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## Enhanced enzymatic saccharification of pretreated biomass using glycerol thermal processing (GTP)

Wei Zhang<sup>a,b</sup>, Noppadon Sathitsuksanoh<sup>c</sup>, Justin R. Barone<sup>d</sup>, Scott Renneckar<sup>e,\*</sup>

<sup>a</sup> Macromolecules and Interfaces Institute, Virginia Tech, Blacksburg, VA 24061, United States

<sup>b</sup> Department of Sustainable Biomaterials, Virginia Tech, Blacksburg, VA 24061, United States

<sup>c</sup> Department of Chemical Engineering, University of Louisville, Louisville, KY 40292, United States

<sup>d</sup> Department of Biological Science and Engineering, Virginia Tech, Blacksburg 24061, United States

<sup>e</sup> Department of Wood Science, University of British Columbia, Vancouver V6T1Z4, Canada

### HIGHLIGHTS

- Glycerol thermal processing enhances saccharification of biomass.
- Higher crystallinity not correlated with saccharification efficiency.
- Lignin removal did not positively impact enzyme digestibility of pretreated biomass.
- Removal of xylan significantly enhanced rate of conversion.

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### ABSTRACT

Biomass was heated (200–240 °C) in the presence of glycerol, for 4–12 min, under shear to disrupt the native cell wall architecture. The impact of this method, named glycerol thermal processing (GTP), on saccharification efficiency of the hardwood *Liquidambar styraciflua*, and a control cellulose sample was studied as a function of treatment severity. Furthermore, the enzymatic conversion of samples with varying compositions was studied after extraction of the structural polymers. Interestingly, the sweet gum processed materials crystallinity index increased by 10% of the initial value. The experiments revealed that the residual lignin was not a barrier to limiting the digestibility of cellulose after pretreatment yielding up to 70% glucose based on the starting wood material. Further xylan removal greatly improved the cellulose hydrolysis rate, converting nearly 70% of the cellulose into glucose within 24 h, and reaching 78% of ultimate glucan digestibility after 72 h.

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### 1. Introduction

A global rise in fuel demand is expected as the population expands by another billion people in the next 12 years. With current petroleum production around 80 million barrels a day, emitting additional CO<sub>2</sub> at increased rates into a closed atmospheric system should be considered carefully. Concern for global warming has motivated interest in the development of renewable biofuels and biomaterials produced from sustainable biomass. First generation biofuels derived from corn-starch or sugarcane through batch fermentation have received scrutiny. Debates and concerns (Agbor et al., 2011) about these first generation biofuels have arisen because of the competition between food and fuels, as well as con-

cern about the additional emissions of greenhouse gases from their production. As an alternative, lignocellulosic biomass is viewed as an attractive feedstock for biofuel production due to its lower environmental footprint and large abundance. Lignocellulosic biomass contains approximately 70% dry weight polysaccharides, which can be enzymatically hydrolyzed to pentoses and hexoses, and further fermented to ethanol and/or butanol. Moreover, abundant biopolymers in lignocellulosics such as lignin and xylan provide potential sustainable polymeric materials to subsidize the cost of biofuel production.

The crux of cellulosic biofuels production is the limited access that enzymes have to the polysaccharide scaffolding that makes up the cell wall. The native structure of lignocellulosic biomass greatly impedes the conversion efficacy for biofuel production and the cell wall must be partially disassembled to allow for efficient conversion. Several structural features have been proposed

\* Corresponding author.

E-mail address: [scott.renneckar@ubc.ca](mailto:scott.renneckar@ubc.ca) (S. Renneckar).

