.66	0.62	8.09	N/A
180	40	1	0.00	2.16	2.07	0.59	3.33	N/A

Temp = temperature; Conc = concentration; HMF = hydroxymethylfurfural; GPB = glucan in pretreated biomass; DPB = digestibility of pretreated biomass; N/A = not available

Table 3: Analysis of Variance for Xylose Regression

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	7	1376.1740	196.596	33.8495
Error	16	92.9273	5.808	**Prob > F**
C. Total	23	1469.1014		<.0001*

DF = degree of freedom

Table 4: Analysis of Variance for Glucose Regression

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	7	149.68582	21.3837	6.1661
Error	16	55.48682	3.4679	**Prob > F**
C. Total	23	205.17263		0.0013*

DF = degree of freedom

Table 5: Analysis of Variance for Glucan Content Regression

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	6	7441.8945	1240.32	17.3052
Error	17	1218.4388	71.67	**Prob > F**
C. Total	23	8660.3333		<.0001*

DF = degree of freedom

Table 6: Analysis of Variance for Digestibility

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	6	2894.7907	482.465	8.9572
Error	9	484.7678	53.863	**Prob > F**
C. Total	15	3379.5585		0.0022*

DF = degree of freedom

Table 7: Regression coefficient and P-values for prediction of xylose, glucose concentration in dilute acid prehydrolyzate of switchgrass, glucan content and digestibility of pretreated switchgrass

Terms	Coefficient Values				*p*-values			
	Xylose (g/L)	Glucose (g/L)	Glucan content (%)	Digestibility (%)	Xylose (g/L)	Glucose (g/L)	Glucan content (%)	Digestibility (%)
β_0	84.83	-14.58	202.52	-118.19	<.0001	0.0016	<.0001	0.0022
β_1	-0.45	0.12	-0.96	1.21	<.0001	0.0001	<.0001	0.0001
β_2	-0.12	NS	NS	NS	0.0168	NS	NS	NS
β_3	NS	NS	-5	NS		NS	0.0101	NS
β_4	NS	NS	NS	-0.04		NS	NS	0.0443
β_5	NS	NS	-0.36	NS		NS	0.0037	NS
β_6	NS	NS	NS	NS		NS	NS	NS
B_7	NS	-0.01	NS	NS		0.0074	NS	NS

NS = Not significant. $P > 0.05$

Figure captions

Figure 1 Effect of dilute acid pretreatment conditions on compounds concentration in switchgrass prehydrolyzate with 0.5% (v/v) H_2SO_4. HMF = hydroxymethylfurfural

Figure 2 Effect of dilute acid pretreatment conditions on compounds concentration in switchgrass prehydrolyzate with 1% (v/v) H_2SO_4. HMF = hydroxymethylfurfural

Figure 3 Effect of dilute acid pretreatment conditions on glucan content in pretreated switchgrass. (A) = 0.5% H_2SO_4, (B) = 1% H_2SO_4

Figure 4 Effect of dilute acid pretreatment conditions on digestibility of pretreated switchgrass. (A) = 0.5% H_2SO_4, (B) = 1% H_2SO_4

Figure 5 Surface plot of predicted xylose and glucose concentration in dilute acid prehydrolyzate of switchgrass, glucan content and digestibility of pretreated switchgrass. Pretreatment time for xylose, glucose, and glucan content was 25 min; acid concentration for digestibility was 0.75% (v/v).

Fig. 1

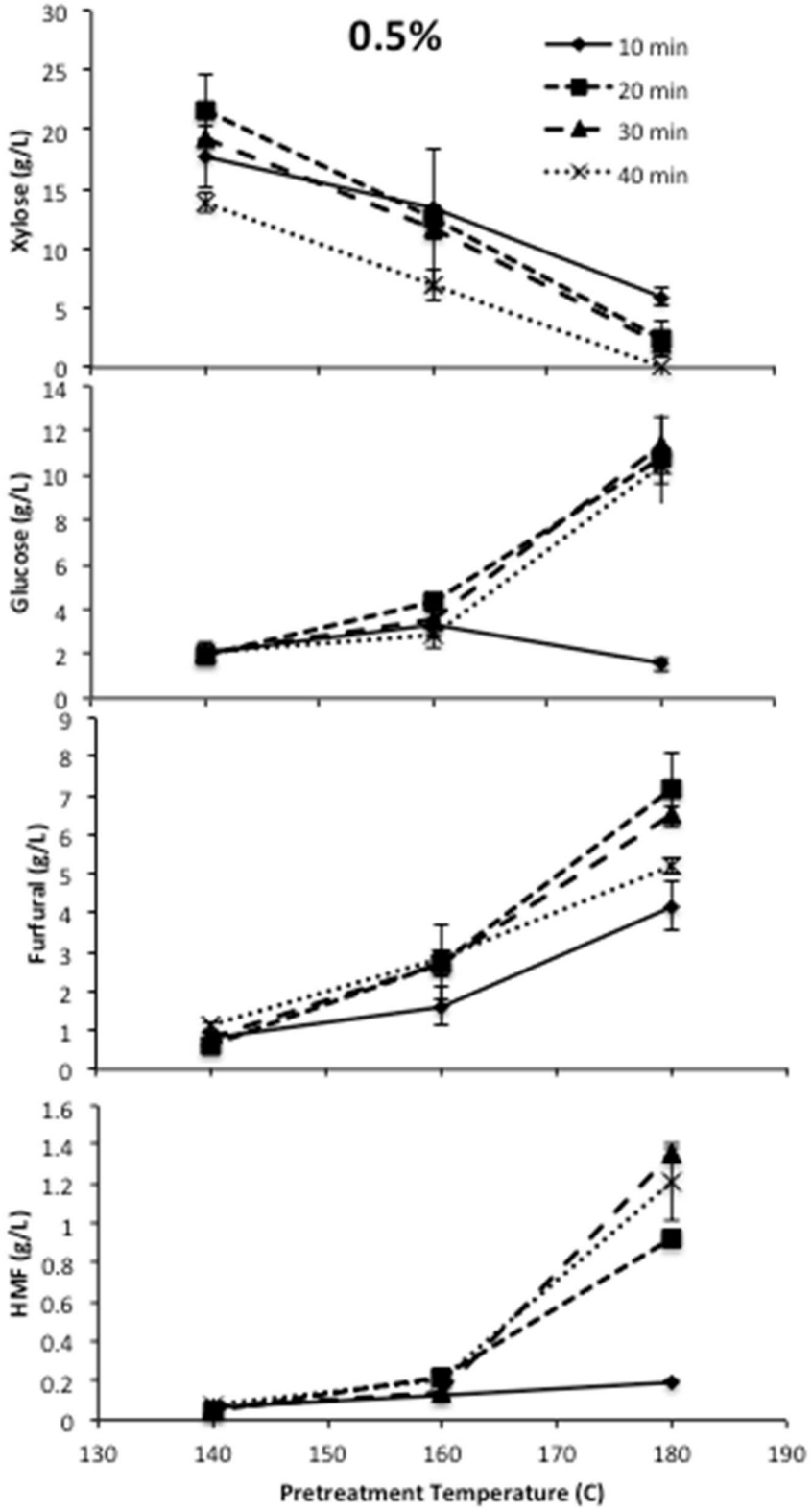

0.5%
10 min
20 min
30 min
40 min
Xylose (g/L)
Glucose (g/L)
Furfural (g/L)
HMF (g/L)
Pretreatment Temperature (C)

Fig. 2

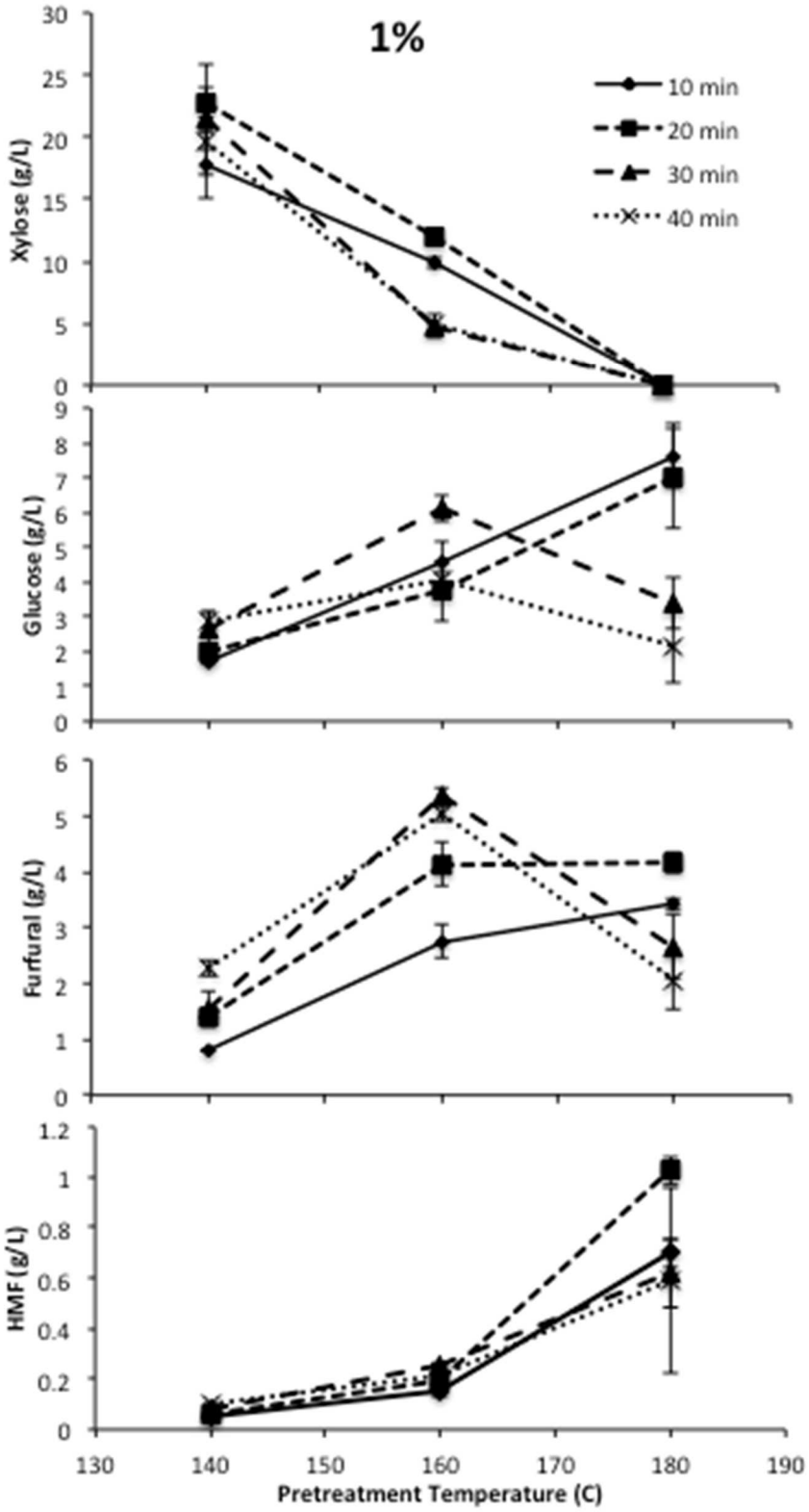

1%
10 min
20 min
30 min
40 min
Xylose (g/L)
Glucose (g/L)
Furfural (g/L)
HMF (g/L)
Pretreatment Temperature (C)

Fig. 3

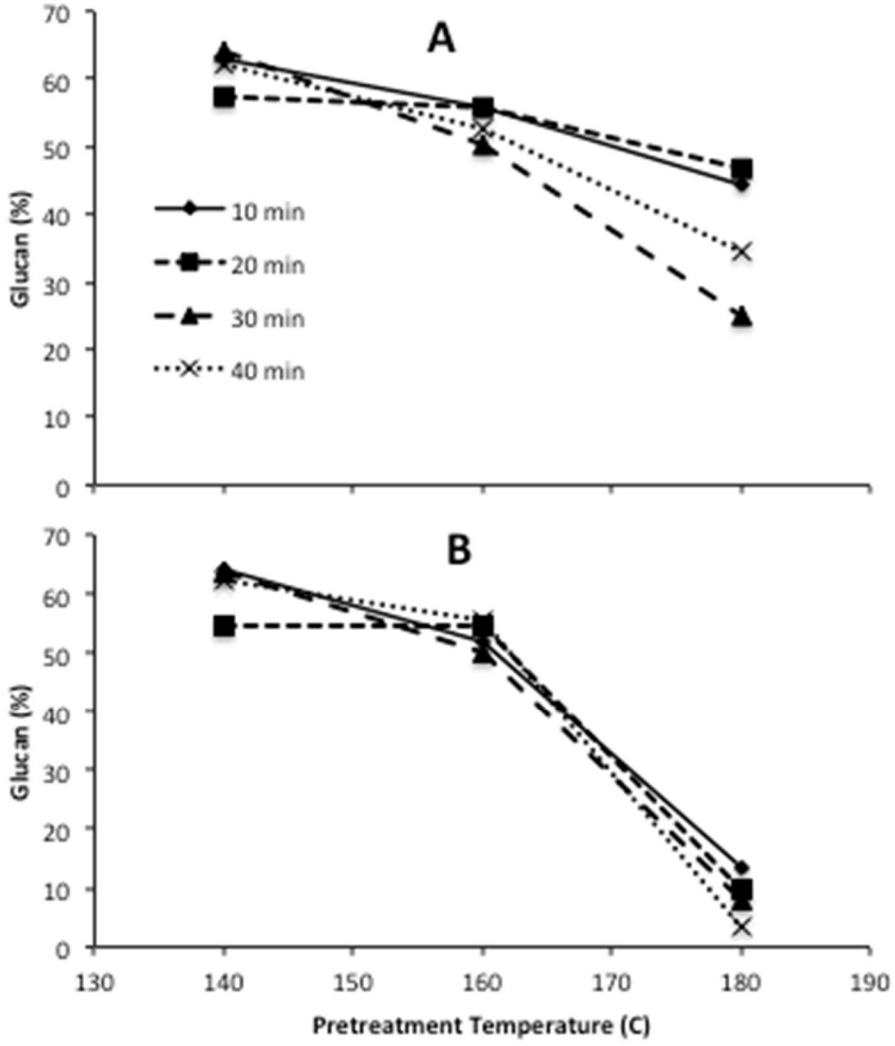

A
B
Glucan (%)
Glucan (%)
10 min
20 min
30 min
40 min
0
10
20
30
40
50
60
70
130
140
150
160
170
180
190
Pretreatment Temperature (C)

Fig. 4

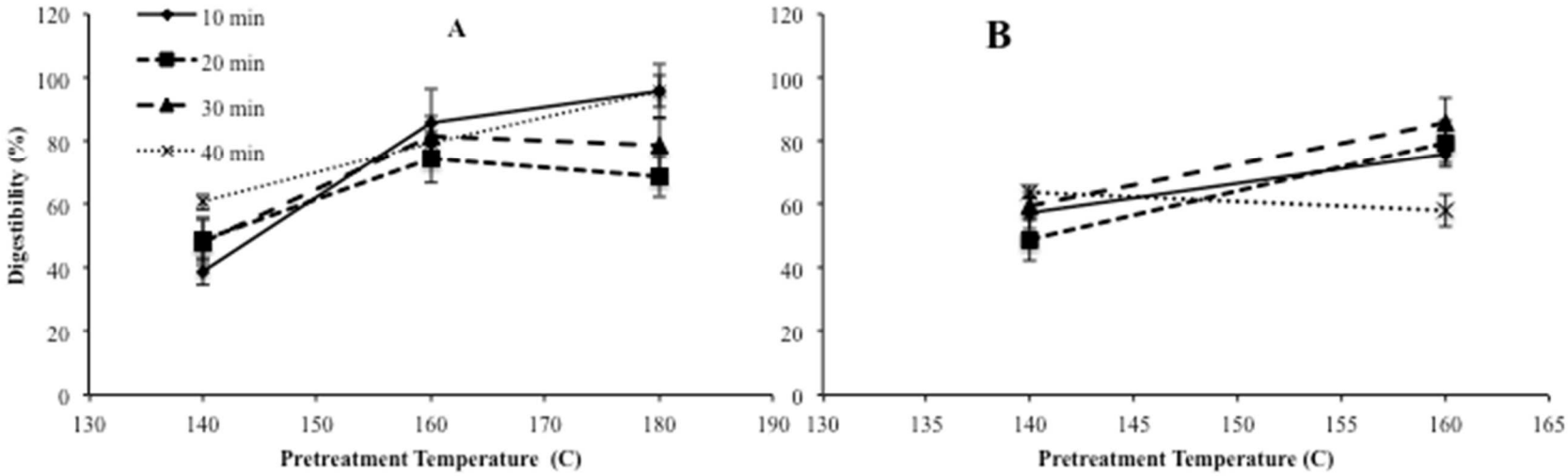
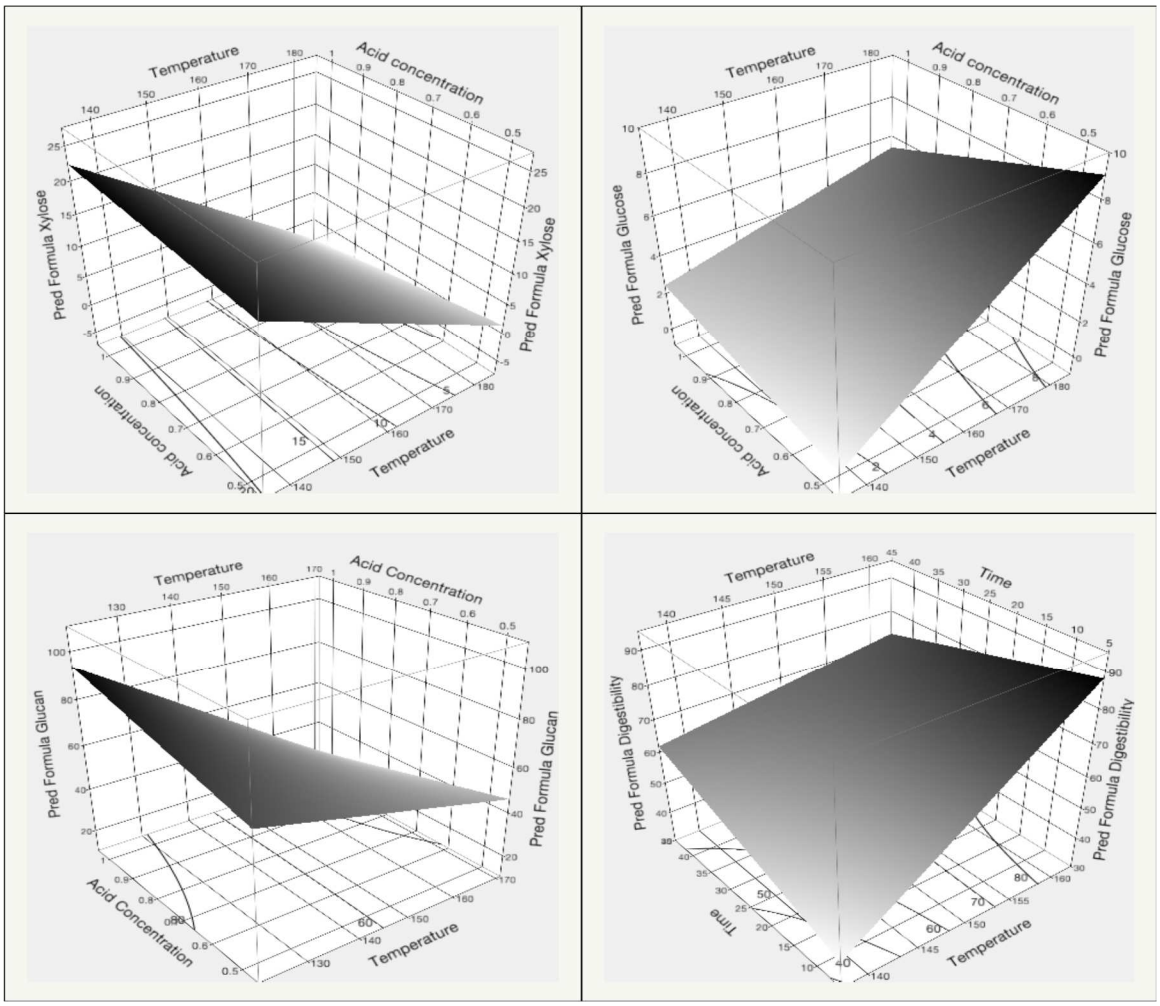

A
B
10 min
20 min
30 min
40 min
Digestibility (%)
Pretreatment Temperature (C)
Pretreatment Temperature (C)

Fig. 5


Temperature
Acid concentration
Pred Formula Xylose
Acid concentration
Temperature
Pred Formula Xylose
Temperature
Acid concentration
Pred Formula Glucose
Acid concentration
Temperature
Pred Formula Glucose
Temperature
Acid Concentration
Pred Formula Glucan
Acid Concentration
Temperature
Pred Formula Glucan
Temperature
Time
Pred Formula Digestibility
Time
Temperature
Pred Formula Digestibility

III. Inhibition of cellulolytic hydrolysis enzyme by switchgrass dilute sulfuric acid prehydrolyzates and ensuing centrifugal partition chromatography fractionated components

Abstract

Identification of compounds, produced during biomass pretreatment, that impede cellulose hydrolysis, would provide insights as to how to improve overall saccharification processes. This current study investigated the effect of switchgrass dilute acid prehydrolyzates on cellulose hydrolysis enzymes. Switchgrass prehydrolyzates were prepared using 24 different pretreatment conditions with temperatures varying from 140°C to 180°C, processing times ranging from 10 to 40 min, and sulfuric acid concentration fluctuating between 0.5% or 1% (v/v). Results showed that, when assayed against cellulose powder, cellobiose, and 4-Methylumbelliferyl β-D-cellobioside, activities of cellulase, β-glucosidase, and exoglucanase, respectively, were significantly reduced by switchgrass prehydrolyzates. Of the three tested enzymes, exoglucanase was the most sensitive with its activity reduced from 57.8% to 88.2%, as compared to controls. The inhibitory effect of switchgrass prehydrolyzates on β-glucosidase and on whole cellulase cocktail ranged from 31.8% to 62.5% and 16% to 41%, respectively. Statistical analysis showed that a significantly strong positive correlation between inhibition of β-glucosidase and concentration of glucose, acetic acid, and furans could be established. Similar results were also observed with exoglucanase inhibition and concentration of phenolic compounds and acetic acid. Inhibitory effect of compound groups identified in the prehydrolyzates and obtained from centrifugal partition chromatography (CPC) of prehydrolyzates prepared at 160°C, 30 min, and 1% acid, were tested against β-glucosidase and exoglucanase. Activity of β-glucosidase was impeded by fractions elevated in monomeric sugars,

while exoglucanase activity was suppressed to 94.7% of that of controls, by fractions rich in phenolic compounds. Moreover, time studies delineated that phenolic compounds deactivated exoglucanase enzymes, as end-product concentrations were consistently 2X higher in control experiments, even after 24 h of incubation time. These results confirm that phenolic-based compounds, which are released during dilute acid pretreatment, impede saccharification.