as major hurdles to both the rate and extent of biomass saccharification (Mansfield et al., 1999). Biomass surface area and pore volume are the primary physical barriers that dictate how biomass can be enzymatically deconstructed. Larger surface area and pore volume facilitates the enzyme catalyzed hydrolysis on accessible cellulose chains, which would in-turn positively impact biomass saccharification (Sathitsuksanoh et al., 2013). Furthermore, a high degree of polymerization and abundant hydrogen bonding between cellulose chains in the microfibril structure requires a number of cellulases that lead to chain scission and end-wise conversion into cellobiose units. Some studies have demonstrated that cellulose crystallinity is a dominant factor and high crystallinity limits cellulose enzyme accessibility whereas reduced crystallinity improves the initial hydrolysis rate (Chang and Holtzapple, 2000). Lignin and hemicellulose surrounding cellulose microfibrils are also proposed to negatively impact biomass saccharification. These two components limit the efficient access of enzymes to the cellulose surface while cellulose crystallinity prevents access of enzymes internally within the cellulose crystallites. Additional routes to access sugars locked into polymers involve lytic polysaccharide monooxygenase enzymes and others in the AA9 group (Žifčáková and Baldrian, 2012) that can bind to, and disrupt crystalline cellulose chains at the surface, whereas other cellulase enzymes can only attack cellulose in non-crystalline forms. Furthermore, the acetyl groups substituted on hardwood hemicellulose are also proposed to limit the biomass digestibility (Grohmann et al., 1989) and specialized enzymes are also known to be involved in deacetylation. Overall, the native structure of the cell wall limits the efficient access of enzymes to convert cellulose into fermentable sugars.

Biomass pretreatment prior to enzymatic hydrolysis is an essential step for overcoming the structural and steric barriers to enzyme access for more efficient conversion in biofuel production. Over the last three decades, extensive research has explored many paths for pretreatment methods to overcome the recalcitrance of the native lignocellulosic biomass for efficient enzymatic saccharification. Dilute acid (Grethlein, 1980), alkaline (Chang et al., 1997), steam-assisted (Grous et al., 1986) and organosolv (Holtzapple and Humphrey, 1984) methods typically occur at high temperatures of 150–200 °C resulting in enhanced biomass surface area and significant removal of lignin and/or hemicellulose (Zhao et al., 2009), and these pretreatments have been widely applied on hardwood, softwood and grasses. When acid catalyzed or auto-catalyzed processes are used for pretreatment, many undesirable side products for fermentation, such as furfurals, are generated and remain a problem (Cantarella et al., 2004). Pretreatments with ionic liquids (Labbe et al., 2012) have expanded green processing technologies for efficient biomass saccharification, but more efforts are still needed to resolve the problems of high cost and recovery/reuse. Although recent work shows that ionic liquids can be recycled 5 times without losing their efficacy to deconstruct biomass.

Continuous pretreatment protocols involving screw-extrusion and other high-shear processing systems have been recently developed for saccharification. Extruders are widely used in the polymer processing industry and offer several features applicable to biomass pretreatment, such as excellent temperature control, the capacity for high solids loading, and simultaneous heating and shearing during processing. The first application of extrusion-assisted pretreatment was reported in 1999 (Dale et al., 1999), where a twin-screw extruder was combined with a liquid ammonia pretreatment to enhance the digestibility of corn stover. Lee et al. (2010) conducted a series of experiments using an extrusion system with different additives for biomass pretreatment, obtaining a maximum glucose yield of 62.4% when enzymatic saccharification was used with ethylene glycol as an additive. The resulting biomass fibers were highly fibrillated and possessed a greatly

expanded surface area after extrusion. Karunanithy et al. (2012) also reported a maximum digestion yield of 65.8% for pine chips when high moisture conditions were used with extrusion processes.

We previously reported adopting the high temperature shearing methods in the presence of glycerol using a batch extruder (glycerol thermal processing) to disrupt the hardwood structure (Zhang et al., 2015). The novelty of the process was based on the dearth of studies in the literature that use anhydrous glycerol at very high temperatures and atmospheric pressure for short times; previously reported studies using glycols under anhydrous conditions only had gone between 180 and 225 °C, for extended periods of time up to 9 h (Demirbağ, 1998). These studies delignified the biomass, such that the degraded lignin products were solubilized into the pulping liquor. In contrast the treatment with GTP only causes the removal of the hemicellulosic side chains into the pulping liquor. Because the limited degree of overall compositional changes, and the ability to remove lignin and xylan, in a step-wise manner, a range of extraction procedures were examined for biopolymer fractionation to reveal how the constitutional components impacted the saccharification efficiency. Hence, the experimental protocols provided a way to investigate the influence of the degree of disruption of the cell wall, as well as the impact of the composition on conversion efficiency, yielding additional experimental insight into key factors of glucan digestibility of biomass. Unlike the previous report on fractionation of GTP biomass, this study reveals that disrupted cell wall walls can achieve high levels of glucan conversion without removal of lignin, and that removal of significant xylan components greatly enhance the hydrolysis rates.

## 2. Methods

### 2.1. Materials

Chemicals and reagents used in this research were purchased from Sigma–Aldrich, Alfa Aesar and MP Biomedicals, and used as received. A mature sweet gum (*Liquidambar styraciflua*) from Blacksburg, VA was debarked, machined to cubes, and stored in a freezer before use. Prior to pretreatment, the biomass was knife milled using a Thomas-Wiley mill model 4, and sorted to a particle size between 40 and 60 mesh on a metal screen (250–420 μm). The extractive-free sweet gum particles were prepared according to ASTM standard D1105 (D1105-96, 2007). Deionized water (DI-water: 18.2 mΩ) was used in all steps in this research.

### 2.2. Methods

#### 2.2.1. Glycerol thermal processing (GTP) pretreatment

Extractive-free biomass (6% MC) was mixed with glycerol (1:3 dry mass basis) using a bench-top internal mixing head with a high intensity shear paddler driven by a C.W. Brabender® Prep-Center® drive as detailed previously (Zhang et al., 2015). The GTP pretreatment severity parameter ( $R_0$ ) was calculated according to an equation developed by Overend and Chornet (1987) to define pretreatment conditions. Both the time and temperatures used

**Table 1**

Glycerol thermal processing conditions and corresponding severity parameters for biomass pretreatment in this research.

Sample label	T (°C)	t (min)	log( $R_0$ )
SG#1	200	4	3.55
SG#4	220	4	4.14
SG#8	240	8	5.03
SG#9	240	12	5.20

during processing and the corresponding logarithm of  $R_0$  in this research are shown in Table 1. After pretreatment, the GTP pretreated biomass was collected from the mixing head and stored at 4 °C until further analysis. Samples for each severity condition were run in triplicate.

A highly purified cellulose, Whatman brand fibrous cellulose CF11 powder, was used for a reference material in this study. Only one GTP condition at 240 °C and 8 min was used to process the cellulose samples in triplicate. After pretreatment, the GTP pretreated cellulose was collected and stored at 4 °C for further analysis.

#### 2.2.2. Water extraction to remove glycerol and water soluble degradation products

The GTP pretreated biomass was water washed to remove glycerol residue and any degraded components as detailed previously (Zhang et al., 2015). After the initial wash of the glycerol soaked biomass with 40 °C water, the samples were resuspended in water, centrifuged and decanted, and this process was repeated approximately 10 times with fresh water. To minimize recrystallization during drying, the water extracted GTP biomass was freeze-dried immediately after processing. The GTP pretreated Whatman CF11 cellulose powder was water washed and freeze-dried using the same methods and no further extractions were conducted on these samples.

#### 2.2.3. Solvent extraction to remove lignin

The water extracted GTP biomass was further solvent extracted with aqueous dioxane 96% wt./wt.% to extract lignin from the pretreated fiber. The solvent extracted GTP biomass was washed with new aqueous dioxane solvent of equal volume, and then subsequently with acetone until the wash was colorless. The samples were washed with DI water until the wash water was colorless, before freeze-drying the biomass.

#### 2.2.4. Alkaline extraction to remove crude xylan

The solvent-extracted GTP biomass was alkali extracted at room temperature for 24 h with 1.0 M NaOH, 4 w/w%, to remove crude xylan. After extraction, the biomass residue was washed using DI water until the wash water was colorless with a neutral pH (approximately 10 times) and the GTP biomass was immediately freeze-dried. The choice of alkali concentration was used based on the literature for xylan isolation.

#### 2.2.5. Biomass structural carbohydrate analysis

The carbohydrate content of the non-pretreated and GTP pretreated sweet gum were analyzed in duplicate according to NREL laboratory analytical procedures (LAP) (Sluiter et al., 2008).