1. Introduction

Naturally abundant lignocellulosic biomass can be converted to fuels and chemicals, enhancing their sustainable production (Jørgensen et al., 2007; Alvira et al., 2010). Through biochemical processes, polymeric carbohydrate fractions of lignocellulosic biomass, especially cellulose, can be hydrolyzed into fermentable monomeric sugars, which can be converted to a plethora of bio-based products (Sun and Cheng, 2002). However, inherent lignocellulose characteristics impede its direct saccharification; this limitation can be overcome by pretreating the material prior to enzymatic hydrolysis (Sun and Cheng, 2002; Mosier et al., 2005; Kumar et al., 2009). Leading pretreatment techniques developed over the years improved lignocellulose saccharification by loosening plant cell wall-derived hemicellulose and lignin fractions, thereby increasing access to cellulose surface areas, while decreasing its crystallinity (Sun and Cheng, 2002; Mosier et al., 2005; Kumar et al., 2009). Unfortunately, pretreatment of lignocellulosic biomass generates by-products, which are known to be inhibitory to fermentation microorganisms (Palmqvist and Hahn-Hägerdal, 2000a,b; Klinke et al., 2004; Jönsson et al., 2013). Although exhaustive research has been conducted on inhibition of microorganisms used in fermentation by degradation compounds generated during pretreatment, underpinning inhibition mechanisms of the enzymatic system remains to be elucidated.

Cellulose is a homo-polysaccharide composed entirely of glucose linked by β -1,4-glucosidic bonds and with a degree of polymerization of up to 10 000 or higher. The linear structure of cellulose chains enables the formation of both intra- and intermolecular hydrogen bonds, resulting in the aggregation of chains into elementary crystalline fibrils of 36 cellulose chains (Jørgensen et al., 2007). Cellulose saccharification, in the biochemical conversion platform, is done with cellulose hydrolysis enzymes, which can be classified into three

categories. Cellulolytic enzyme categories include: 1) β-1,4- endoglucanases (EC 3.2.1.4), which hydrolyze internal β-1,4-glucosidic bonds randomly in the cellulose chain; 2) β -1-4- exoglucanase (cellobiohydrolase, EC 3.2.1.91), which attack the cellulose chain from reducing and non-reducing ends, cleaving off cellobiose units; and 3) 1,4-β -glucosidases (EC 3.2.1.21), which complete the hydrolytic process by catalyzing the hydrolysis of cellobiose residues to glucose. All these enzymes work synergistically to hydrolyze cellulose by creating new accessible sites and easing product inhibition (Mansfield et al., 1999; Jørgensen et al., 2007).

The nature and levels of degradation products produced during biomass pretreatment are dependent on the type of biomass, pretreatment techniques, and conditions used (Du et al., 2010). In general, pretreatment-generated by-products originate from complete or partial hydrolysis of hemicellulosic and lignin fractions. Like cellulose, hemicellulose is a polysaccharide, and, depending on the species, displays a xylan or a mannan backbone, which is decorated with sugars, such as glucose and arabinose (Jørgensen et al., 2007). Hydrolysis of hemicellulose results in the formation of xylose and xylooligomeres; xylose can further degrade into furfural and then to formic acid through a dehydration process. Due to the fact that hemicellulose is acetylated, acetic acid is released during its hydrolysis (Palmqvist and Hahn-Hägerdal, 2000b; Jørgensen et al., 2007). In contrast, lignin is a complex network formed by polymerization of phenyl propane units and constitutes the most abundant non-polysaccharide fraction of lignocellulose. The three most common lignin monomers are *p* -coumaryl alcohol, coniferyl alcohol and sinapyl alcohol, which are linked through ether bonds (Jørgensen et al., 2007). It is believed that lignin hydrolysis leads to the generation of phenolic compounds, which are detected in biomass prehydrolyzate (Palmqvist and Hahn-Hägerdal, 2000b). Furthermore

under acidic conditions, the recombination of lignin-generated-phenolic compounds with glucose has been reported (Olsson et al., 1978; Xiang et al., 2004).

Prior to saccharification, pretreated biomass is separated from the liquid prehydrolyzate and washed with colossal volumes of water to remove pretreatment degradation molecules. If not rinsed, pretreated biomass is not fully saccharified (Frederick et al., 2014; Rajan and Carrier, 2014a). The usage of such colossal amounts of water would not be economically feasible and environmentally friendly in large-scale biorefineries (Frederick et al., 2014; Rajan and Carrier, 2014a; Cantarella et al., 2004). Additionally, to improve and simplify overall biomass-to-fuels/chemicals conversions, it would be beneficial to supplement the output of saccharification processes with additional sugars derived from liquid prehydrolyzates and, if possible, to eliminate all together biomass rinsing steps (Tengborg et al., 2001). Therefore, identification of pretreatment degradation products that impede cellulose saccharification and determination of their mechanism of inhibition would be beneficial to biomass-to- fuels/chemicals conversion processes, as it would provide insight in designing process to mitigate their effects.

Recent investigations determined that by-products generated from pretreatment of agricultural residue or mixed hardwood, using leading pretreatment techniques, such as dilute acid, liquid hot water, and steam explosion pretreatment, inhibited cellulolytic enzyme activity (Kothari and Lee, 2011; García-Aparicio et al., 2006; Cantarella et al., 2004; Arora et al., 2012; Rajan and Carrier, 2014b; Ximenes et al., 2010, 2011; Kim et al., 2011, 2013). However, because these degradation compounds were present in liquid prehydrolyzates in the form of mixtures, requiring complex separation problems, enzymatic inhibition studies were conducted with crude prehydrolyzates or commercial reference compounds, as opposed to compounds derived from authentic prehydrolyzates. In addition, the inhibition action was evaluated on crude

commercial cellulase cocktail, rendering the determination of inhibition mechanisms on individual cellulolytic enzyme challenging (Mhlongo et al., 2015).

Centrifugal partition chromatography (CPC) is a technique that can be used to separate complex crude prehydrolyzates. It uses two immiscible liquids, one as mobile phase and the other as stationary phase, to fractionate or purify compounds (Berthod and Amstrong, 1988a,b). CPC benefits from multiple examples for the separation of phenolic compounds from plant natural extracts (Uppugundla et al., 2009; Abbott et al., 2010). Most recently, CPC has also been used to fractionate hydrolyzates generated from hemicellulose hydrolysis (Lau et al., 2013; Bunnell et al., 2015; Chen et al., 2015). This separation technique can be used to fractionate prehydrolyzates into specific compound groups, such that their inhibitory effect on saccharification enzymatic systems can be determined. The objective of this study was to determine the inhibitory effect of switchgrass dilute acid prehydrolyzate, as well as their fractionated compounds, on the cellulase enzyme cocktail, as well as on individual cellulolytic enzymes.

2. Materials and Methods

2.1. Liquid prehydrolyzates

Liquid prehydrolyzates were prepared by pretreating switchgrass with dilute sulfuric acid, using 24 different combinations of temperature, time, and acid concentration, as outlined in Chapter 2 of the thesis. All 24 pretreatments conditions are listed in Table 1. After pretreatment, pH of all prehydrolyzates was adjusted to 4.8 $\pm$ 0.03 with 5 N ammonium hydroxide from Sigma-Aldrich (St Louis, MO); pH was determined with a pH meter from Mettler Toledo (Columbus, OH). Adjusted prehydrolyzates were filtered through 0.45 μm PTFE membranes attached to syringe filters (VWR International, Radnor, PA). Filtered prehydrolyzates were

immediately placed at -20°C until they were used for characterization or in enzyme inhibition assays.

2.2. Centrifugal partition chromatography

2.2.1. Solvent preparation

HPLC grade butanol, methanol, and filtered water from a Direct-Q system (Millipore, Billerica, MA) with 18.2 MΩ resistivity, were used to prepare a biphasic solvent system as described in Lau et al. (2011). Butanol, methanol, and water were mixed in a 2-L separatory funnel at a ratio of 5:1:4 (v/v/v). Mixture was allowed to separate for at least 2 h into: 1) an upper organic rich phase and 2) a bottom aqueous phase. Each phase was carefully collected in 1-L glass bottle; the aqueous phase was used as the stationary phase while the organic phase was the mobile phase during the CPC run.

2.2.2. Sample preparation

Liquid prehydrolyzate (P15) prepared at 160°C, 30 min, and 1% acid was dried under pressure and no heat with a Savant SpeedVac Concentrator SPD 1010 (Thermo Scientific, Ashville, NC) set at 7 Torr. Drying was completed when solid content reached a constant weight. CPC sample was obtained by dissolving 2 g of solid from P15 prehydrolyzate in 5 mL of solvent organic phase and 5 mL of aqueous phase. CPC sample was filtered through 5 μm PTFE membranes.

2.2.3. CPC run

CPC sample was fractionated with a bench scale SCPC-250 system from Armen Instruments (Saint-Avé, France) equipped with a Prep-scale HPLC pump and controlled by Trilution® software (Gilson, Middleton, WI). Fractionation was based on methods from Lau et al. (2013) and Chen et al. (2015). In brief, the stationary phase (mostly water) was loaded at 10

mL/min in the rotor spinning at 500 RPM for 30 min. Rotor speed was then increased to 2300 RPM at which point the mobile phase (mostly butanol) was introduced in the rotor at 8 mL/min until equilibrium between the two immiscible phases was observed. At equilibrium, 110 mL of stationary phase and 140 mL of mobile phase were present in the 250-mL capacity rotor. Equilibrium was determined when the mobile phase started eluting from the rotor, and this occurred after approximately 22 min. After equilibrium, the sample was injected through a 10-mL sample loop. The fractionation process lasted 105 min after sample injection; fraction collection started 30 min after sample injection. Fractions were collected every minute, using a Foxy R1 (Teledyne Isco, Lincoln, NE) fraction collector. Eluent was monitored with a UV detector set at 280 and 300 nm. All the resulting fractions were dried in a SpeedVac and then reconstituted with 0.5 mL of filtered Millipore water for further analysis.

2.3. Characterization of liquid prehydrolyzates and CPC fractions

2.3.1. HPLC – analysis

Monosaccharides in prehydrolyzates were analyzed with a Waters 2695. Separations module (Milford, MA) equipped with a Shodex precolumn (SP-G, 8 μm, 6 x 50 mm) and Shodex column (SP0810, 8 μm x 300 mm). Millipore filtered water (0.2 mL/min) was the mobile phase; the column was heated to 85˚C with an external heater. Sugars were detected with a Waters 2414 Refractive Index Detector (Milford, MA).

Furans and aliphatic acids present in prehydrolyzate were analyzed with a Waters 2695 Separations module equipped with a Bio-Rad Aminex HPX-87H Ion Exclusion 7.8 mm X 30 mm column, heated to 55˚C. The mobile phase was 0.005 M H_2SO_4 flowing at 0.6 mL/min. Compounds were detected with a UV index using the Waters 2996 Photodiode Array detector set at 210 nm.

Monomeric phenolic compounds were analyzed with an Acquity Ultra Performance Liquid Chromatography (UPLC) system equipped with a BEH C18 (1.7 μm × 2.1 mm × 50 mm) analytical column and an Acquity VanGuard guard column (Waters, Milford, MA, U.S.). The mobile phases were: A) 0.1 % formic acid (v/v) in water and B) 100% methanol mixed in a gradient of 88.5:11.5 to 30:70, during a run time of 3.5 min. The column was heated to 50 °C and the samples were eluted at a flow rate of 0.4 mL/min. Compounds were detected using a photodiode array detector, set to 220, 267, 280 and 300 nm.

Compound concentrations were determined using linear calibration curved prepared with commercial standards. Commercial standards of glucose and arabinose were purchased from Alfa-Aesar (Ward Hill, MA); xylose, HMF, furfural, *p*-coumaric acid, *trans*-ferulic acid, syringaldehyde, 4-hydroxybenzoic acid, and vanillin were from TCI chemicals (Montgomeryville, PA); vanillic acid, acetic acid, and formic acid were obtained from Amresco (Solon, OH)

2.3.2. Folin-Ciocalteau (F-C) assay

The concentration of total monomeric phenolic (TP) compounds in the prehydrolyzates was determined according to a method developed by Ainsworth and Gillespie (2007). In brief, the sample was diluted with filtered water to 1 g/L of solid concentration. Aliquot of diluted sample (100 μL) was mixed with 200 μL of Folin Ciocalteau (F-C) reagent (Sigma-Aldrich, St Louis, MO). Mixture was incubated in the dark for 5 min before addition of 700 μL of 7.5% sodium carbonate (Sigma-Aldrich, St Louis, MO) solution. Final mixture was incubated in the dark, at room temperature for two hours. Absorbance was read at 765 nm with a spectrophotometer (Model 517601, Beckman Coulter Inc., Indianapolis, IN). Gallic acid standards of concentrations between 0.05 g/L and 1.25 g/L, treated exactly as samples were used

to prepare a linear standard curve. Concentrations of total phenolics of liquid prehydrolyzates were expressed as gallic acid equivalents (GAE).

2.4. Enzyme activities

Cellulase and β-glucosidase activities were determined according to the standards of the International Union of Pure and Applied Chemistry (IUPAC), as described in Ghose (1987). Accellerase® 1500, a cellulose hydrolysis enzyme cocktail, generously donated by Dupont Industrial Biosciences (Cedar Rapids, IA) was utilized for this investigation. Cellulase cocktail activity on filter paper was determined to be 25 FPU/mL.

β-glucosidase enzyme produced by *Aspergillus niger* was acquired from Megazyme (Wicklow, Ireland). β-glucosidase activity on cellobiose was determined to be 103 CBU/mL. Glucose released in the assays was measured with a glucose analyzer, YSI 2900 (YSI Inc., Yellow Springs, OH).

Exoglucanase enzyme produced by *Trichoderma reesei* was purchased from Infinite Enzyme (Jonesboro, AR). Its activity on 4-Methylumbelliferyl β-D-cellobioside (MUC) was 154 U/mL, as determined by the manufacturer. Exoglucanase activity was defined by the amount of enzyme necessary to release one micromole of 4-Methylumbelliferyl (MU) per minute (Boschker and Cappenberg, 1994). MU is the fluorescent substrate released during the hydrolytic cleavage of MUC. The substrate, MUC, was purchased from Sigma-Aldrich (St. Louis, MO).

2.5. Enzyme inhibition

All the 24 liquid prehydrolyzates and the consolidated CPC fractions (total of six) were individually used as treatment in enzyme inhibition assays. Consolidated CPC fractions were reconstituted with 50 mM citrate buffer; experimental control consisted of using solely buffer in the enzyme assay.

2.5.1. Cellulase

Microcrystalline cellulose powder (MP Biomedicals, Solon, OH), assay solvent, and cellulase enzyme were mixed in a 13 x 100 mm glass tube, such that a 2% cellulose solution was obtained. Assay solvent was either 50 mM citrate buffer (pH 4.8) or filtered liquid prehydrolyzates. Dosage of the cellulase enzyme in the assay was 15 FPU/g cellulose powder. Corresponding blanks, cellulose solution without enzyme, and enzyme + buffer, were also prepared in a similar manner. All samples were incubated for 60 min in a water bath (Thermo Electron Corporation) set at 50°C. Reaction was stopped by heating up samples in boiling water for 5 min, then transferring them to iced water prior to glucose analysis. Glucose was measured with a glucose analyzer, YSI 2900. The glucose released from hydrolysis of cellulose powder was calculated by subtracting the amount of glucose in blank samples from glucose in enzyme assay.

2.5.2. β-glucosidase

Cellobiose solutions of 15 mM were prepared by dissolving cellobiose (Sigma-Aldrich, St Louis, MO) in 50 mM citrate buffer (pH =4.8), in liquid prehydrolyzates (pH = 4.8), or CPC fraction. β-glucosidase enzyme was added to the cellobiose solution at a dosage of 40 CBU/g cellobiose. Corresponding assay blanks, cellobiose solution with no enzyme, and enzyme + buffer, were also prepared. All assays were incubated for 30 min in a water bath set at 50°C. Assay reaction was stopped and analyzed for glucose in a similar manner as in cellulase assay.

2.5.3. Exoglucanase

A 0.005 M substrate stock solution was prepared by dissolving 25 mg of MUC in 1 mL of dimethyl sulfoxide (Sigma-Aldrich, St Louis, MO) and 9 mL of 50 mM acetate buffer (Sigma-Aldrich, St Louis, MO) (pH = 5.0). Substrate solution (25 µL), diluted enzyme (8 µL), and assay

solvent (92 µL) were mixed in flat-bottom 96-wells microplate with lid (Corning®, Radnor, PA). Assay solvent was either 50 mM acetate buffer (pH 5.0) as control, liquid prehydrolyzates (pH 4.8) or CPC fractions as treatments. Diluted enzyme was obtained by diluting the original enzyme solution by a factor of 20X. Corresponding assay blanks, MUC solution with no enzyme, and enzyme + buffer, were also prepared. The 96-wells microplate was covered with a plate lid and incubated at 50°C in a water bath, for 60 min. Reaction was stop by mixing 25 µL of assay with 225 µL of 0.2 M sodium carbonate solution in a flat-bottom 96-wells black reading plates (FluoroNunc™, Fischer Scientific, Pittsburg, PA). Assay fluorescence was analyzed with a Synergy HT (BioTek Instruments, Winooski, VT) micro-well plate reader (excitation 360 nm and emission 460 nm). Fluorescence values of MUC hydrolysis were determined by subtracting fluorescence obtained for blank readings from those of the samples.

2.6. Statistical analysis

Enzyme activity inhibition experiments were performed in duplicate. Statistical analysis, such as ANOVA, Dunnett's control test, or Student' *t*-test were executed in JMP Pro 11 (SAS Institute). Significance for all the analysis was established for p-values < 0.05.