#### 2.2.6. Enzymatic saccharification

Non-pretreated and GTP pretreated sweet gum (water, solvent and alkaline extracted) biomass was enzymatic hydrolyzed to a glucan consistency of 1%. Hydrolysis using a CTec2 enzyme cocktail in a pH 4.8, 0.05 M sodium citrate buffer with 0.02% (w/v)  $\text{Na}_3\text{N}$  to prevent the growth of microorganisms during the digestion (Selig et al., 2008) was performed in a water bath shaker at 50 °C and 180 rpm for 72 h with a dry solid to liquid loading of 0.01 g to 0.025 g per ml of buffer. The protein content of CTec2 was measured by BCA assay using a Pierce™ bicinchoninic acid (BCA) method (Thermo Scientific, Rockford, IL) standardized using bovine serum albumin. The CTec2 cocktail (protein content of 188 mg/ml) was generously donated by Novozymes (Franklinton, NC) and used as received. The enzyme loading for hydrolysis was based on 20 mg protein/g cellulose to avoid insufficient digestibility due to limited enzyme loading (demonstrated by enzymatic hydrolysis of Avicel, data not shown). At predetermined time intervals, well-mixed 2-ml aliquots were removed from the digest, micro-

centrifuged at 13,000 rpm for 15 min to remove the solid biomass, and the clear supernatant was used to determine glucose released by the enzymatic treatment. The non-pretreated and GTP pretreated CF11 cellulose was also enzymatically hydrolyzed using the same method.

#### 2.2.7. Determination of released glucose

Glucose released during enzymatic hydrolysis was determined on a Metrohm ion chromatograph (IC) and a Shimadzu high performance liquid chromatograph (HPLC). When tested by IC, the 1 ml supernatant (pre-passed 0.2  $\mu\text{m}$  filter) was diluted to 50 times of its original concentration. The glucose in diluted aliquot was measured using a pulsed amperometric detector (PAD) after separation on a Hamilton RCX-30 (250  $\times$  4.6 mm) column with DI-water as eluent as detailed in previous research (Zhang et al., 2015). Samples assayed by HPLC were separated using a Bio-Rad Aminex® HPX-87H column (300  $\times$  7.8 mm) using a 5 mM sulfuric acid mobile phase with a constant flow rate of 0.5 ml/min. The sample injection volume was 15  $\mu\text{l}$  and an RID-10A refractive index detector was used for glucose detection. A 6 point calibration curve using pure glucose standards was run prior to each batch of tests ( $R^2 > 0.999$ ).

The overall enzymatic glucan digestibility (%) was calculated as follows:

$$\% \text{digestibility} = \frac{G_h}{(180/162) \times G_i} \times 100\% \quad (1)$$

where  $G_h$  = the amount of soluble glucose after enzymatic hydrolysis;  $G_i$  = the initial added glucan in the biomass before enzymatic hydrolysis.

#### 2.2.8. X-ray diffraction (XRD) of GTP pretreated biomass

The crystallinity index of the water extracted GTP biomass and cellulose was measured on a Bruker D8 Discover X-ray diffractometer with a Cu K $\alpha$  radiation source ( $\lambda = 0.154 \text{ nm}$ ) generated at 40 kV and 40 mA. A 1 mm slit was used and a locked couple 2-theta and theta scan was performed from 10° to 50° at a scan speed of 4°/min. The biomass samples were flattened on a quartz slide with a thickness of 1–2 mm to collect the diffraction profile. Non-pretreated sweet gum and CF11 cellulose were used as the control and reference samples, respectively.

The crystallinity index (CrI) of the different biomass samples tested was calculated according to the methods developed by Segal and coworkers (Segal et al., 1959):

$$\text{CrI} = \frac{I_{200} - I_{AM}}{I_{200}} \times 100\% \quad (2)$$

where  $I_{200}$  is the maximum intensity of the 200 lattice diffraction and  $I_{AM}$  is the minimum intensity between the 200 peak and the 101 peak.

#### 2.2.9. Morphology of GTP pretreated biomass

The topology of the water extracted GTPSG was imaged using a NeoScope JCM-5000 scanning electron microscope (SEM). Prior to analysis, biomass samples were vacuum dried at 40 °C over  $\text{P}_2\text{O}_5$  (5.4 mmHg) for 48 h. Sample biomass particles were then sputter-coated with 3.5–4.0 nm gold–palladium before imaging. Extractive-free sweet gum particles without GTP pretreatment were used as reference samples.

### 3. Results and discussion

Compositional analysis of non-pretreated and GTP pretreated sweet gum (SG) used in the enzymatic saccharification revealed significant changes as a function of severity level (Table 2). An

**Table 2**

Compositional analysis wt% of non-pretreated and GTP pretreated biomass for enzymatic saccharification with the standard deviation shown in parentheses. Note, composition is based on relative content within biomass.

Sample ID	Glucan %	Xylan %	Klason lignin%	Arabinan %	Galactan %	Mannan %
SG control	40.11 (0.11)	20.61 (0.07)	21.87 (0.17)	0.58 (0.01)	0.56 (0.01)	1.95 (0.00)
Water extracted GTPSG 4	47.60 (0.16)	21.25 (0.05)	23.61 (0.18)	0.35 (0.00)	0.32 (0.00)	1.87 (0.01)
Water extracted GTPSG 8	47.23 (0.54)	20.14 (0.33)	23.94 (0.33)	0.14 (0.00)	0.18 (0.00)	1.54 (0.01)
Water extracted GTPSG 9	49.00 (0.26)	20.71 (0.10)	24.72 (0.24)	0.10 (0.01)	0.15 (0.00)	1.39 (0.04)
Solvent extracted GTPSG 4	49.00 (0.12)	21.58 (0.11)	19.15 (0.21)	0.35 (0.00)	0.31 (0.00)	1.90 (0.03)
Solvent extracted GTPSG 8	56.74 (0.82)	24.16 (0.63)	12.90 (0.31)	0.15 (0.01)	0.18 (0.00)	1.85 (0.09)
Solvent extracted GTPSG 9	58.41 (0.32)	24.97 (0.41)	11.68 (0.02)	0.09 (0.00)	0.13 (0.01)	1.81 (0.08)
Alkaline extracted GTPSG 9	83.65 (0.17)	9.34 (0.01)	8.43 (0.36)	0.08 (0.00)	0.14 (0.01)	2.43 (0.01)

additional mass balance study revealed that cellulose was fully conserved after GTP processing and after subsequent extractions (Zhang et al., 2015). The water extracted GTP biomass still contained a significant amount of lignin relative to the solvent extracted GTP biomass (Table 2). However, GTP biomass had a major disruption of the native cell wall network as evidenced by reduced arabinan, galactan, and mannan with increasing severity parameter (Table 2). Subsequent solvent extraction reduced the concentration of lignin in the GTP biomass, especially at high GTP severities. Further extraction using aqueous alkali resulted in GTP biomass with only 20% of the initial xylan in the wood and an additional ~15% removal of the initial lignin, resulting in a cellulose-rich substrate. It is important to note that this extraction procedure is not suggested to be scaled; the methods and solvents were utilized for analytical purposes only. This issue is because aqueous dioxane provides a route to fractionate lignin with the same solvent used in ball milled near native lignin extraction, while 1.0 M NaOH is used in the literature for xylan extraction from wood.

### 3.1. Enzymatic digestibility of biomass after GTP pretreatment and water washing

Enzymatic saccharification of water extracted GTP biomass revealed increased digestibility as a function of time (Fig. 1a). In contrast to the minimal digestibility of non-pretreated SG, the digestibility of GTP pretreated SG was greatly enhanced. There was a positive trend of increased digestibility when processing the biomass at higher severity treatments (Fig. 1a). A maximum 20-fold increase was observed at severity level 9 [ $\log(R_0) = 5.20$ ] for the 72 h hydrolysis, corresponding to 68% digestibility of the original material. Moreover, water extracted GTP biomass still contained similar amounts of lignin and xylan in amounts comparable to the non-pretreated SG (Table 2). Hence, the enzymatic digestibility was enhanced after GTP processing at more severe levels, but the overall composition was only slightly altered, related to the loss of the side-chain hemicelluloses responsible for lignin-carbohydrate (LCC) linkages. At the highest levels more than three quarters of the arabinan and galactan were removed

by the GTP process. Hence, GTP treatment caused disruption of the cell wall network and this correlated with the degree of digestibility of the cellulose.