3. Results and Discussion

3.1. Liquid prehydrolyzate composition

The compositions of the 24 liquid prehydrolyzates (P1 to P24) are presented in Table 1. Prehydrolyzate compounds monitored included xylose, glucose, acetic acid, formic acid, hydroxymethylfurfural (HMF), and furfural. The concentrations of total phenolic compounds, expressed as GAE, were also determined. The concentration of identifiable compounds in the prehydrolyzate varied as a function of pretreatment conditions. For most of the prehydrolyzates, xylose was the compound with the highest concentration, with its maximum level of 21.90 g/L

registered in P6 (140°C, 20 min, 1% acid). Glucose concentration varied from 1.67 g/L to 9.86 g/L; the highest concentrations were found in prehydrolyzates prepared at 180°C. Highest concentrations for acetic acid, formic acid, HMF, and furfural were 11.04 g/L, 6.08 g/L, 1.02 g/L, and 4.01 g/L, respectively, and these concentrations were determined in P20, P15, P20, and P18 prehydrolyzates, respectively. Total phenolic concentrations were on average 1 g/L for the majority of the prehydrolyzates. Variations in glucose, xylose, acetic acid, formic acid, HMF, and furfural found in the 24 prehydrolyzates allowed for testing a wide range of concentrations on enzymatic hydrolysis inhibition.

3.2. Effect of switchgrass prehydrolyzates on activity of cellulose hydrolysis enzyme

3.2.1. Cellulase

Cellulase activity on microcrystalline cellulose powder was investigated in medium consisting of either citrate buffer, as control, or switchgrass prehydrolyzates, as treatments. As shown in Table 2, an analysis of variance showed that switchgrass prehydrolyzate composition significantly affected cellulase activity. Using the Dunnett's control test, glucose released from cellulose hydrolysis, in each of the 24 prehydrolyzates, was compared to that of the control. It was determined that glucose concentrations released from all treatments were significantly lower than those of the control. These results demonstrated that the 24 liquid prehydrolyzates were inhibitory to cellulase activity.

After establishing that switchgrass prehydrolyzates significantly inhibited cellulase activity, the magnitude of the inhibition from each treatment was calculated as shown in Equation (1).

$$Inhibition(\%) = \frac{Glucose_C - Glucose_P}{Glucose_C} \times 100 \qquad (1)$$

where $Glucose_C$ = glucose formed in control assay and $Glucose_p$ = glucose formed in treatment assay.

Inhibition percentages of cellulase activity by treatments were compared with a Student's *t*-test and significance was established for $\alpha = 0.05$ and *t*-value = 2.06. Figure 1 presents cellulase inhibition as a function of the 24 prehydrolyzates. Treatments displaying similar letters were not statistically different.

Cellulase activity was reduced by at least 16% when measured in prehydrolyzates. The highest inhibition percentage, calculated as 41%, occurred using P20; however, the effect from P20 was not statistically different than that observed from 12 other treatments. Most of the treatments affected cellulase activity in a similar manner ($p > 0.05$). Seventy five percent (18 out 24) of the prehydrolyzates had a statistically similar inhibition percentage between 29% and 39%. Of the 24 treatments, only P20 (41%), P4 (32.5%), P14 (23.5%), and P23 1(5.7%) were found to have distinct, significant different inhibition effects.

Using the composition and the inhibition percentage of P20, P4, P14, and P23, a regression analysis showed that there were no significant ($p > 0.05$) correlations between inhibition percentage and concentrations of identified compound in these prehydrolyzates. The fact that none of the identified compounds in the prehydrolyzates could be correlated to differences in cellulase inhibition possibly indicates that the important players, leading to cellulase inhibition, remain unidentified. On the other hand, it may be possible that cellulase inhibition could be the product of interactions among the identified compounds, and such interaction could not be measured in this investigation.

Inhibition of cellulase activity has been observed from degradation products that were contained in prehydrolyzates obtained from agricultural residue to mixed hardwood, using

leading pretreatment techniques, such as dilute acid, liquid hot water, and steam explosion pretreatment (Kothari and Lee, 2011; García-Aparicio et al., 2006; Cantarella et al., 2004; Arora et al., 2012; Rajan and Carrier, 2014b; Ximenes et al., 2010; Kim et al., 2011). Because degradation compounds are simultaneously present in prehydrolyzates, it is difficult to evaluate their individual contribution to inhibition of cellulase activity. Therefore, synthetic solutions containing commercially available degradation products have been used to investigate reduction in cellulase activity. Arora et al. (2012) observed that formic acid at 10 g/L reduced cellulase activity by 81% and 97% when activity was tested on cellulose powder and dilute acid pretreated poplar, respectively. Cantarella et al. (2004) showed that 11.5 g/L of formic acid deactivated cellulase during saccharification of steam-exploded poplar wood. Both investigators reported that furfural did not have a significant role in suppressing cellulase activity. While Kim et al. (2011; 2013), and Ximenes et al. (2010; 2011) showed that monomeric phenolic compounds did not significantly affect cellulase activity, they reported that polyphenolic compounds, such as tannins, were strong cellulase enzyme deactivators. García-Aparicio et al. (2006), Kothari and Lee, (2011) and Qing et al. (2010) demonstrated that hemicellulose-derived sugars, glucose, and cellobiose were potent cellulase inhibitors. Cellulase activity decreased by 80% after addition of 15 g/L of glucose; whereas addition of 20 g/L of xylose, arabinose, galactose, or mannose decreased enzyme activity by 35%, 13%, 11.5%, and 5% respectively (García-Aparicio et al., 2006). Qing et al. (2010) observed that 12.5 mg/ml of xylooligomers lowered initial hydrolysis rates of Avicel by 82% and the overall final hydrolysis yield by 38%.

Unfortunately, the results obtained in this work could not elucidate which specific compounds present in dilute acid prehydrolyzates were responsible for cellulase inhibition. As highlighted by Du et al. (2010), the establishment of a correlation between degradation products

present in biomass prehydrolyzate and cellulase inhibition depends upon the capability to identify these products. Nevertheless, using authentic prehydrolyzates, as opposed to synthetic solutions, has the advantage of not overestimating the inhibition effect of individual degradation products or erroneously attributing effects to specific compounds. This could justify García-Aparicio et al. (2006) and Kothari and Lee (2011) observing that summative inhibition effect from synthetic solutions are lesser than the effect from authentic prehydrolyzate, pointing to action of unidentified compounds.

3.2.2. β-glucosidase

Similar to cellulase inhibition studies, statistical impact of the prehydrolyzates on β-glucosidase activity were established with an ANOVA analysis, as shown in Table 3. A Dunnett's control test revealed that glucose formed in assays from all the treatments was significantly lower than that of the control. This difference highlighted the fact that β-glucosidase activity was significantly inhibited by liquid prehydrolyzates. The magnitude of β-glucosidase inhibition by switchgrass prehydrolyzate was calculated using Equation (1). Figure 2 presents β-glucosidase inhibition as a function of the 24 prehydrolyzates. Inhibition percentages from treatments were compared with Student's *t*-test; significance was determined for $\alpha = 0.05$ and *t*-value = 2.06. Treatments not connected by similar letters were significantly different.

As compared to cellulase activity, the 24 prehydrolyzates further affected the β-glucosidase system. The highest inhibition effect, 62.5%, was observed while testing P19, whereas P23 had the lowest inhibitory percentage of 31.8%. On the other hand, as for the cellulase system, P23 and P19 yielded the lowest and the highest inhibitory effect, respectively. As observed for cellulase activity, the range in inhibitor response (31.8% to 62.5%) was also narrow. Significant differences were observed between P19, P17, P22, P10, and P23

prehydrolyzates. The most statistically common inhibition effect of 39% was found in P10, as well as in 16 other prehydrolyzates. Prehydrolyzates P17, P22, and P23 inhibited β-glucosidase activity by 55.6%, 46.3%, and 31.8%, respectively.

Prehydrolyzates P19, P17, P22, P10, and P23 were used to determine if any linear correlation could be traced between β-glucosidase inhibition and compound concentrations. Compounds with significant ($p < 0.05$) linear relation are shown in Fig. 3. The strength of the linear relationship was measured with the Pearson correlation coefficient, and its squared value (R^2) corresponding to each compound is also presented in Fig. 3. Significance of Pearson coefficient was validated through ANOVA analysis. Results presented in Fig. 3 indicated that there was a strong, significant linear correlation between β-glucosidase inhibition and the concentration of: 1) glucose; 2) acetic acid, 3) HMF, and 4) furfural. Inhibition of β-glucosidase intensified with increases in glucose, acetic acid, HMF, and furfural concentrations. Glucose had the strongest ($R^2 = 0.94$) and the most significant ($p = 0.007$) correlation with β-glucosidase inhibition, followed by HMF with $R^2 = 0.92$ and $p = 0.010$. R-squared values for acetic acid and furfural were 0.89 and 0.86, respectively, with $p = 0.02$ for both compounds. Results in Fig. 3 point to the fact that glucose, HMF, acetic acid, and furfural are powerful and significant β-glucosidase inhibitors. There have been previous reports pertaining to β-glucosidase inhibition. Xiao et al. (2004) observed a 30% decrease in cellobiose hydrolysis when glucose concentrations were increased from 0 g/L to 20 g/L. Dekker et al. (1986) reported that, at glucose concentrations of 102 g/L, β-glucosidase activity was reduced by 37%; furthermore, hydrolysis times were increased from one hour to 22 hours when glucose concentrations were increased to the levels previously stated. Cantarella et al. (2004) reported that furfural and HMF at 2 g/L decreased β-

glucosidase activity by 15%. Mhlongo et al. (2015) determined that acetic and formic acids at 75 mM and 18.75 mM, respectively, strongly inhibited β-glucosidase.

3.2.3. Exoglucanase

Similar to cellulase and β-glucosidase inhibition studies, the effect that the 24 prehydrolyzates had on exoglucanase activity was determined. Results in Table 4 present the significant impact of the treatments on exoglucanase activity, as analyzed by ANOVA. The Dunnett's control test confirmed that all 24 prehydrolyzates significantly inhibited exoglucanase activity. The percentage inhibition of exoglucanase was determined as illustrated by Equation (1), where, instead of glucose, the differences in fluorescence were measured. Inhibition percentages from treatments were compared with Student's *t*-test; significance was determined for $\alpha = 0.05$ and *t*-value = 2.06. Figure 4 presents exoglucanase inhibition as a function of the 24 prehydrolyzates. Treatments connected with identical letters were not statistically different.

Of all the enzymatic systems tested in this study, the exoglucanase system was the most sensitive to inhibition produced by prehydrolyzates. The highest value of inhibition, 88.2%, was obtained with prehydrolyzate P1. Interestingly, the inhibition percentage of prehydrolyzate P1 was not statistically similar to that of P19, which had the highest inhibition effect on cellulase and β-glucosidase. The lowest inhibition percentage recorded for exoglucanase was 57.8% with prehydrolyzate P23; this prehydrolyzate also yielded the lowest inhibition values for the two other enzyme systems. Seven prehydrolyzates with distinct significantly different inhibition effects were identified as P1 (88.2%), P2 (83%), P3 (78.8%), P6 (74%), P5 (70.2%), P8 (63.1%), and P23 (57.8%). With the exception of P23, which resulted from switchgrass pretreated at 180°C, prehydrolyzates P1, P2, P3, P5, P6, and P8 were obtained at a pretreatment temperature of 140°C. Fifteen prehydrolyzates out of 24 inhibited exoglucanase activity by a

percentage of at least 68%. As for cellulase and β-glucosidase enzyme systems, the range representing exoglucanase inhibition was approximately ten percentage points.

Prehydrolyzates P1, P2, P3, P5, P6, P8, and P23 were used to determine if there were correlations between exoglucanase inhibition and prehydrolyzate-generated-compound concentrations. Results showed that only phenolic compounds, acetic acid, glucose, and furfural displayed a significant linear relationship with exoglucanase inhibition; results are presented in Fig. 5. The *p*-values were calculated as 0.01, 0.02, 0.03, and 0.03 for total phenolic compounds, acetic acid, glucose, and furfural, respectively. Total phenolic compounds and acetic acid exhibited marginally strong ($R^2 = 0.7$) and positive linear relationships with exoglucanase inhibition. A positive correlation reflected the fact that, as prehydrolyzate concentration of total phenolic compounds and acetic acid increased, so was inhibition to the exoglucanase system. Such results inferred that phenolic compounds and acetic acid are important factors in conferring inhibition in the exoglucanase system. On the other hand, glucose and furfural had an inverse relationship with exoglucanase inhibition, where inhibition decreased with increasing glucose and furfural concentrations. Caution should be exerted while interpreting this negative correlation, as it is possible that glucose and furfural could delay inhibition of exoglucanase activity.

Inhibition and deactivation of cellulase enzyme have been associated to polyphenolic molecules (Ximenes et al., 2010, 2011; Kim et al., 2011, 2013). Mhlongo et al. (2015) attributed such response to the sensitivity of exoglucanase enzyme present in the cellulase system. It was determined that polyphenols, such as tannic acid at 1.0 mM, would completely shut down exoglucanase activity (Mhlongo et al., 2015). Positive correlation between acetic acid concentration and exoglucanase inhibition observed in this work also paralleled findings in

Mhlongo et al. (2015) where a 53% drop in exoglucanase activity was observed when acetic acid concentration increased from 18.75 mM to 75 mM. On the other hand, results from Mhlongo et al. (2015) showed that increasing furfural concentration from 0.75 g/L to 3 g/L did not significantly change the magnitude of exoglucanase inhibition, which is in contrast with the results obtained in this study. Moreover, Mhlongo et al. (2015) reported that furfural at 3 g/L would suppress exoglucanase activity by 40%. The differences between this study and that of Mhlongo et al. (2015) could be attributed to the nature of the prehydrolyzate solutions, one being authentic, while the other being synthetic.

3.3. Centrifugal partition chromatography (CPC)

In order to further understand the inhibitory effect of degradation compounds produced during pretreatment on cellulolytic enzyme systems, a prehydrolyzate was fractionated by CPC. Prehydrolyzate P15, prepared at 160°C, 30 min, 1% H_2SO_4, was selected due to the fact that it impeded cellulase, β-glucosidase, and exoglucanase by 35.00% ± 0.03, 55.96% ± 0.03, and 68.52% ± 0.02, respectively (Figs. 1, 2, and 4). Moreover, pretreatment conditions employed to prepare P15 also yielded pretreated biomass with glucan content of 50.2% and digestibility of 86.12% ± 7.85 (See Chapter 2 of thesis). Because of these characteristics, P15 was deemed a good test candidate for CPC fractionation.

The CPC solvent system, butanol: methanol: water; 5:1:4 (v/v/v) (BMW), was developed by Lau et al. (2011). This solvent system has been successfully used to fractionate by CPC xylose oligomers derived from birchwood xylan (Lau et al., 2013), switchgrass hemicellulose (Bunnell et al., 2015), and miscanthus (Chen et al., 2015). The BMW solvent system was deemed appropriate for CPC fractionation of prehydrolyzate P15 due to its elevated monomeric sugars and furan content, as shown in Table 1.

A total of 44 fractions were obtained from the CPC fractionation of prehydrolyzate P15. An HPLC analysis of the collected fractions showed that some displayed similar chromatogram profiles, and hence, could be combined. The consolidation of the 44 CPC fractions resulted into the establishment of six major fractions (F1 to F6); their order of elution from the CPC process and composition are shown in Fig. 6 and Table 5, respectively. Monomeric phenolic compounds present in the prehydrolyzate eluted in the first 10 min of the CPC fractionation process and were represented by major fractions F1 and F2. Monomeric phenolic compounds identified in F1 and F2 included 4-hydroxybenzoic acid (HBA), vanillic acid (VA), syringaldehyde (SY), *p*-coumaric (PC), ferulic acid (FA), and salicylic acid (SA) with concentration, varying from 0.49 g/L to 0.05 g/L. The difference between F1 and F2 was essentially that concentrations of phenolic compounds were lower in F2 than that of F1, and that HBA and PC detected in F1 were not present in F2. Because of their concentration differences, F1 and F2 remained as two distinct fractions. Monomeric sugars and aliphatic acids eluted from the 39^{th} min to the 60^{th} min of the fractionation process, and were grouped as fractions F3 and F4. Specifically F3, which was obtained from fractions collected between 39 min and 46 min, contained glucose, xylose, arabinose, formic acid, and acetic acid at concentrations of 54.18 g/L, 15.42 g/L, 3.78 g/L, 12.11 g/L, and 3.2 g/L, respectively. F4, containing 11.07 g/L and 6.79 g/L of glucose, and formic acid, respectively, was collected between 46 min and 53 min. Traces of xylose were present in fractions F4 and F5 at average concentrations of 3.42 g/L ± 0.41. The highest concentration of arabinose was 10.36 g/L, and was obtained in fraction F5, which was collected between 54 min and 60 min. F6, collected between 61 min and 74 min, contained 0.30 g/L of xylose and 0.03 g/L arabinose. Although fraction F6 contained lower concentrations of carbohydrates than fractions F3, F4 and F5, its total solid concentration was 99.7 g/L, which, at this point, remained as

unidentified compounds. Interestingly furans, originally present in the liquid prehydrolyzate, were not recovered after the CPC process, and this was most likely due to the fact that they evaporated during sample preparation using vacuum drying.

With the exception of F3, identified compounds in CPC fractions F1, F2, F4, F5, and F6 represented at most 6% of the total solid content of the corresponding fraction. Known compounds in F3 constituted 39.85% of solid content. This indicated that there were numerous compounds in P15 that remained to be identified. Work from Du et al. (2010) reported that more than 40 degradation compounds, classified as aliphatic acids, aromatic acid, aldehyde and ketone, could be identified by high performance liquid chromatography in combination with UV spectroscopy or mass spectrometry detection in prehydrolyzate ensuing from biomass pretreatment with leading techniques. Such characterization could definitely improve the purity of the CPC fractions obtained in this investigation. Additionally, although the CPC process successfully isolate the phenolic molecules in F1 and F2, monomeric sugars and aliphatic acid co-eluted in F3. Therefore, testing for individual effect of these compound groups would not be possible.

3.4. Effect of CPC fractions on activity of cellulose hydrolysis enzyme

3.4.1. β-glucosidase

Individual CPC fractions, prepared at solid concentrations of 25 g/L, were used as treatments in the β-glucosidase assay using 15 mM of cellobiose. Control experiments consisted of cellobiose hydrolysis in citrate buffer. An analysis of variance (Table 6) showed that the amount of glucose formed during cellobiose hydrolysis was significantly influenced by the assay medium. Figure 7 reflects the response of cellobiose hydrolysis with respect to CPC fractions and control. Comparison of treatments with a Student's *t*-test showed that glucose released in

assays treated with fractions F2, F3, F4, F5, and F6 were significantly lower than that of the control, as indicated in Fig. 7 by treatments with different letter. These results indicated that fractions F2, F3, F4, F5, and F6 contained inhibitory compounds that impeded β-glucosidase activity. The fact that F1, which contained more monomeric phenolic compounds than F2, did not significantly impede β-glucosidase activity indicated that phenolics were not critical for inhibiting this enzyme system. Moreover, inhibitory effect from F2 was not significantly different than what was observed for F5 and F6, in which no monomeric phenolic compounds could be detected. The fractions F3, F4, F5, and F6 yielded statistically similar inhibitory effects of 56%, 56%, 50%, and 48%, respectively. Fractions F3, F4, F5, and F6 displayed higher monomeric sugar concentrations when compared to those of F1 and F2. Specifically, F3 and F4 contained higher concentrations of glucose and xylose, while F5 displayed higher concentrations of arabinose. Composition of F6 was not elucidated, where only traces of xylose and arabinose were detected, yet this fraction inhibited the β-glucosidase system enzyme with a magnitude similar to that of fractions F3, F4, F5, and F6. It is more than likely that the unidentified compounds of fraction F6 could be attributed to the observed β-glucosidase inhibition

Inhibition of β-glucosidase by sugar-rich fractions F3, F4, F5, and F6 corroborated the earlier results where strong positive correlation ($R^2 = 0.93$) was observed between glucose concentration and β-glucosidase inhibition. Similarly, insensitivity of β-glucosidase to F1, also confirmed the lack of correlation between phenolic compounds and inhibition of this enzyme system. Ximenes et al. (2010, 2011) attributed such insensitivity to the origin of the enzyme system, reporting that β-glucosidase from *A. niger* would only be affected by phenolics at concentrations 10X higher than those required to inhibit enzyme from *T. reesei.*

3.4.2. Exoglucanase

Fractions F1, F2, F3, F4, F5, and F6, prepared at a concentration of 25 g/L, were tested for their inhibitory effect on the exoglucanase system, using MUC as a substrate. The control assay was performed in 50 mM acetate buffer. Fluorescence intensity from the assay, due to the release of MU, was significantly impacted by assay medium, as indicated by an ANOVA analysis presented in Table 7. All CPC fractions significantly reduced exoglucanase activity, which was correlated by the fact that fluorescence intensities from CPC fractions were lower than the intensity obtained from the control (Fig. 8). Fractions F1 and F2 arrested exoglucanase activity by decreasing MUC hydrolysis by 94.67% and 90.87%, respectively, while fractions F3 to F6 inhibited the enzyme system by 64.22% to 72.84%. Such results indicated that exoglucanase was more sensible to phenolic compounds than any other degradation products in the liquid prehydrolyzate. Again, this finding corroborates the previously established positive correlation between phenolic compounds and exoglucanase inhibition.