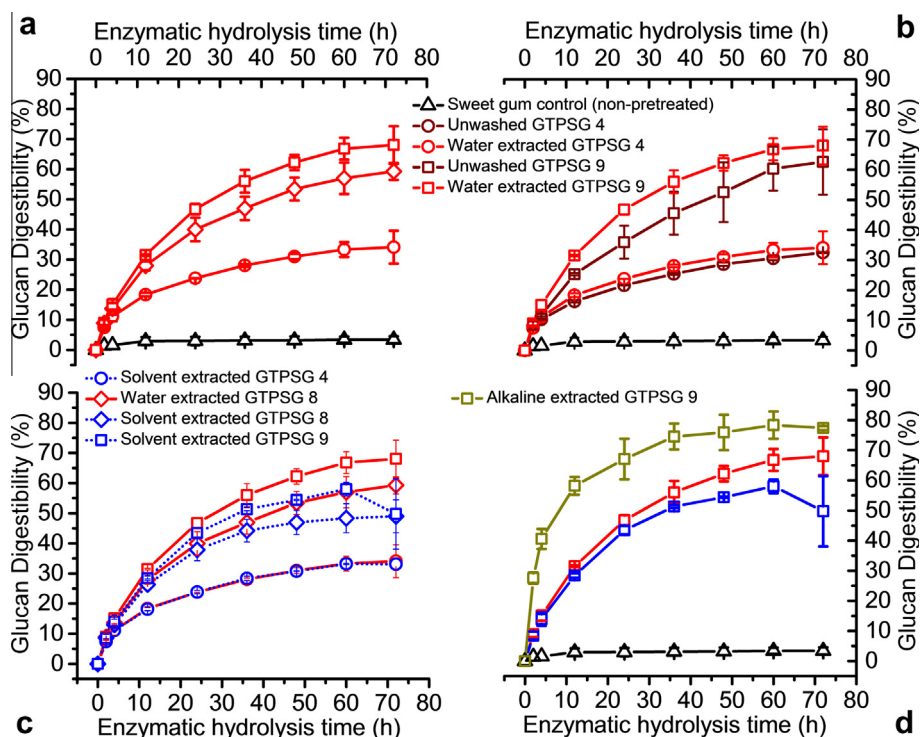
A control sample of Whatman cellulose CF 11 (cotton derived fibrous substrate) was processed under similar conditions to the SG. As seen in Fig. 2, the GTP pretreated cellulose had only a 2-fold increase in glucan digestibility after 72 h of hydrolysis (Fig. 2) compared to the 18-fold increase in biomass digestibility of the sweetgum under the same GTP conditions (Fig. 1a). Thus, the GTP pretreatment did not impact the structural characteristics for the conversion of the pure cellulose sample to the same degree as in SG. The initial saccharification rate is similar for the two samples in the first 5 h, while the non-pretreated sample quickly levels off. After 12 h the GTP cellulose sample continues to be converted into glucose at a reduced rate. A recent study on the investigation of Avicel hydrolysis revealed that amorphous cellulose at the surface is readily hydrolyzed and then hydrolysis occurred in a layered fashion (Gao et al., 2014). The incremental conversion of the Whatman cellulose powder suggests a similar trend and the difference with GTP cellulose may lie in additional overall surface area of the fiber.

### 3.2. XRD crystallinity of water-extracted GTP biomass and cellulose

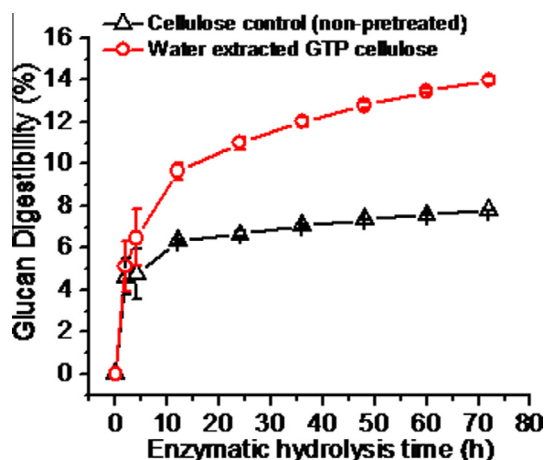
A comparison of the crystallinity index (CrI) of the GTP pretreated cellulose and biomass revealed surprising results (Table 3). The pure cellulose had a relatively high CrI of 72.6% as expected, but increased to 92% after GTP pretreatment. The SG had a lower CrI than the control sample, but also had a slight increase of crystallinity after the GTP pretreatment increasing from 54% to 60% after GTP pre-treatment. A similar increase in crystallinity has been reported for steam-exploded biomass (Ibrahim et al., 2010) because of the degradation of amorphous cellulose and significant removal of hemicellulose. In the present case, limited main chain hemicellulose loss was observed during the GTP processing and water washing. As hemicellulose branches that bond to lignin were cleaved during processing and the material was exposed to high heat and shear in a plasticized state, the enhanced mobility of any amorphous zones would allow these cellulose chains to orient in the shear field and crystallize, a common phenomenon during the extrusion processing of high molecular weight polymer molecules. Furthermore, additional crystallinity changes of GTP treated biomass would therefore most likely be partially restrained by the presence of xylan and mannan. The more digestible biomass substrate after GTP pretreatment confirms that the core chain crystallinity is not a dominant reason restricting the biomass saccharification and may be related to a surface accessibility effect of the native structure.

### 3.3. SEM morphology structures of water-extracted GTP biomass

Particle size, porosity and surface area have been proposed as the primary properties which govern biomass saccharification (Puri, 1984). Extractive-free SG particles were pre-milled and screened to a particle size of 250–420  $\mu\text{m}$  for the GTP pretreatment and morphological observation of biomass using SEM before and after GTP pretreatment was performed to qualitatively evaluate the changes in biomass structure associated with GTP processing. The vascular elements were fractured transversely through the cross-section of the non-pretreated SG during the milling process in sample preparation, whereas the longitudinal surface was split to reveal a relatively smooth and well-preserved lumen surface. After a moderate severity GTP pretreatment [ $\log(R_0) = 4.14$ ], smaller wood particles were produced. The GTPSG 4 sample showed that the tracheids and vessel elements of the wood particle disintegrated leaving residual ray parenchyma, tracheids, vessels and



**Fig. 1.** Enzymatic hydrolysis of (a) non-pretreated and water extracted GTP biomass at different severity levels; (b) of non-pretreated and GTP pretreated SG before and after water extraction at different severity levels; (c) of water and solvent extracted GTP biomass at different severity levels; and (d) of water, solvent and alkaline extracted GTP biomass at the highest severity level 9.



**Fig. 2.** Enzymatic hydrolysis of non-pretreated and water extracted GTP cellulose at severity 8.

**Table 3**

XRD crystallinity index of water extracted biomass and pure cellulose samples after GTP pretreatment. Standard deviation shown in parentheses.

Sample ID	$\log(R_0)$	CrI
Cellulose	–	72.6 (1.6)
GTP cellulose	5.03	92.0 (0.1)
SGC <sup>*</sup>	–	53.7 (1.8)
GTPSG 1	3.55	58.6 (2.6)
GTPSG 4	4.14	63.9 (1.5)
GTPSG 8	5.03	59.5 (0.1)
GTPSG 9	5.20	62.7 (3.1)

<sup>\*</sup> Non-pretreated sweet gum.

fiber tissue. Ray tissue was more susceptible to treatment and degraded more readily as would be expected since the ray parenchyma cell walls are relatively thin compared to fiber cell walls. However, with increasing GTP severity to  $\log(R_0) = 5.03$  (GTPSG 8), more cellular debris with size around 10  $\mu\text{m}$  is observed as the ray tissue was further disintegrated. At the highest levels of treatment, the initial wood particle was severely destroyed into fragments.

Additionally, the longitudinal cellulose fibers were observed to separate from wood bundles, especially for the pretreated wood at the highest severity conditions, because of the severe disintegration of the wood surface structure. As a result, the porosity and surface area were increased, which was demonstrated by the Brunauer–Emmett–Teller (BET) surface area analysis tripling in specific surface area (Zhang et al., 2015). Thus, the cellulose microfibrils become more exposed rendering the polysaccharide structure more susceptible to enzyme mediated hydrolysis.