3.5. Time effect on CPC enzyme inhibition

It has been reported in the literature that a decrease in cellulose hydrolysis rate during biomass saccharification could be due to deactivation of cellulolytic enzyme (Ximenes et al., 2010, 2011; Kim et al., 2011, 2013). Ximenes et al. (2011) defined enzyme deactivation as irreversible enzyme inhibition and determined that it occurred from prolong exposure of enzyme to pretreatment degradation products. Such effect was tested for β-glucosidase and exoglucanase, using CPC fractions from which they showed high sensitivity.

3.5.1. β-glucosidase

As fractions F3 to F6 strongly inhibited β-glucosidase activity, fraction F4 was selected to further determine the effect of reaction time on the cellobiose hydrolysis system. Within the

first 30 min of the assay and at a solid concentration of 15 g/L, fraction F4 reduced cellobiose hydrolysis (Fig 9A). Initial hydrolysis rate of cellobiose, determined by the slope of the linear relationship between glucose released by hydrolysis and reaction time, was twice that of when the assay was performed in citrate buffer. However, as the hydrolysis reaction progressed, β-glucosidase seemed to recover from the effect of fraction F4 and yielded almost an identical concentration of glucose (5 g/L) as that of the control in less than three hours (Fig. 9B).

3.5.2. Exoglucanase

Similarly, the phenol rich fraction, F1, prepared at a 4 g/L solid concentration, reduced initial hydrolysis rate of MUC by exoglucanase enzymes (Fig. 10A). The linear expression of the fluorescence intensity generated by the hydrolysis of MUC with respect to time, showed that MUC was hydrolyzed twice as fast in acetate buffer than in F1 solution, within the first 45 min of the reaction. Contrary to β-glucosidase, exoglucanase did not recover from the effect of F1. Fluorescence intensity of the control assay was always two times greater than that of the F1 treatment, even after 24 h of reaction (Fig. 10B). These results point to the fact that the deactivation of the cellulase cocktail, as reported in the literature, could be due to the deactivation of exoglucanase system in the cellulase cocktail.

4. Conclusion

Inhibition of cellulolytic enzyme by switchgrass dilute acid prehydrolyzate was studied using 24 different prehydrolyzates and CPC fractions of prehydrolyzate. This study established that monomeric sugars, as well as degradation compounds produced during dilute acid pretreatment, are detrimental to cellulolytic enzyme activity. The intensity of the inhibitory effect was dependent on the enzyme system, compounds nature, and concentration. Although not explicitly studied here, the origin of the enzyme system could also be a significant factor to

inhibition. The majority of prehydrolyzates displayed statistically similar inhibition effects on the activity of cellulolytic enzymes and phenolic compounds were the most detrimental constituents identified in the prehydrolyzates. In sum, any detoxification method to diminish inhibitory potential of dilute acid prehydrolyzates should reduce concentration of aromatic compounds in prehydrolyzate.

5. References

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Table 1: Composition of dilute acid prehydrolyzate of switchgrass obtained at different combinations of temperature, time, and sulfuric acid concentration

Samples	Temp (°C)	Time (min)	Acid Conc. (%V)	pH	Xylose (g/L)	Glucose (g/L)	Acetic Acid (g/L)	Formic Acid (g/L)	HMF (g/L)	Furfural (g/L)	TP (g/L)
P1	140	10	0.5	4.76	19.58	1.70	4.07	0.03	0.11	0.42	1.14
P2	140	20	0.5	4.85	19.71	1.87	4.50	1.97	0.12	0.68	1.22
P3	140	30	0.5	4.72	19.25	2.15	4.21	2.24	0.12	0.81	1.15
P4	140	40	0.5	4.75	14.44	1.78	3.40	2.07	0.11	0.68	0.77
P5	140	10	1	4.71	15.88	1.67	3.93	1.66	0.12	0.82	0.85
P6	140	20	1	4.79	21.94	1.89	4.34	1.45	0.12	0.85	1.00
P7	140	30	1	4.79	18.29	2.35	4.77	1.98	0.14	1.32	0.95
P8	140	40	1	4.77	20.27	2.61	2.72	3.66	0.14	1.65	0.85
P9	160	10	0.5	4.80	13.29	2.91	3.45	2.47	0.18	1.26	1.26
P10	160	20	0.5	4.75	3.85	4.37	3.04	2.12	0.21	2.45	0.82
P11	160	30	0.5	4.80	11.06	4.01	8.51	5.48	0.28	2.06	1.39
P12	160	40	0.5	4.78	5.99	2.41	2.03	1.55	0.22	1.74	1.00
P13	160	10	1	4.75	9.56	4.99	7.94	4.31	0.24	2.42	1.29
P14	160	20	1	4.80	11.64	3.11	3.58	2.60	0.20	1.79	1.03
P15	160	30	1	4.81	4.62	6.36	6.90	6.08	0.26	3.12	1.07
P16	160	40	1	4.81	5.57	3.88	7.06	5.94	0.19	2.83	0.75
P17	180	10	0.5	4.80	1.19	8.97	8.95	4.66	0.70	3.91	1.33
P18	180	20	0.5	4.77	1.37	6.79	9.56	4.58	0.63	4.01	1.41
P19	180	30	0.5	4.79	1.29	8.58	8.95	3.56	0.83	3.83	1.36
P20	180	40	0.5	4.79	1.84	9.86	11.04	4.25	1.02	3.50	1.51
P21	180	10	1	4.79	1.56	8.27	10.33	4.88	0.66	3.36	1.57
P22	180	20	1	4.79	1.33	5.97	7.08	3.77	0.51	2.07	1.35
P23	180	30	1	4.79	1.05	2.88	2.72	1.53	0.26	1.07	0.90
P24	180	40	1	4.76	0.00	2.92	10.35	4.46	0.33	1.68	1.77

Temp = temperature; conc = concentration, HMF = hydroxymethylfurfural; TP = total phenolic compounds as gallic acid equivalent

Table 2: Analysis of variance of the effect of switchgrass dilute sulfuric acid prehydrolyzates on the inhibition of cellulase initial hydrolysis rate of crystalline cellulose powder

Source	**DF**	**Sum of Squares**	**Mean Square**	**F Ratio**
Model	24	0.35483108	0.014785	10.8891
Error	25	0.03394361	0.001358	**Prob > F**
C. Total	49	0.38877469		<.0001*

DF = degree of freedom

Table 3: Analysis of variance of the effect of switchgrass dilute sulfuric acid prehydrolyzates on the inhibition of β-glucosidase initial hydrolysis rate of cellobiose

Source	**DF**	**Sum of Squares**	**Mean Square**	**F Ratio**
Model	24	0.63708074	0.026545	24.5110
Error	25	0.02707462	0.001083	**Prob > F**
C. Total	49	0.66415536		<.0001*

DF = degree of freedom

Table 4: Analysis of variance of the effect of switchgrass dilute sulfuric acid prehydrolyzates on the inhibition of exoglucanase initial hydrolysis rate of 4-Methylmebelliferyl cellobioside

Source	**DF**	**Sum of Squares**	**Mean Square**	**F Ratio**
Model	24	1.3109789	0.054624	155.0574
Error	25	0.0088071	0.000352	**Prob > F**
C. Total	49	1.3197860		<.0001*

DF= degree of freedom

Table 5: Composition of switchgrass dilute sulfuric acid prehydrolyzate fractions obtained by centrifugal partition chromatography (CPC)

CPC Fractions	Solid (g/L)	HBA (%)	VA (%)	V (%)	SY (%)	P-C (%)	FE (%)	SA (%)	GL (%)	XY (%)	AR (%)	FA (%)	AA (%)
F1	46.5	0.36	1.05	0.31	0.25	0.11	0.12	0.35					
F2	56.6		0.16	0.08	0.14		0.10	0.08					
F3	222.6								24.34	6.93	1.70	5.44	1.44
F4	404.15								2.74	0.92		1.68	
F5	198.1									1.58	5.23		
F6	99.7									0.37	0.03		

HBA = 4-Hydroxybenzoic Acid, VA = Vanillic Acid, V = Vanillin, SY = Syringaldehyde, P-C = *P*-Coumaric, FE = Ferulic Acid, SA= Salicylic Acid, GL = Glucose, XY = Xylose, AR = Arabinose, FA = Formic Acid, AA = Acetic Acid

Table 6: Analysis of variance of the effect of CPC fractions of switchgrass dilute sulfuric acid prehydrolyzate on the inhibition of β-glucosidase initial hydrolysis rate of cellobiose

Source	**DF**	**Sum of Squares**	**Mean Square**	**F Ratio**
Model	6	5.1701429	0.861690	20.0660
Error	7	0.3006000	0.042943	**Prob > F**
C. Total	13	5.4707429		0.0004*

DF= degree of freedom

Table 7: Analysis of variance of the effect of CPC fractions of switchgrass dilute sulfuric acid prehydrolyzate on the inhibition of exoglucanase initial hydrolysis rate of 4-MUC

Source	**DF**	**Sum of Squares**	**Mean Square**	**F Ratio**
Model	6	1355387.7	225898	183.4970
Error	7	8617.5	1231	**Prob > F**
C. Total	13	1364005.2		<.0001*

DF = degree of freedom

Figure captions

Figure 1 Effect of switchgrass dilute acid prehydrolyzates on cellulase activity. Prehydrolyzates were obtained at 24 different combinations of temperature (140°C, 160C, 180°C), time (10,20,30, 40 min) and H_2SO_4 concentration (0.5%, 1%). Data and error bars are means and standard deviation of 2 replications, respectively. Treatments not connected by the same letter are significantly different ($p < 0.05$)

Figure 2 Effects of switchgrass dilute acid prehydrolyzates on β-glucosidase activity. Prehydrolyzates were obtained at 24 different combinations of temperature (140°C, 160C, 180°C), time (10,20,30, 40 min) and H_2SO_4 concentration (0.5%, 1%). Data and error bars are means and standard deviation of 2 replications, respectively. Treatments not connected by the same letter are significantly different ($p < 0.05$)

Figure 3 Linear correlation between degradation products in switchgrass prehydrolyzates and inhibition of β-glucosidase activity

Figure 4 Effect of switchgrass dilute acid prehydrolyzates on exoglucanase activity. Prehydrolyzates were obtained at 24 different combinations of temperature (140°C, 160C, 180°C), time (10,20,30, 40 min) and H_2SO_4 concentration (0.5%, 1%). Data and error bars are means and standard deviation of 2 replications, respectively. Treatments not connected by the same letter are significantly different ($p < 0.05$)

Figure 5 Linear correlation between degradation products in switchgrass prehydrolyzates and inhibition of exoglucanase activity

Figure 6 Centrifugal partition chromatography profile of dilute sulfuric acid switchgrass prehydrolyzate

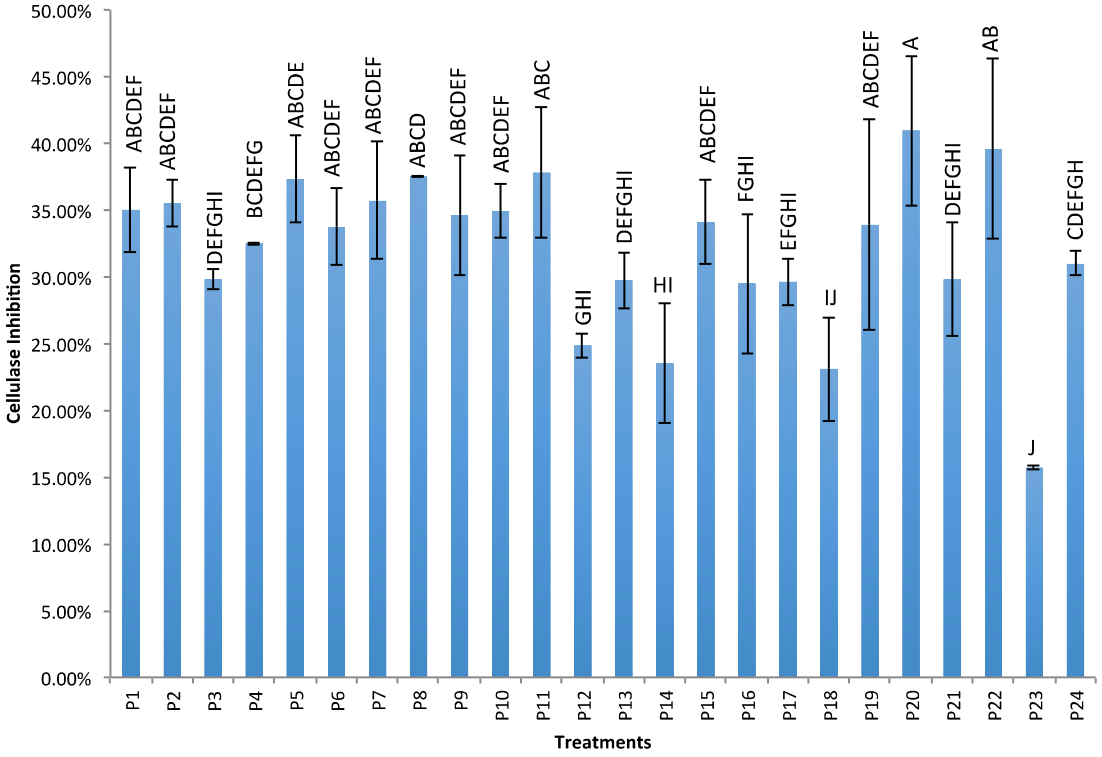
Figure 7 Effect of centrifugal partition chromatography (CPC) fractions of switchgrass dilute acid prehydrolyzate on β-glucosidase activity. C = control, F1-F6 = CPC fractions. Data and error bars are means and standard deviation of 2 replications, respectively. Treatments not connected by the same letter are not significantly different ($p < 0.05$)

Figure 8 Effect of centrifugal partition chromatography (CPC) fractions of switchgrass dilute acid prehydrolyzate on exoglucanase activity. C = control, F1-F6 = CPC fractions. Data and error bars are means and standard deviation of 2 replications, respectively. Treatments not connected by the same letter are not significantly different ($p < 0.05$)

Figure 9 Time effect on β-glucosidase inhibition by sugar-rich CPC fraction of switchgrass dilute sulfuric acid prehydrolyzate. CPC = centrifugal partition chromatography. Data and error bars are means and standard deviation of 2 replications, respectively

Figure 10 Time effect on exoglucanase inhibition by phenolic-rich CPC fraction of switchgrass dilute sulfuric acid prehydrolyzate. CPC = centrifugal partition chromatography. Data and error bars are means and standard deviation of 2 replications, respectively

Fig. 1


Cellulase Inhibition
50.00%
45.00%
40.00%
35.00%
30.00%
25.00%
20.00%
15.00%
10.00%
5.00%
0.00%
ABCDEF
ABCDEF
DEFGHI
BCDEFG
ABCDE
ABCDEF
ABCDEF
ABCD
ABCDEF
ABCDEF
ABC
GHI
DEFGHI
I
ABCDEF
FGHI
EFGHI
I
ABCDEF
A
DEFGHI
AB
J
CDEFGH
P1
P2
P3
P4
P5
P6
P7
P8
P9
P10
P11
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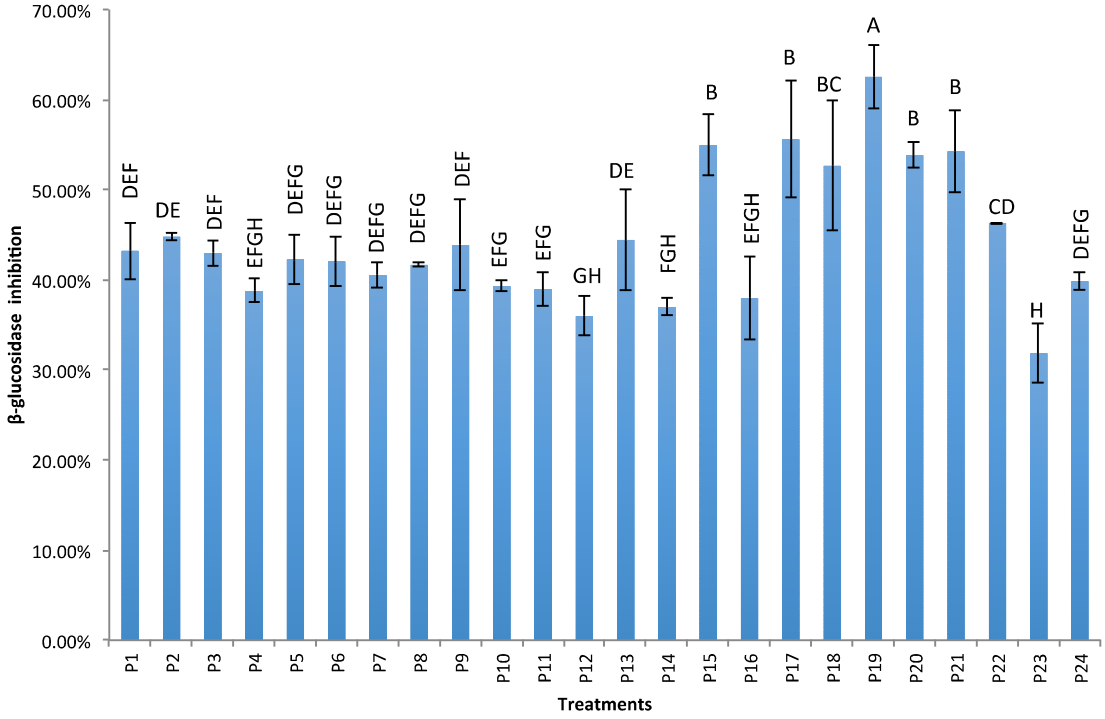
P23
P24
Treatments

Fig. 2


70.00%
60.00%
50.00%
40.00%
30.00%
20.00%
10.00%
0.00%
β-glucosidase inhibition
DEF
DE
DEF
EFGH
DEFG
DEFG
DEFG
DEFG
DEF
EFG
EFG
GH
DE
FGH
B
EFGH
B
BC
A
B
B
CD
H
DEFG
P1
P2
P3
P4
P5
P6
P7
P8
P9
P10
P11
P12
P13
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P16
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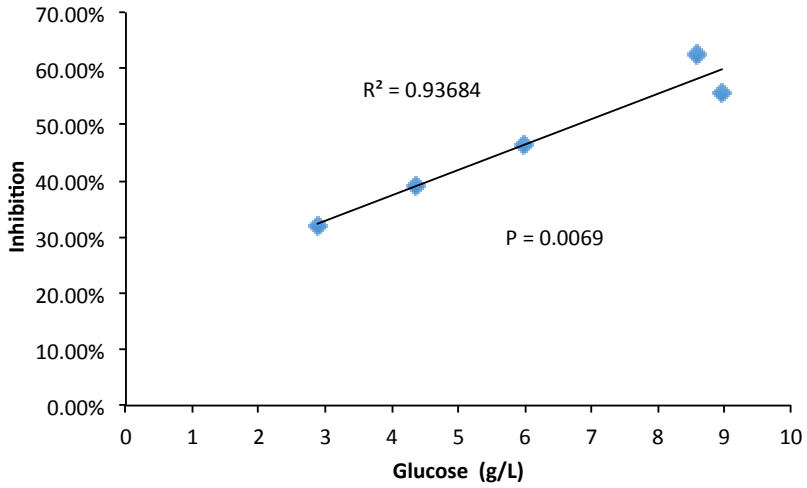
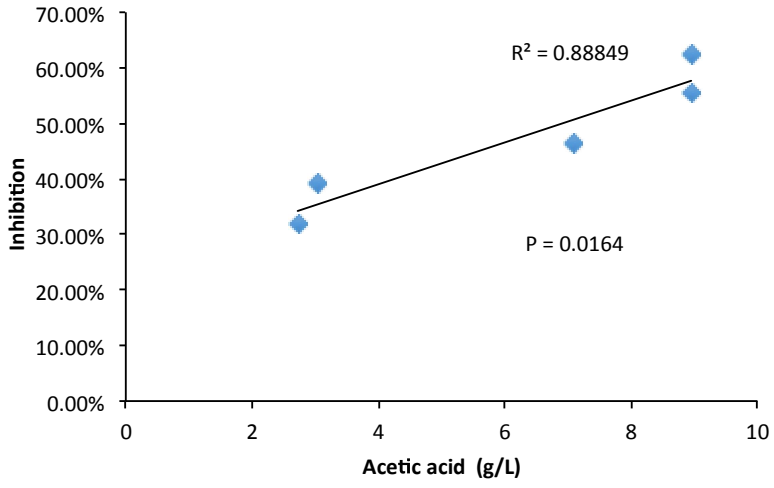
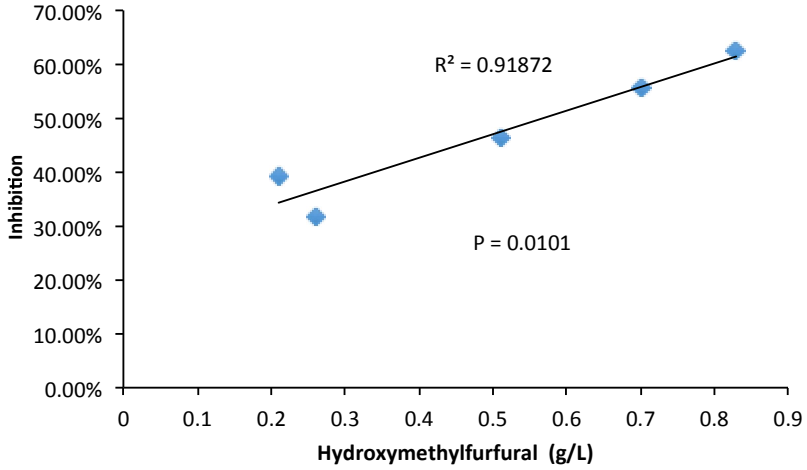
P18
P19
P20
P21
P22
P23
P24
Treatments

Fig. 3


70.00%
60.00%
50.00%
40.00%
30.00%
20.00%
10.00%
0.00%
Inhibition
R² = 0.93684
P = 0.0069
0 1 2 3 4 5 6 7 8 9 10
Glucose (g/L)


70.00%
60.00%
50.00%
40.00%
30.00%
20.00%
10.00%
0.00%
Inhibition
R² = 0.88849
P = 0.0164
0 2 4 6 8 10
Acetic acid (g/L)


70.00%
60.00%
50.00%
40.00%
30.00%
20.00%
10.00%
0.00%
Inhibition
R² = 0.91872
P = 0.0101
0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9
Hydroxymethylfurfural (g/L)

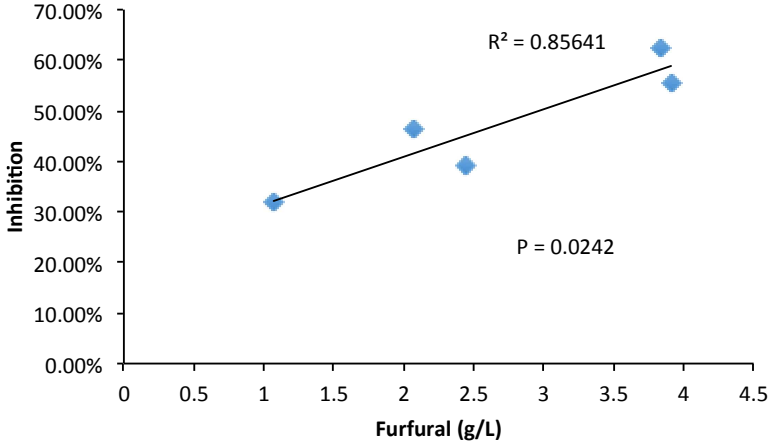
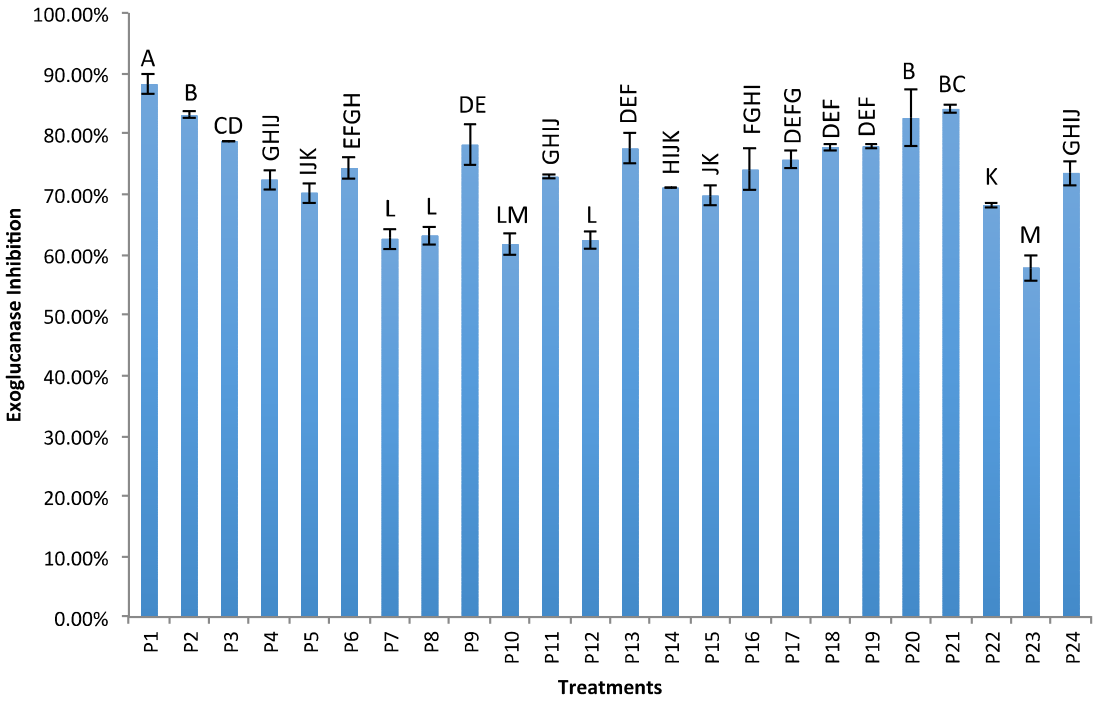

70.00%
60.00%
50.00%
40.00%
30.00%
20.00%
10.00%
0.00%
Inhibition
R² = 0.85641
P = 0.0242
0 0.5 1 1.5 2 2.5 3 3.5 4 4.5
Furfural (g/L)

Fig. 4


100.00%
90.00%
80.00%
70.00%
60.00%
50.00%
40.00%
30.00%
20.00%
10.00%
0.00%
Exoglucanase Inhibition
A
B
CD
GHIJ
IJK
EFGH
L
L
DE
LM
GHIJ
L
DEF
HIJK
JK
FGHI
DEFG
DEF
DEF
B
BC
K
M
GHIJ
P1
P2
P3
P4
P5
P6
P7
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P23
P24
Treatments

Fig. 5

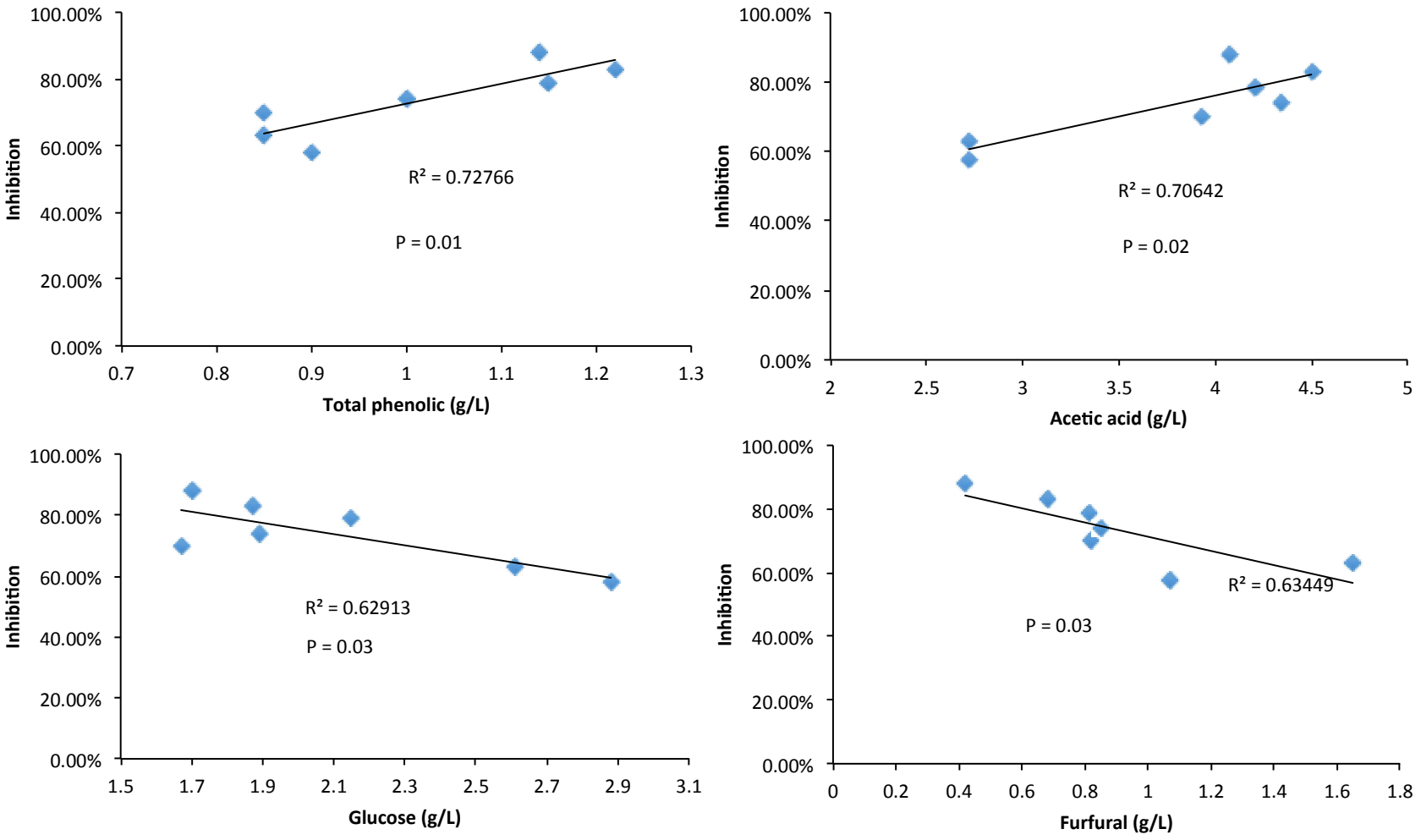

100.00%
80.00%
60.00%
40.00%
20.00%
0.00%
Inhibition
R² = 0.72766
P = 0.01
0.7
0.8
0.9
1
1.1
1.2
1.3
Total phenolic (g/L)
R² = 0.70642
P = 0.02
2
2.5
3
3.5
4
4.5
5
Acetic acid (g/L)
R² = 0.62913
P = 0.03
1.5
1.7
1.9
2.1
2.3
2.5
2.7
2.9
3.1
Glucose (g/L)
R² = 0.63449
P = 0.03
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
Furfural (g/L)

Fig. 6

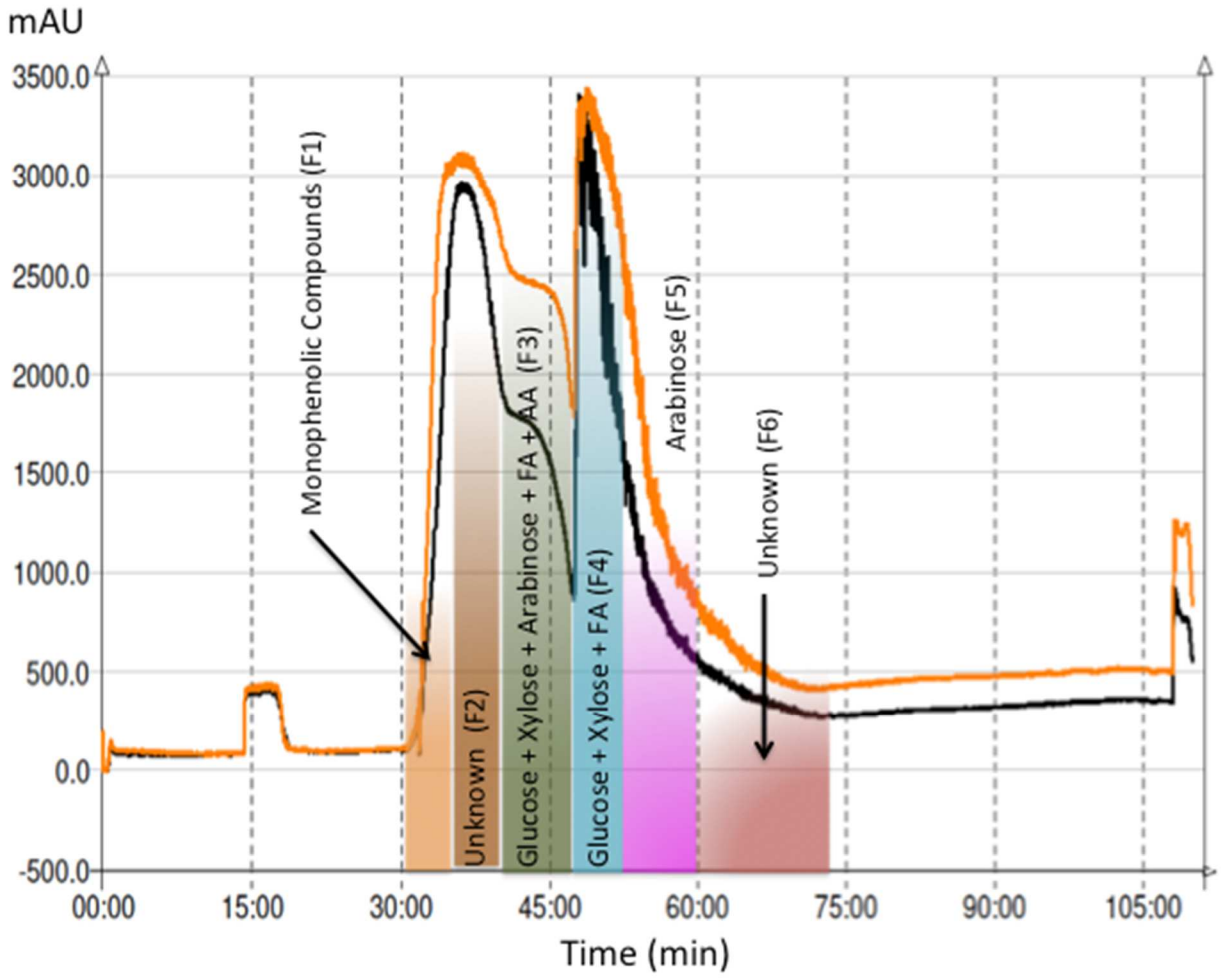

mAU
3500.0
3000.0
2500.0
2000.0
1500.0
1000.0
500.0
0.0
-500.0
00:00
15:00
30:00
45:00
60:00
75:00
90:00
105:00
Time (min)
Monophenolic Compounds (F1)
Unknown (F2)
Glucose + Xylose + Arabinose + FA + AA (F3)
Glucose + Xylose + FA (F4)
Arabinose (F5)
Unknown (F6)

Fig. 7

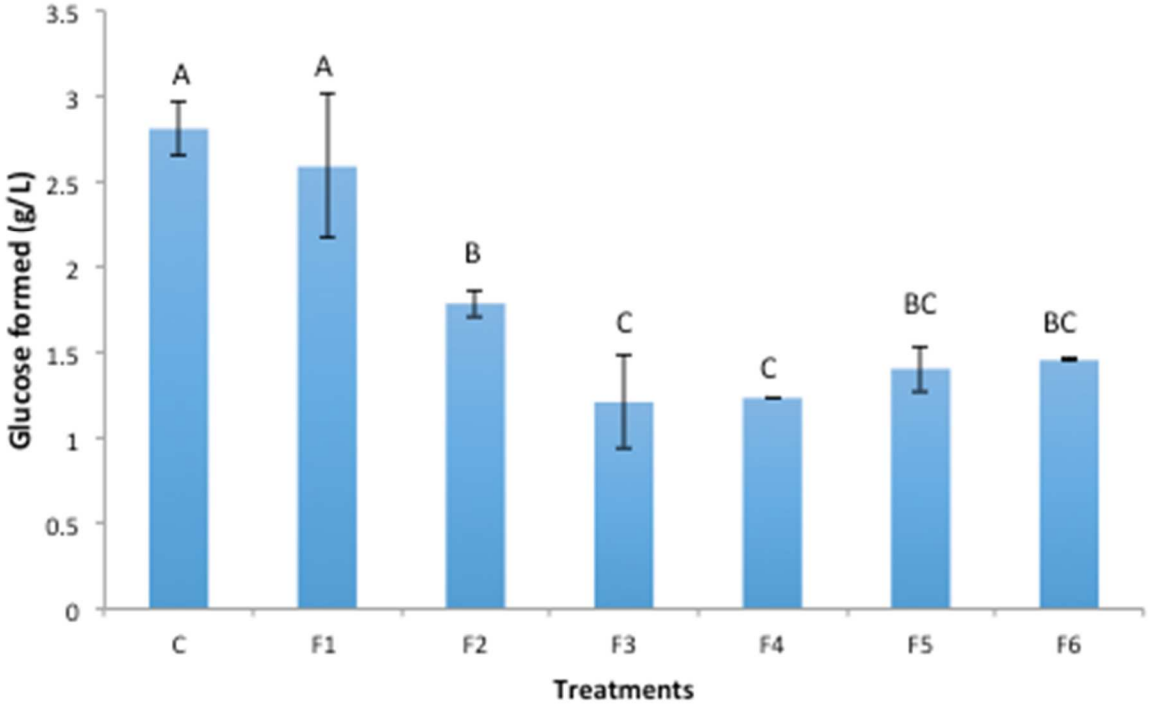

3.5
3
2.5
2
1.5
1
0.5
0
Glucose formed (g/L)
A
A
B
C
C
BC
BC
C
F1
F2
F3
F4
F5
F6
Treatments

Fig. 8

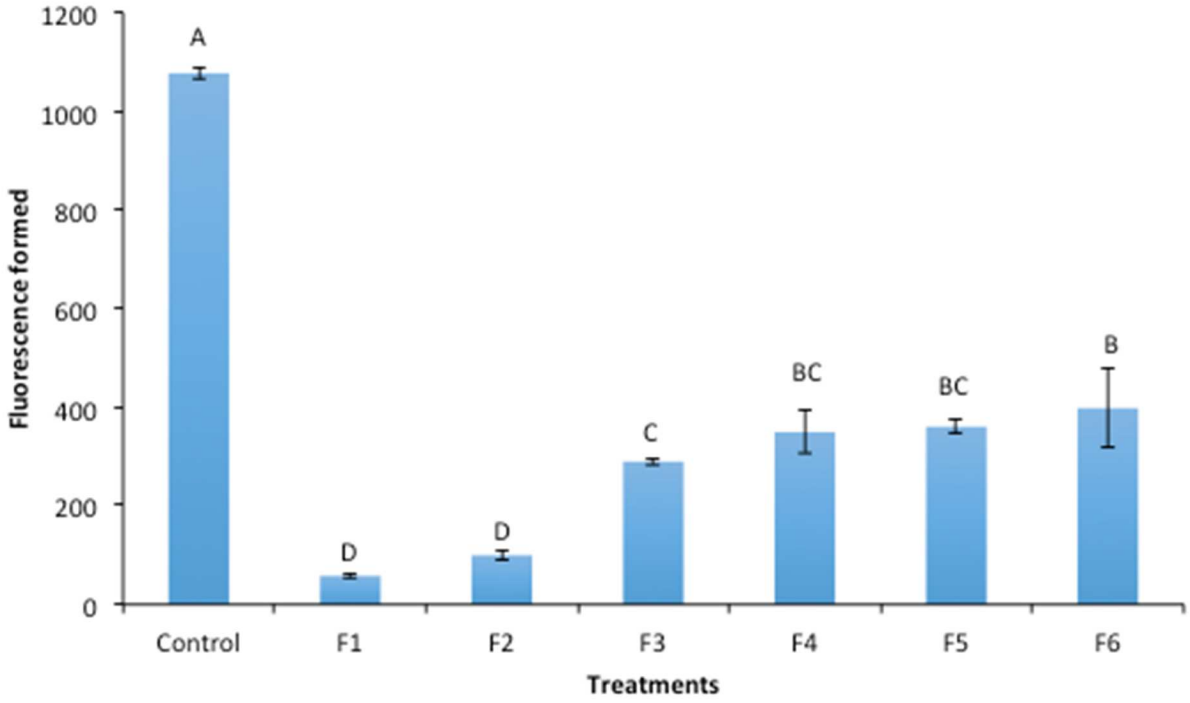

1200
1000
800
600
400
200
0
Fluorescence formed
A
D
D
C
BC
BC
B
Control
F1
F2
F3
F4
F5
F6
Treatments

Fig. 9

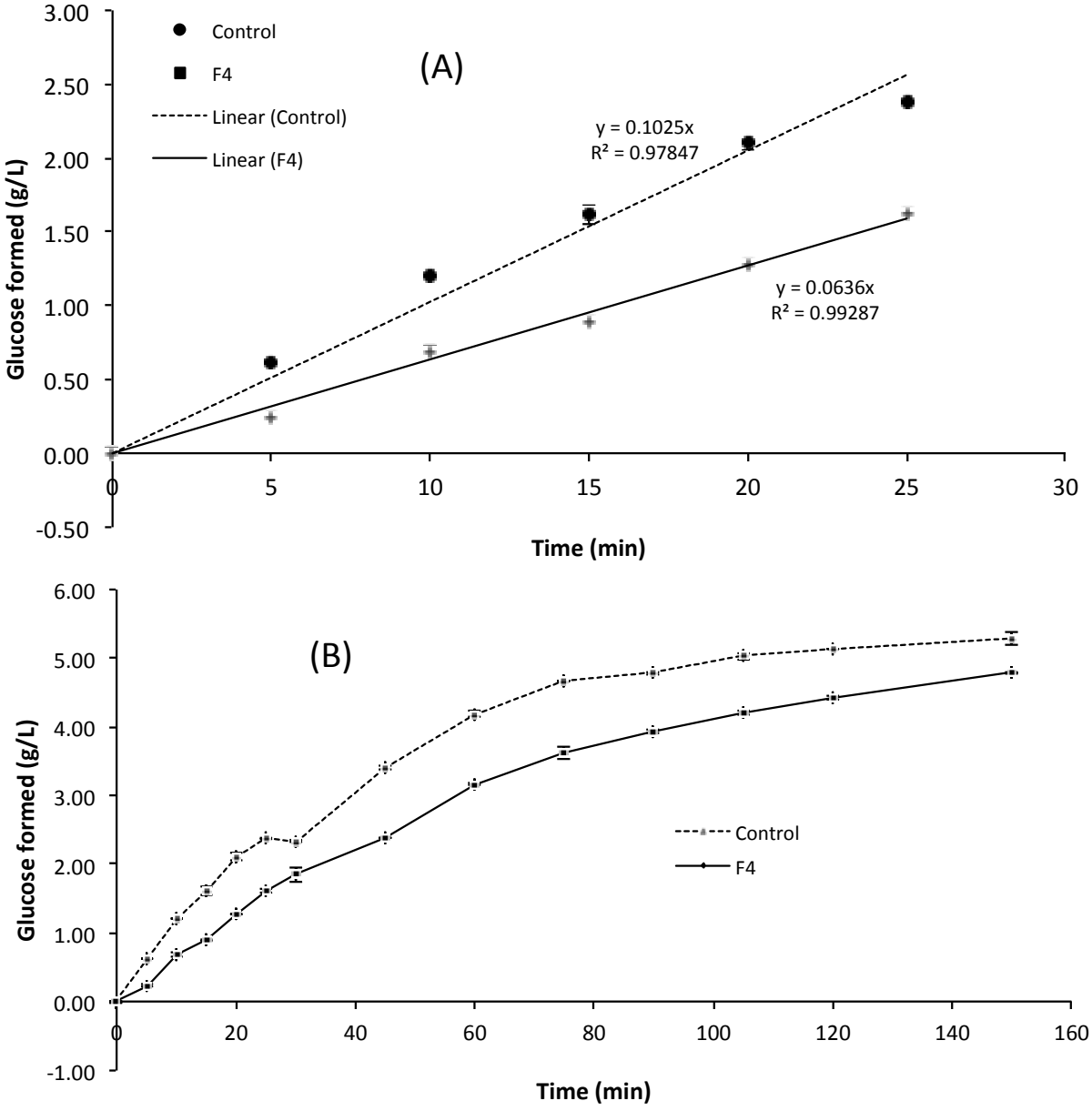
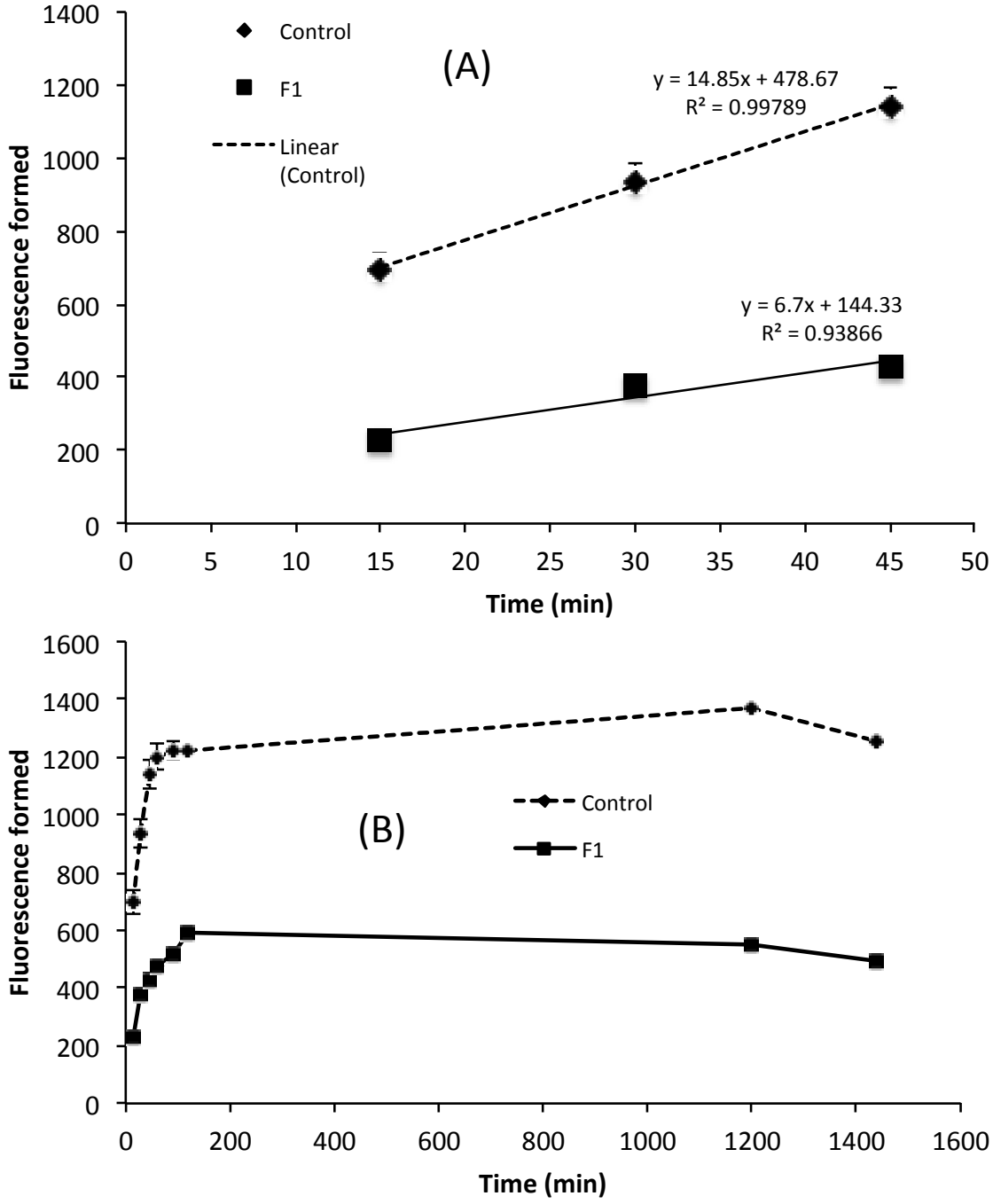

(A)
Control
F4
Linear (Control)
Linear (F4)
y = 0.1025x
R² = 0.97847
y = 0.0636x
R² = 0.99287
Glucose formed (g/L)
Time (min)
(B)
Control
F4
Glucose formed (g/L)
Time (min)

Fig. 10


(A)
Control
F1
Linear (Control)
y = 14.85x + 478.67
R² = 0.99789
y = 6.7x + 144.33
R² = 0.93866
Fluorescence formed
Time (min)
0
200
400
600
800
1000
1200
1400
0
5
10
15
20
25
30
35
40
45
50
(B)
Control
F1
Fluorescence formed
Time (min)
0
200
400
600
800
1000
1200
1400
1600

IV. Sweetgum Bark water extract: biological activity and inhibitory effect on cellulolytic enzyme

Abstract

Sweetgum bark extract was investigated for its antimicrobial, antioxidation activity, and inhibitory effect on cellulose hydrolysis enzyme. Bark extract was prepared in a stirring reactor with 85°C water, for one h. Three fractions (F1 to F3) of the bark extract were obtained by centrifugal partition chromatography (CPC) with a solvent system consisting of ethyl acetate: ethanol: water at a 2:1:2 (v/v/v) ratio. Antimicrobial activity of bark extract and its CPC fractions was tested against *Staphylococcus aureus* using the disc diffusion method. The ability of the bark extract and its CPC fractions to inhibit copper induced peroxidation of low-density lipoprotein was determined using thiobarbituric acid reactive substance (TBARS) assay. Results showed that 12% of solid content in the bark extract consisted of phenolic compounds, of which gallic acid was found in highest concentration. It was determined that bark extract and CPC fractions F1 and F2 impeded growth of *S. aureus*, resulting in zone of inhibition measuring 15, 13, and 17 mm, respectively. Results from TBARS assays indicated that bark extract and F2 displayed antioxidant activity, as they prevented significant formation of TBARS over 24 h. On the other hand, when tested against cellulase, sweetgum bark extract, at 4 g/L solid content, reduced the initial hydrolysis rate of cellulose powder by 66%. Cellulase inhibition increased with time and reached 82.32% ± 7.27 after 48 h. Removal of biomass extractives prior to pretreatment could represent a financial-profitable way to reduce inhibitory compounds in biomass prehydrolyzate, while generating compounds that have societal benefits.

1. Introduction

Forest residues, such as bark and foliage, account for a significant portion of forest biomass, and are usually overlooked by forest-based industries. In most cases, bark is usually burned to produce energy, necessary to operate pulp and paper mills. However, forest residues contain natural compounds with biological activities that can be of great interest to drugs and food industries. There are numerous studies reporting on antioxidant and antimicrobial activities of phytochemicals contained in plant extracts and forestry residues that are attracting attention, as natural and renewable sources for such compounds are sought (Rice-Evans et al., 1996; Cowan, 1999; Panda et al., 2009; Tambe et al., 2014; Das et al., 2014).

A biorefinery is a facility that converts biomass into fuels, chemicals, and power through biochemical or thermochemical routes. A forest-based biorefinery uses as feedstock forest biomass, such as wood and saw dust. Forest-based biorefineries can be built on capital equipment from an existing paper/pulp mill, either by integrating the biorefinery into the mill or by complete conversion of the mill (Van Heiningen, 2006; Söderholm and Lundmark, 2009). One important operation already in place in the paper/mill industries is the removal of bark from trees. This unit operation produces a considerable quantity of bark that could be possibly exploited in a biorefinery setting in terms of its phytochemical content.

The economic existence of biorefineries is strongly dependent on the degree of diversification of its product portfolio (Van Heiningen, 2006; Lynd et al., 2005). Such diversity could be accomplished in a forest-based biorefinery by using all parts of the harvested tree to produce high value co-products. Currently, the main research efforts that are centered on identification of biorefinery co-products are carbohydrate and lignin inspired products. Little

attention is paid to extractive components present in bark (Zhang et al., 2008; Huang et al., 2010).

Carrier and Clausen (2008) described how an extraction operation to harness phytochemical from biomass could be added prior to the biomass conversion in a biorefinery. The proposed scheme would use water as solvent to prevent loss of structural carbohydrate during extraction and ensure that the extracted biomass could still be safely converted. Additionally, water would have the advantage to be cheaper and easier to dispose than organic solvents, usually used for phytochemical extraction. Such scenario could be easily applied to forestry residues where phytochemicals will be extracted with water from bark before the extracted bark is burned to produce power.

Liquidambar styraciflua L. (sweetgum) is a hardwood tree that grows voluntarily as understory in southern pine forests. In addition to its timber and ornamental value, sweetgum can also be a potential feedstock in a biorefinery. Djioleu et al. (2012) and Torget et al. (1991) reported that sweetgum's polymeric carbohydrate constituents could be saccharified into fermentable sugars. Moreover, sweetgum extractives have been shown to contain valuable phytochemicals that can be used in the synthesis of medicine to treat the flu (Enrich et al., 2008; Martin et al., 2010), pain (El-Readi et al., 2013), and Alzheimer disease (Rashed et al., 2014). Therefore, a biorefinery using sweetgum as feedstock would have the possibility to derived a variety of valuable compounds from this biomass.

Although work has already been conducted to demonstrate biological activity of sweetgum extract, most studies used organic solvent, such as methanol, which could not be easily integrated in a biorefinery setting. Investigation of extract production using an environmentally friendly solvent, such as water, could certainly benefit all aspects of future

biorefineries. . Therefore, this current study investigates antimicrobial and antioxidation activity of water extract of sweetgum bark and its ensuing purified fractions.

2. Materials and Methods

2.1. Biomass

Sweetgum bark was separated with a chain flail debarker from mature trees harvested with a chainsaw from a pine plantation understory in Drew County, AR. The sampled trees were 15 to 20 years old with a height of 7 to 10 m and a diameter of 20 to 25 cm at the root collar, which is the base of the tree. The bark biomass was milled to pass through a 20 mesh (0.84 mm) screen using a Wiley Mini Mill (Thomas Scientific, Swedesboro, NJ) and stored in an air-sealed container at 4°C until used.

2.2. Chemicals

Water used for all experiments was obtained from a Direct-Q filtering system from Millipore (Billerica, MA) that had 18.2 ΩM resistivity. Analysis grade ethanol, ethyl acetate, methanol, and formic acid from VWR international (Radnor, PA) were also employed for experiments. Yeast extract and Tryptic Soy Broth were from Becton Dickison (Sparks, MD). Tris buffer, 2-thiobarbituric acid (TBA), 1,1,3,3-tetraethoxypropane (TEP), copper sulfate, and butylated hydroxytoluene (BHT), Folin & Ciocalteau's (F-C) phenol reagent, and potassium sodium tartrate tetrahydrate were purchased from Sigma-Aldrich (St. Louis, MO). Sodium chloride was purchased from Mallinckrodt Baker (Phillipsburg, NJ). Sodium hydroxide was obtained from Fisher Scientific (Milford, MA). Trichloroacetic acid (TCA) was purchased from MP Biomedicals (Santa Ana, CA). Accellerase® 1500, a cellulose hydrolysis enzyme cocktail was generously donated by Dupont Industrial Biosciences (Cedar Rapids, IA).

2.3. Water Extraction

Twenty-five grams of milled sweetgum bark was extracted with 250 mL of Millipore water in a 1-L Parr 4525 reactor (Moline, IL). Extraction was carried out at 85°C for 60 min and slurry was agitated in the reactor at 144 RPM. Measurement of extraction time started at the moment the temperature inside the reactor reached 85°C. The ensuing slurry was separated into a solid and liquid portion through a perforated Büchner funnel lined with a Whatman N° 1 filter paper using vacuum filtration. A 50-mL aliquot of the liquid portion was dried down until constant weigh under pressure without heat with a Savant SpeedVac Concentrator SPD 1010 (Thermo Scientific, Ashville, NC) set at 7 Torr. The bark extract was reconstituted with 10 mL of water and stored at -20°C until used for analysis, fractionation, and biological assays.

2.4. Centrifugal Partition Chromatography (CPC)

Bark water extract was fractionated using a bench scale SCPC-250 system from Armen Instruments (St Ave, France) equipped with CherryOne Beta (C1) countercurrent chromatography control system (Chicago, IL). The CPC method was adapted from Uppugundla et al. (2009), using a solvent system consisting of ethyl acetate: ethanol: water at a 2:1:2 (v/v/v) ratio. When prepared, the solvent system was allowed to separate for at least two hours in a 2-L separatory funnel (VWR International, Radnor, PA) into two immiscible phases: 1) an upper rich organic phase and 2) an aqueous bottom phase. Each phase was carefully collected in a 1-L glass bottle and immediately used. The 5 mL reconstituted bark sample was mixed with 12.5 mL of solvent organic rich phase and 12.5 mL of solvent aqueous rich phase. The organic rich phase was loaded as the stationary phase in the 250-mL CPC rotor at 10 mL/min, with the rotor spinning at 500 RPM for approximately 30 min. After loading the stationary phase, the speed of the rotor was increased to 2500 RPM at which point the mobile phase (aqueous bottom phase of

the solvent system) was loaded into the rotor at 8.33 mL/min until equilibrium was reached. A total of 115 mL of stationary phase was present in the CPC rotor by the time the two phased reached equilibrium. The bark sample was then filtered through a 5 μm PTFE syringe filter (Thermo Scientific National, Rockwood, TN) prior to injection. After equilibration, the bark sample was injected in the 30-mL sample loop. CPC fractionation lasted 80 min from the time of sample injection; fraction collection started after 20 min and a total of 60 fractions were collected. Collection was done every minute using a Foxy R1 (Teledyne Isco, Lincoln, NE) fraction collector. Ensuing fractions were monitored through an evaporative light scattering (ELSD). All collected fractions were dried down with a SpeedVac Concentrator. Afterwards, fractions were reconstituted with 0.5 mL of water for prior to HPLC analysis. CPC fractionation was successfully accomplished twice; however, only biological activities of fractions from the 2nd run are reported.

2.5. Gravimetric analysis

Total solids content in samples was determined by a gravimetric method, using an EL204 analytical balance from Mettler-Toledo, LLC (Columbus, OH). Known volumes of samples were placed in 13 x 100 mm pre-weighted test tube (VWR International, Houston, TX). Sample-containing tubes were dried down with the Savant SpeedVac Concentrator until constant weigh under vacuum with no heat. The mass of the tubes after drying was recorded and the difference between before and after masses were calculated as total solid content of sample.

2.6. Folin-Ciocalteau Assay

The protocol was similar to that described in Chapter 3 of this thesis. Concentration of total phenolic compounds was expressed as gallic acid equivalent (GAE).

2.7. Ultra Performance Liquid Chromatography (UPLC) analysis

Monomeric phenolic compounds in samples were analyzed by UPLC as described in Chapter 3 of this thesis. Compounds were detected at 270 nm.

2.8. Disc diffusion assay

Antimicrobial activity of samples in terms of inhibiting growth of *Staphylococcus aureus* was determined using the disc diffusion assay as in Adams et al. (2014). Culture of *S. aureus* were passed every 24 h in Tryptic Soy Broth supplemented with Yeast Extract and placed in a 37°C incubator for at least 48 h prior to being used for the assay. The original inoculum was 8.7 logs CFU (colonies forming unit). Agar plates were prepared with Mueller-Hinton agar from HiMedia Laboratories (Mumbai, India). Plates were inoculated with *S. aureus* using the streaking method and a sterile cotton-tipped applicator manufactured by Puritan Medical Products (Guilford, ME). Inoculated plates were allowed to dry for approximately 5 to 10 minutes before applying the 6 mm diameter blank sterile paper discs (Becton Dickinson, Sparks, MD), using sterile forceps. Discs were gently pressed to ensure contact with the agar. Discs were impregnated with 20 μL of sample and plates were allowed to rest for 15 min before being inverted and placed in a 37° C incubator for 18 to 24 hours. After the incubation period, plates were removed from the incubator and zones of inhibition were measured using a ruler. Experiment was run in duplicate.

2.9. Thiobarbituric acid reactive substances (TBARS) assay

Antioxidation capacity of the bark sample was determined using copper induced TBARS assay on human low-density lipoproteins (LDL). The method was essentially performed as described by Uppugundla et al. (2009). Human LDL from Biomedical Technologies Inc. (Stroughton, MA) was dialyzed using dialysis tubes (10 000 molecular weight cutoff, Pierce,

Rockford, IL) in EDTA-free TRIS (pH 7.4) buffer for 24 h at 4°C. Exactly, 10 µL of test samples, with 100 µL of dialyzed LDL, and 10 µL of 55 µM copper sulfate were pipetted into the wells of a 96-well-assay plate (Becton Dickinson, Franklin Lanes, NJ). Pipetting of each test samples was done in duplicate. Immediately after pipetting, 10 µL of BHT (1 mM) was added to a well representative of each testing condition in order to arrest any oxidation reaction. Plates were covered with a breathable sterile tape from Nalge Nunc International (Rochester, NY) and incubated at 37°C for 24 h in a water bath. After the 24 h incubation period, 10 µL of BHT was added to all the remaining wells to stop the reaction. Exactly 50 µL of 50%(w/v) TCA and 75 µL of 1.3%(w/v) TBA were then added to all wells. The plate was recovered with the breathable sterile tape and placed at 60°C for 40 min in a water bath. After the 60 min incubation period, plates were removed from the water bath. The amount of TBARS present in wells was calculated using the difference of absorbance from each well read at 600 nm and 530 nm. Absorbance was measured using a microplate reader (BioTek, Winooksi, VT). A standard curve prepared with TEP was used to estimate the concentration of TBARS formed. TBARS assay was triplicated.

2.10. Cellulase Inhibition assay

Cellulase inhibition was performed as that described in Chapter 3 of this thesis.

2.11. Statistical Analysis

Data from TBARS were subjected to ANOVA and student's *t*-test using JMP Pro 11 from SAS Institute (Cary, NC). Significance was established for p-value < 0.05.

3. Results and Discussion

3.1. Sweetgum bark water extract and CPC fractionation

Extraction of sweetgum bark with 85°C water in a stirred reactor resulted in the production of a dark brown liquid extract. Solid content in bark extract was originally 32 g/L;

after concentration in the SpeedVac concentrator, the solid content increased to 159.75 g/L (Table 1). Phenolic compounds, determined as GAE, accounted for 12.02 % of the solid content of the extract. UPLC chromatogram of the bark extract is shown in Fig. 1 and at the detection wavelength of 270 nm, ten peaks were detected. It was determined that peaks 1, 2, and 8 corresponded to shikimic acid, gallic acid, and salicylic acid, respectively. Gallic acid was the major constituent identified in the bark extract. All the other peaks remained unknown, as they did not correspond to any standards available in our collection.

Detection of shikimic and gallic acid observed in the aqueous bark extract parallels findings in literature. Martin et al. (2010) reported that 65°C water extraction would yield 1.7 mg of shikimic acid from one g of sweetgum bark. Using a similar extraction process, Enrich et al. (2008) obtained 2.4 to 3.7% (w/w) of pure shikimic acid from sweetgum seed. Spencer and Choong (1977) observed that gallic acid was one of the major constituents present in ethanolic extract of sweetgum bark. They proposed that gallic acid would be formed from the sugar pool in the shikimic acid pathway, substantiating why shikimic acid was present in the extract at low concentration (Spencer and Choong, 1977). Other phenolic compounds, not identified in this investigation, have also been found in sweetgum extracts. Spencer and Choong (1977) identified ellagic acid as another major constituent in ethanolic sweetgum bark extract and suspected that ellagic acid could be methylated. Eid et al. (2015) reported the presence of isorugosin B, casuarictin, quercetin-3-O-b-D-4C1-glucopyranoside, myricetin-3-O-a-L-1C4-rhamnopyranoside (myricetrin), quercetin, and myricetin in sweetgum leaf ethanolic extract.

Sweetgum bark water extract was fractionated using ethyl acetate: ethanol: water (2:1:2, v/v/v) as solvent system. This solvent system has been previously used by Uppungundla et al. (2009) to purified rutin and quercetin from switchgrass extract prepared with 90°C water.

Therefore, the same solvent system was utilized to fractionate phenolic compounds present in sweetgum bark extracts. CPC of the bark extract generated 60 fractions. ELSD signal indicated that compound elution terminated 30 min after the collection was initiated. UPLC analysis of the fractions showed that some fractions had identical profiles and could be combined. Accordingly, similar fractions were combined, dried down, and reconstituted with water. A total of three fractions, F1, F2, and F3, were obtained from the consolidation operation. The order of fraction elution from the CPC rotor is presented in Fig. 2. F1 represented fractions collected between the 40^{th} and 56^{th} min; F2 was obtained from consolidating the following seven fractions; and, F3 resulted from the consolidation of fractions collected between the 64^{th} and 70^{th} min. UPLC profiles for F1, F2, and F3 are shown in Fig. 3. Shikimic acid, present in the bark extract, was collected in F1 and F2. Although F1 did not show any major peak at 270 nm, its solid and total phenolic concentration was determined to be 145.0 g/L and 11.29 g/L (Table 1), respectively. Gallic acid was mainly collected in F2. Solid content and phenolic compounds concentration in fraction F2 were 97.5% g/L and 14.44 g/L, respectively. Fraction F3 exhibited only traces of gallic acid; its solid content and phenolic concentration were 8.75 g/L and 1.14 g/L, respectively.

3.2. Biological activity of bark extract and CPC fractions

Antimicrobial activity of bark extract, F1, F2, and F3 was determined using the disc diffusion assay as the capability to inhibit growth of *S. aureus*. Clear zones of inhibition were observed around discs impregnated with bark extract, as well as from CPC fractions. These results indicated that aliquots from bark extract, F1 and F2 contained compounds that were inhibitory to the growth of *S. aureus.* Diameters of observed zone of inhibition are presented in Table 2. Growth inhibition was concentration dependent; diameter of the zone of inhibition resulting from bark extract, at solid concentration of 32 g/L, was 9 mm, which was lower than 15

mm obtained when solid concentration was 5X higher. F1 and F2, with solid concentration as indicated in Table 1, produced zone of inhibition measuring 13 and 17 mm, respectively. F3 did not present antimicrobial activity against *S. aureus*. This could be due to the fact that F3 did not contain any antimicrobial active compounds or that the F3 fraction was not sufficiently concentrated to display inhibitory effect.

Similarly, the potential of bark extract and its CPC fractions to prevent lipid peroxidation was determined using the TBARS assay. Bark extract, F1, F2, and F3, with solid concentration as indicated in Table 1, were diluted ten times to avoid their color to interfere with the assay. Samples were found to exhibit activity if no significant ($p > 0.05$) difference was determined between TBARS concentration at 0 h and 24 h. Results of TBARS assay are presented in Fig. 4; water was used as negative control. An analysis of variance demonstrated that TBARS concentration at 0 h was significantly different to that at 24 h for the control and for fraction F3, as indicated by an asterix in Fig. 4. Significant increases in TBARS concentration indicated that compounds present in F3 did not have any anti-peroxidation potential or that their concentration was not sufficiently elevated to prevent copper induced peroxidation of human LDL. On the other hand, the bark extract and fraction F2 inhibited significant formation of TBARS over a 24 h period. Results for F1 were not conclusive; although the concentration of TBARS at 24 h was not significantly different than that at 0 h, the difference in magnitude of TBARS concentration between the two time points was 5.97 mM.

Antimicrobial and antioxidation activity observed from plant extracts have been associated with phenolic and flavonoid compounds present in those extracts (Rice-Evan et al., 1996; Cowan, 1999). Biological active compounds have been found in sweetgum leaf and stem (El-Readi et al., 2013; Eid et al., 2015). Current results indicate that sweetgum bark could be a

natural potential source for such compounds as its water extract prevented microbial growth and copper induced LDL peroxidation. Unfortunately, it was not possible to attribute biological activities observed from the bark extract to either shikimic or gallic acid, as neither one has been reported to exhibit such activity (Aruoma et al., 1993; Cholbi et al., 1991; Chung et al., 1993, Lingbeck et al., 2015).

The fact that bark water extract exhibit valuable biological activities opens new economical avenues for a forest-based biorefinery, which could opt to integrate a phytochemical extraction in its conversion process (Devappa et al., 2015). Phytochemicals could find applications in food, drugs, and cosmetic industries as food preservatives, dietary supplement, agent against cancer, inflammatory disease, cardiovascular, viral infection, and to prevent skin damage and aging (Dillard and German, 2000; Kole et al., 2005). In addition, usage of water as solvent extraction, as opposed to the usual organic solvent, present the advantage to use an environmentally friendly solvent and allow the extracted biomass to maintain its combustibility uses without being a hazard.

3.3. Cellulase inhibition of bark extract

In recent publications, phenolic compounds derived from biomass deconstruction, have been highlighted as potent inhibitors and deactivators of cellulolytic enzymes (Ximenes et al., 2010; 2011; Kim et al., 2011; 2013). Gallic acid was found responsible to 20–80% deactivation of cellulolytic enzyme when incubated with the enzyme for 24 h prior to testing enzyme activity (Ximenes et al., 2011). Mhlongo et al. (2015) and results presented in chapter 3 of this thesis indicated that exoglucanase, a category of cellulolytic enzyme, was very sensitive to phenolic molecules. Kim et al. (2011) suggested that such sensitivity could be caused to enzyme

precipitation, corresponding to an effect observed from tannin, a polyphenolic found in plant extract (Cowan, 1999).

Because the proposed scheme is to integrate phytochemicals extraction in a biochemical conversion platform, it becomes important to evaluate how compounds that are to be derived from bark extracts could interact with saccharification unit operations, which are inherent to conversion processes. When assayed against cellulose powder, it was determined that bark water extract strongly inhibited cellulase activity. Figure 5 shows a linear relationship between time and glucose formed during cellulose hydrolysis in 50 mM citrate buffer (control) and bark extract at solid concentration of 4 g/L. The initial hydrolysis rate of cellulose, defined by the slope of the linear curve relating time and glucose formed, was 66% higher in control experiment than those of bark extracts. Moreover, Fig. 6 illustrates that cellulolytic enzyme did not recover from the detrimental effect of sweetgum bark extract during a 120 h incubation period. The highest amount of glucose formed when the reaction was incubated with bark extract was 1.21 g/L, whereas 13.4 g/L was obtained from the control. Inhibition of cellulase by bark extract rose with time (Fig. 7), increasing from 0 to 63.24% ± 6.12 in 2 h, and plateauing to 82.32% ± 7.27 after 48 h. These results imply that it will be critical to prevent any cross contamination between phytochemical extractions and biomass saccharification in a biochemical conversion platform.

Although not specifically investigated in this current study, it could be possible that a phytochemical extraction as proposed here could also improve the overall efficiency of the conversion process. In general, extractives contained in the biomass are not usually removed from the biomass prior to pretreatment and can be found in biomass prehydrolyzates. Biomass prehydrolyzates were shown to inhibit cellulase activity in chapter 3 of this thesis.

4. Conclusion

It is likely that phenolic compounds derived from biomass extractive could be partially responsible for the negative effect of biomass prehydrolyzates on cellulolytic enzyme. However, these phytochemicals displayed interested biological activities, warranting the fact that a phytochemical extraction prior to biomass pretreatment could represent a financial-profitable way to mitigate prehydrolyzate inhibitory effects, while generating compounds that have societal benefits.

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Zhang, Y. P. (2008). Reviving the carbohydrate economy via multi-product lignocellulose biorefineries. J. Ind. Microb. Biotechnol. 35(5), 367-75.

Table 1: Concentration of total solids and total phenolics in sweetgum bark water extract and its CPC fractions (F1 to F3)

Samples	Total Solid (g/L)	Total Phenolic as GAE (g/L)
Bark extract	159.75	19.20
F1	145.00	11.29
F2	97.50	14.44
F3	8.75	1.14

CPC = centrifugal partition chromatography
GAE = gallic acid equivalent

Table 2: Antimicrobial activity of sweetgum bark water extract and CPC fractions (F1 to F2) on *Staphylococcus aureus*

Samples	Zone of Inhibition (mm)
Pure Extract	15 ± 0
F1	13 ± 0
F2	17 ± 0
F3	0 ± 0

CPC = centrifugal partition chromatography. Data are mean ± standard deviation of two replications

Figure captions

Figure 1 Ultra performance liquid chromatography profile of sweetgum bark water extract with detection at 270 nm. 1= shikimic acid; 2 = gallic acid; 8 = salicylic acid

Figure 2 Centrifugal partition chromatography profile of sweetgum bark water extract

Figure 3 Ultra performance liquid chromatography profiles of fractions (F1 to F3) of sweetgum bark water extract. Fractions were obtained from centrifugal partition chromatography. Peaks were detected at 270 nm with 1= shikimic acid; 2 = gallic acid; 8 = salicylic acid

Figure 4 Anti-peroxidation activity of sweetgum bark water extract and its centrifugal partition chromatography fraction (F1 to F3). Water was the negative control. Astride indicates significant difference ($p < 0.05$) between TBARS concentration at 0 h and 24 h. Data are average of three replications ± standard deviation

Figure 5 Comparison of initial hydrolysis rate of cellulose powder in 50 mM citrate buffer (control) and sweetgum bark water extract

Figure 6 Comparison of cellulose powder hydrolysis in 50 mM citrate buffer and sweetgum bark water extract over time

Figure 7 Inhibition of cellulase activity on cellulose powder by sweetgum bark water extract over time. Data are average of two replications with standard deviation as error bar

Fig. 1

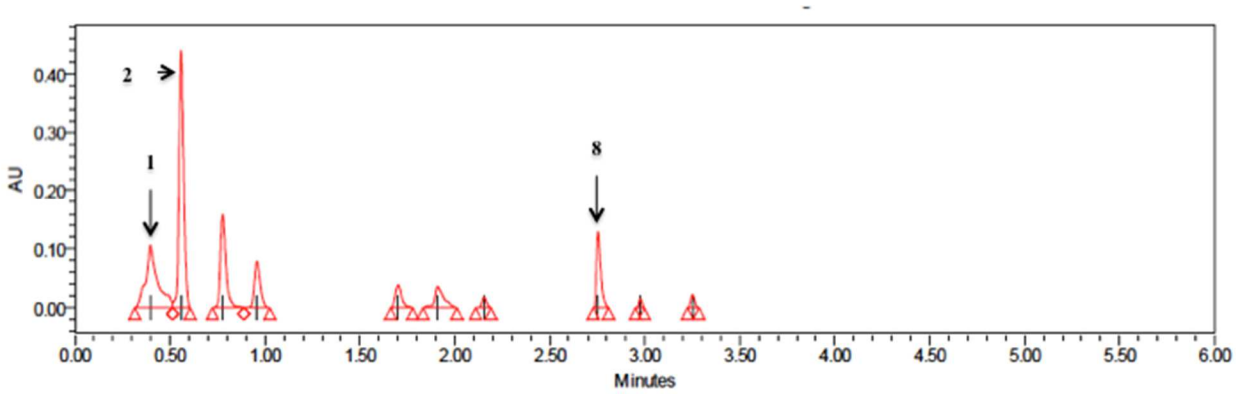

2
1
8
AU
0.40
0.30
0.20
0.10
0.00
0.00
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
4.50
5.00
5.50
6.00
Minutes

Fig. 2

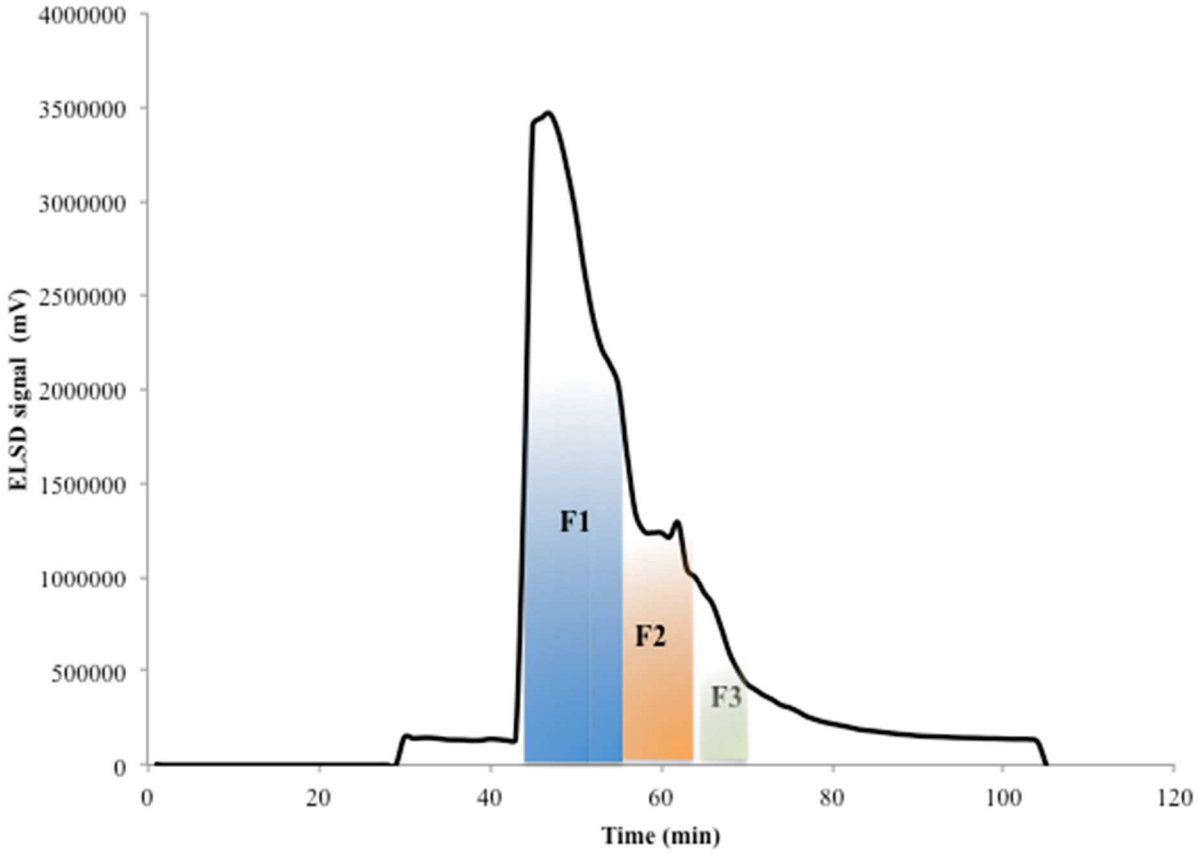

4000000
3500000
3000000
2500000
2000000
1500000
1000000
500000
0
ELSD signal (mV)
F1
F2
F3
0
20
40
60
80
100
120
Time (min)

Fig. 3

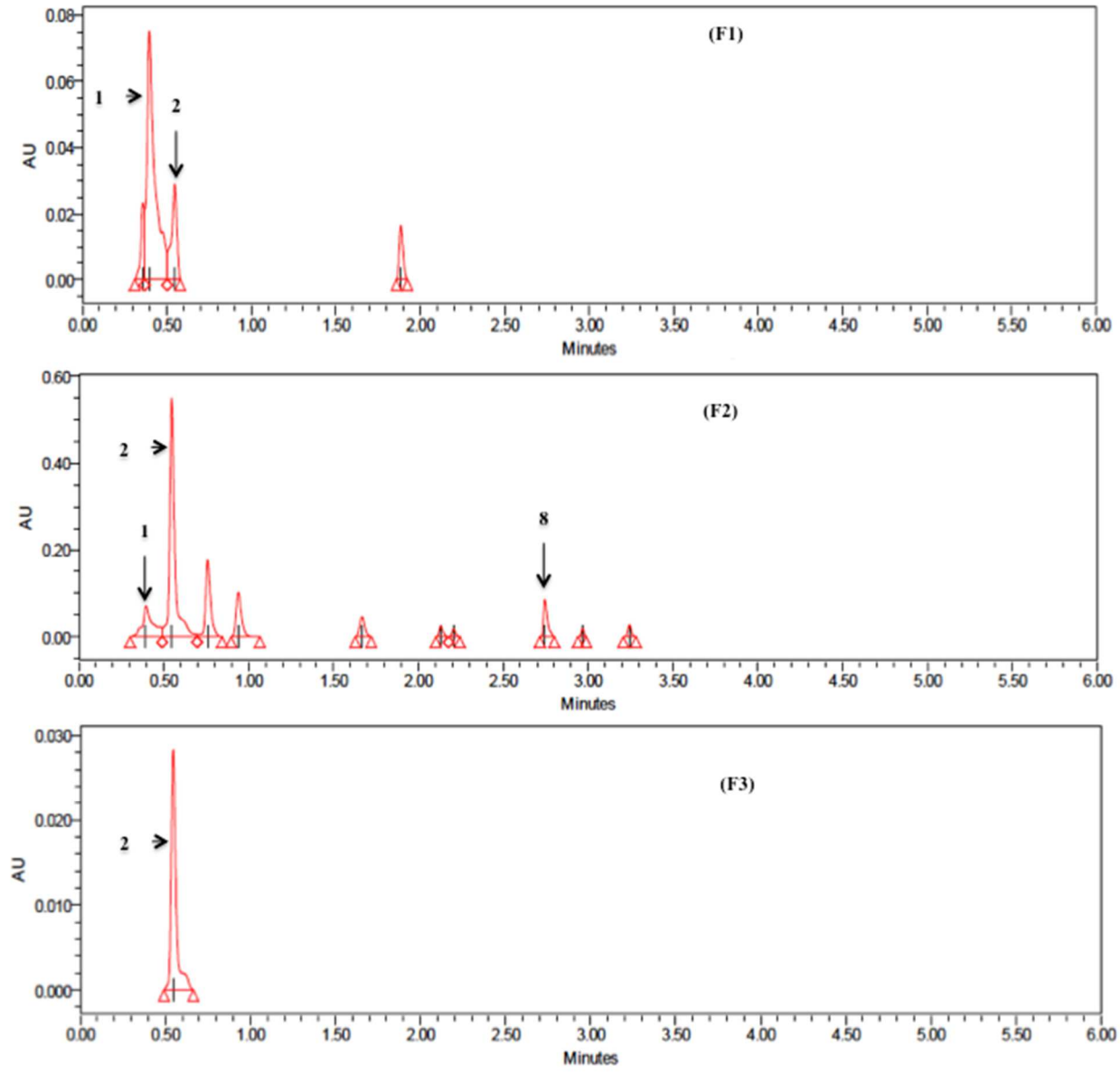


Fig. 4

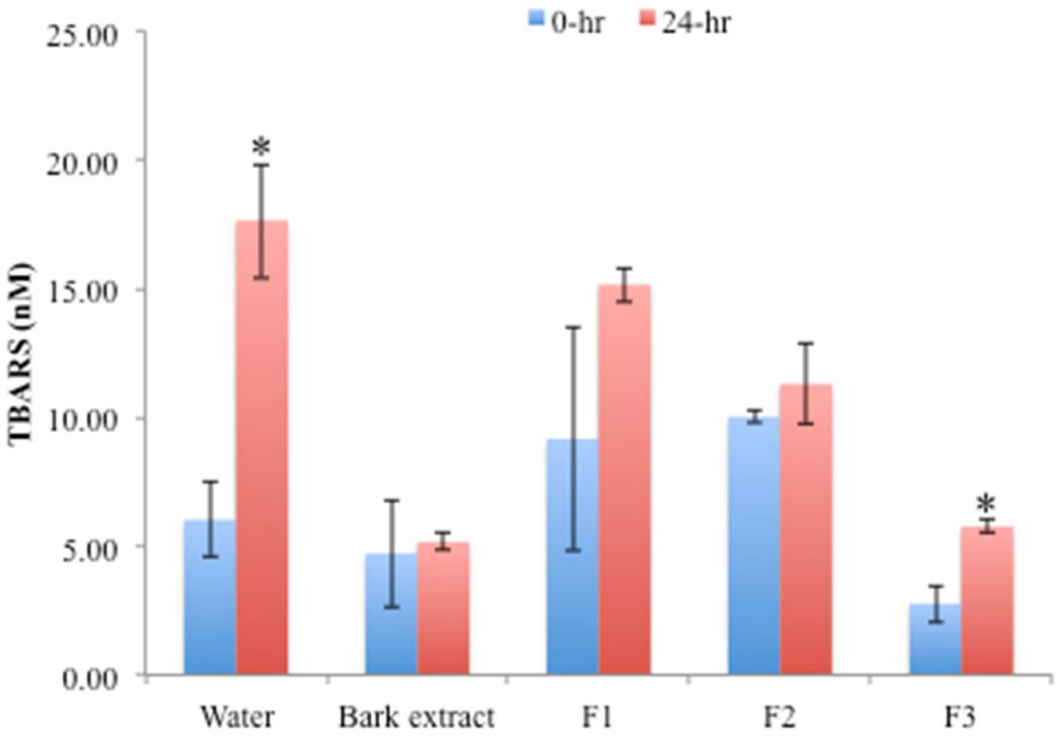

0-hr
24-hr
25.00
20.00
15.00
10.00
5.00
0.00
TBARS (nM)
*
*
Water
Bark extract
F1
F2
F3

Fig. 5

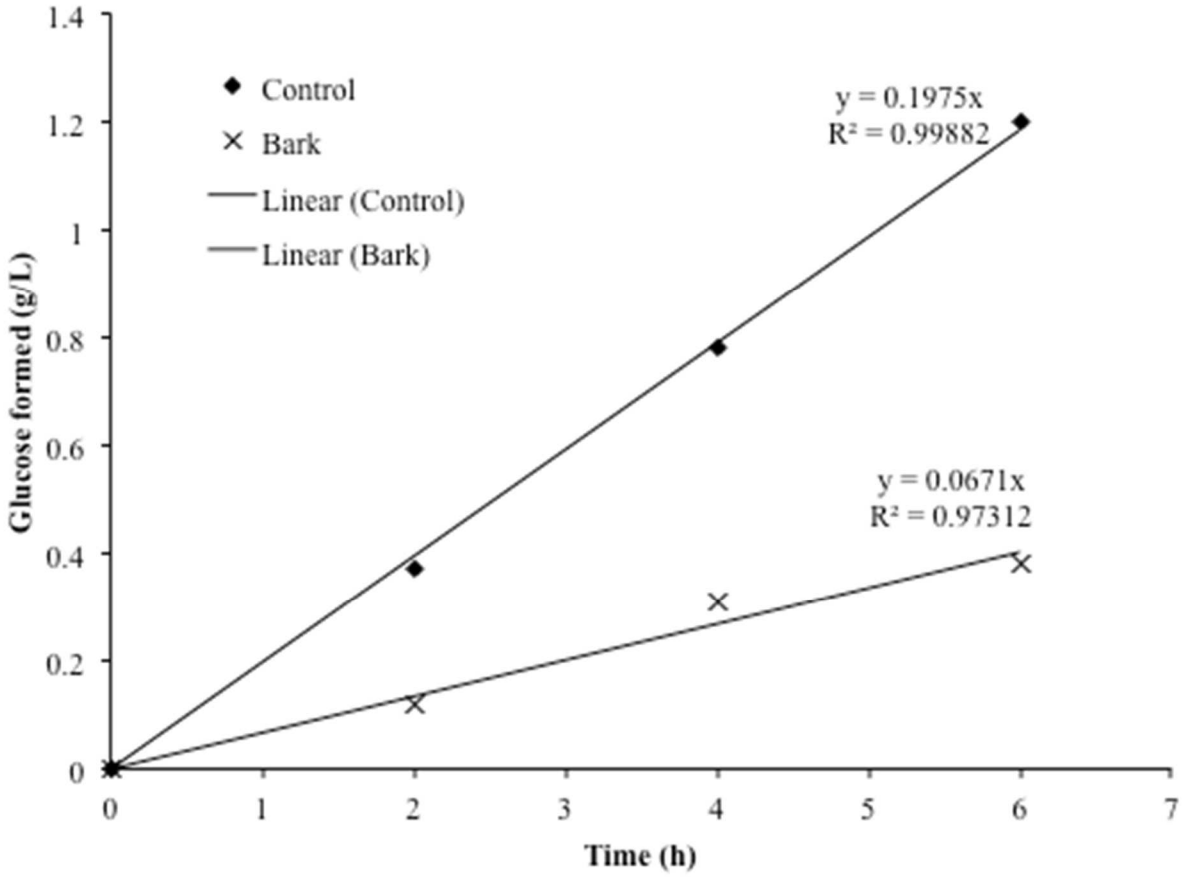

Control
Bark
Linear (Control)
Linear (Bark)
y = 0.1975x
R² = 0.99882
y = 0.0671x
R² = 0.97312
Glucose formed (g/L)
Time (h)
0
0.2
0.4
0.6
0.8
1
1.2
1.4
0
1
2
3
4
5
6
7

Fig. 6

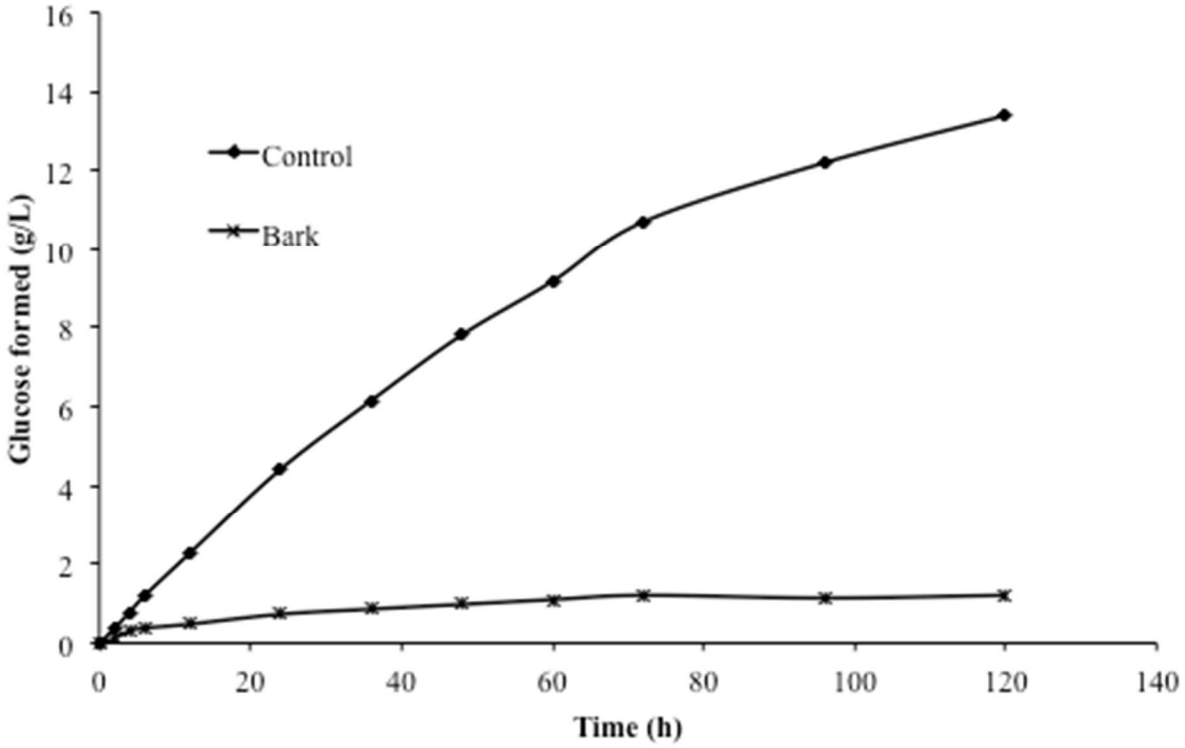
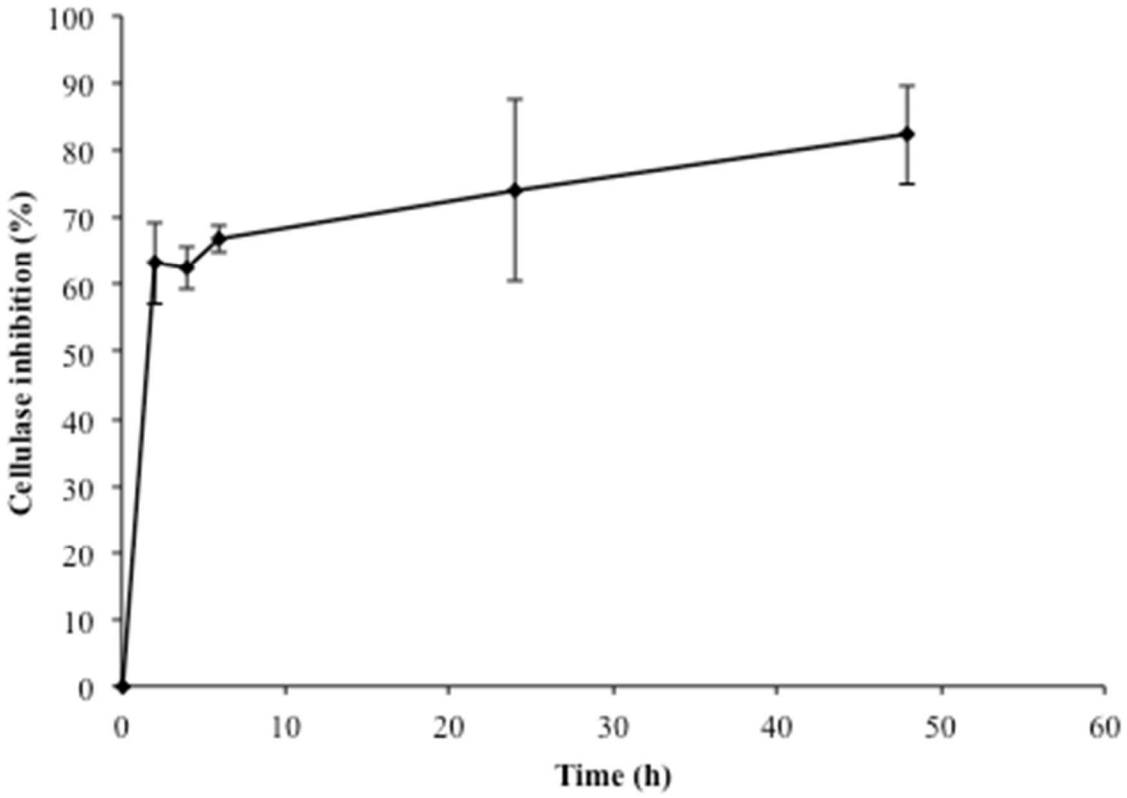

Glucose formed (g/L)
Time (h)
Control
Bark
0
2
4
6
8
10
12
14
16
0
20
40
60
80
100
120
140

Fig. 7


Cellulase inhibition (%)
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
Time (h)

V. Conclusion

Pretreatment of lignocellulosic biomass is a necessary operation for the conversion of lignocellulosic biomass to fuel and chemicals. Understanding how pretreatment-generated compounds affects downstream operations is critical to improve the overall efficiency of conversion processes. This investigation showed that dilute acid pretreatment of switchgrass produced compounds that are inhibitory to cellulolytic enzyme systems. The nature and concentration of the generated compounds were governed by pretreatment conditions. Enzyme systems could be ranked in order of increasing sensibility to switchgrass prehydrolyzate as follows: exoglucanase > β-glucosidase > cellulase cocktail. Phenolic compounds were more detrimental to exoglucanase, whereas β-glucosidase was mostly affected by glucose and furans. Additionally, phytochemicals extracted from sweetgum bark were significantly damaging to cellulose hydrolysis enzymes. On the other hand, phytochemicals extracted from sweetgum bark, mostly phenolics, inhibited the growth of *Staphylococcus aureus* and reduced copper-induced peroxidation of human low-density lipoprotein. Biomass prehydrolyzate detoxification strategies should target phenolic compounds, as they were shown to inhibit exoglucanase as well as the cellulsase cocktail. Removal of biomass extractives, such as phenolics, prior to pretreatment presents the possibility of reducing inhibitory compounds present in biomass prehydrolyzates while generating compounds with societal benefits that could enhance the economic viability of the biorefinery.

Future work

In light of the results from this project, the scientific community in biochemical conversion of lignocellulosic material could benefits from studies to determine:

1. The inhibition mechanism of phenolic compounds to cellulolytic enzyme systems

2. The effect of removing biomass extractives on sugar yields from biochemical conversion of lignocellulosic biomass
3. Potential values in phytochemicals extracted from leading energy crop as well as biomass residue
4. The integration of phytochemical removal in biochemical conversion processes

VI. Appendix

UNIVERSITY OF ARKANSAS

Office of Research Compliance

February 13, 2015

MEMORANDUM

TO: Dr. Danielle Carrier

FROM: W. Roy Penney
Institutional BioSafety Committee

RE: IBC Protocol Approval

IBC Protocol #: 15007

Protocol Title: "Testing pine essential oil for growth inhibition of Listeria"

Approved Project Period: Start Date: February 12, 2015
Expiration Date: February 11, 2018

The Institutional Biosafety Committee (IBC) has approved Protocol 15007, "Testing pine essential oil for growth inhibition of Listeria" You may begin your study.

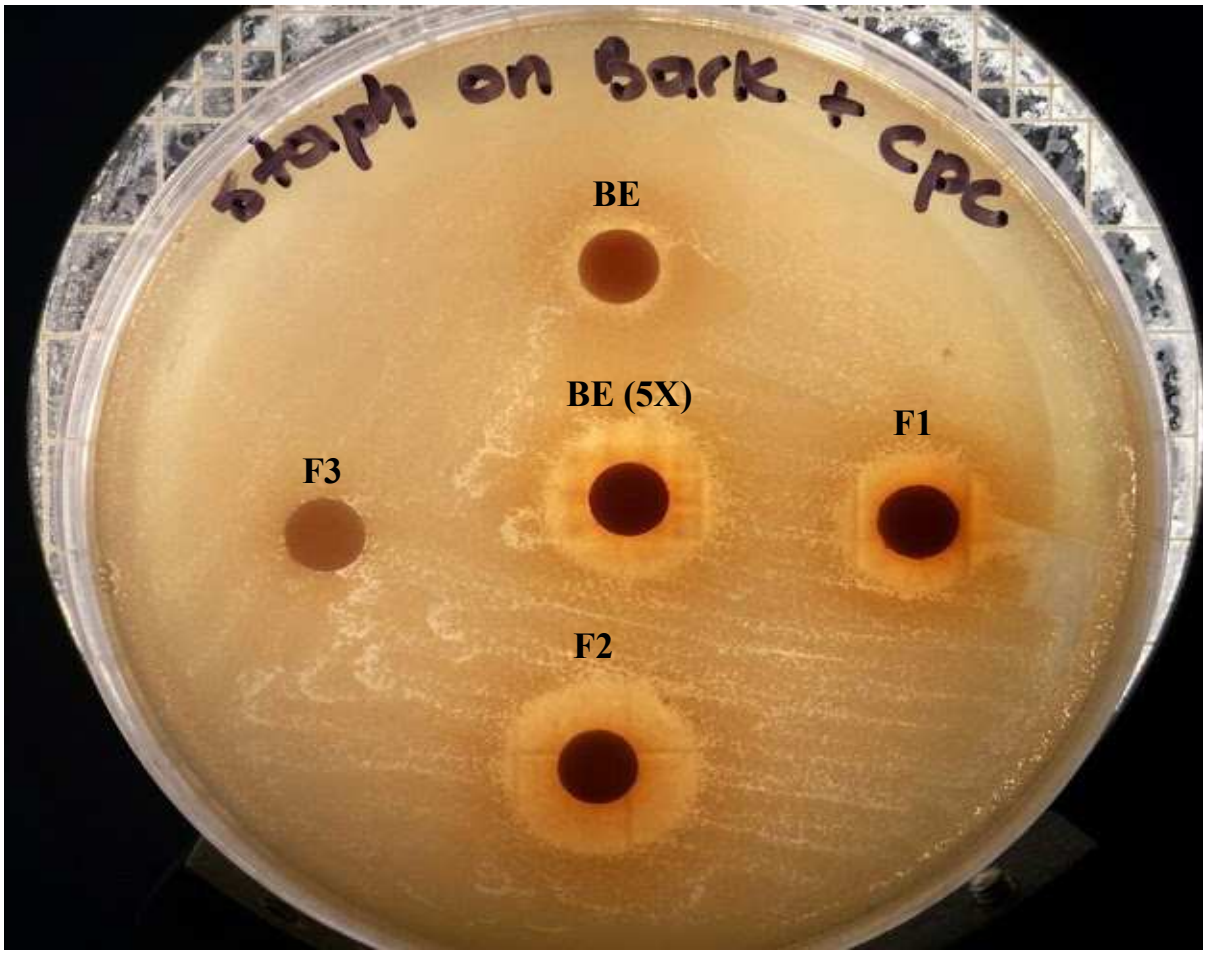
If further modifications are made to the protocol during the study, please submit a written request to the IBC for review and approval before initiating any changes.

The IBC appreciates your assistance and cooperation in complying with University and Federal guidelines for research involving hazardous biological materials.

Administration Building 210 • 1 University of Arkansas • Fayetteville, AR 72701-1201 • 479-575-4572
Fax: 479-575-3846 • http://vpred.uark.edu/199.php

Protocol approval for microbial testing

Sweetgum bark water extract.



Disc diffusion assay: sweetgum bark water extract and its centrifugal partition chromatography fraction (F1 to F3) on *Staphylococcus aureus* growth. BE = bark extract at 32 g/L of solid. BE (5X) = bark extract 5 times concentrated