### 3.4. Influence of water washing on biomass saccharification

Inhibitory by-products formed during pretreatment could potentially impede both the enzymatic hydrolysis and fermentation steps. Several types of inhibitors have been proposed as either toxic to fermentation organisms or that have the ability to competitively or non-competitively inhibit enzyme action (Bhatt and Shilpa, 2014). These include monosaccharides, furan derivatives associated with the degradation of pentoses or hexoses, phenolic compounds from degraded lignin, and organic acids such as acetic acid, formic acid, and levulinic acid (Kim et al., 2011). The concentration and type of inhibitors greatly depend on the pretreatment method, severity, and acid loading. Several pretreatment methods have been demonstrated to produce these inhibitors. For instance, steam-explosion, hydrothermal and dilute acid pretreatment generate inhibitors and water washing is needed to remove these compounds before the enzymatic digestion (Meshartree and

Saddler, 1983). Thus, an effective pretreatment for production of digestible biomass substrate should also avoid the formation of inhibitors to as great an extent as possible.

Enzymatic hydrolysis of GTPSG before and after water extraction was conducted to reveal any inhibitory effects of the GTP processing on the enzyme cocktail used. At a moderate GTP severity [GTPSG 4,  $\log(R_0) = 4.14$ ], the enzymatic hydrolysis profile of water extracted GTPSG closely tracks that of the unwashed GTP biomass during the 72 h hydrolysis (Fig. 1b). At the most severe GTP processing conditions [GTPSG 9,  $\log(R_0) = 5.20$ ], the final enzymatic digestibility of the unwashed GTPSG was comparable to that of the water extracted biomass at the end of the 72 h hydrolysis period. Although the hydrolysis rate decreased in the unwashed GTPSG 9 sample after the first 4 h, this lag recovered by the 72 h period to be statistically the same as the water extracted sample. These results indicate no significant inhibitory compounds that would affect the enzyme cocktail used were produced during GTP pretreatment, especially when using low and moderate GTP conditions. Although GTP processing for digestibility occurred at a similar, or even higher, severity than steam-exploded or hydrothermal pretreatments, glycerol did not behave in the same manner as auto-ionized water in steam pretreatment, where hydronium ions catalyze the degradation of glycosidic bonds and acetyl groups in hemicellulose resulting in degraded furan compounds (Garrote et al., 1999). More than 80% of initial xylan was preserved after GTP processing demonstrating the mild thermal effect on the hemicellulose at these elevated temperatures. Additionally, glycerol left in the GTP pretreated biomass had no negative effect on the subsequent enzymatic hydrolysis and it was 5% by weight of the hydrolysis mixture. Thus, a water extraction (washing) step, which is widely conducted in steam pretreatment, is not necessary after GTP processing for hydrolysis. The GTP pretreated biomass can be potentially used directly for enzymatic hydrolysis. However, no fermentation studies were conducted as part of this research, so it is unknown if any inhibitory compounds may have been generated that would reduce the efficiency of organisms to ferment the released sugars.

### 3.5. Effect of delignification on GTP biomass saccharification

Lignin is known to impede biomass enzymatic saccharification by interfering with cellulose enzyme binding via non specific adsorption as well as limiting access to cellulose surfaces (Ooshima et al., 1990). Several studies (Varnai et al., 2010) have demonstrated that delignification can improve enzymatic saccharification. However, Rollin et al. (2011) proposed that overall increased cellulose accessibility was a more important factor in enhancing saccharification than delignification. In the present study, water extracted GTP biomass was solvent extracted to reduce lignin content and the partially delignified, solvent-extracted GTP biomass was then used as a substrate for enzymatic saccharification. With increased GTP pretreatment severity, more lignin was removed during the mild solvent extraction resulting in a 50% reduction of lignin in the solvent extracted biomass residue compared to that in the water extracted GTP biomass (Table 2). Additionally, no obvious hemicellulose loss was observed in this step and overall, relative xylan concentration increased in the pretreated biomass because of the loss of lignin. As indicated in Fig. 1c, the hydrolysis profile of the solvent extracted GTP biomass, at moderate GTP conditions [GTPSG 4,  $\log(R_0) = 4.14$ ], completely overlapped with that of water extracted biomass. The water extracted GTPSG 4 contained 23.6% of Klason lignin, whereas after solvent extraction, the lignin concentration decreased to 19.2% (Table 2). Although the relative percentage of lignin was partially reduced, the enzymatic digestibility was not further enhanced. With increasing GTP severity, the lack of effect of lignin removal

on biomass digestibility is more obvious. The initial hydrolysis rate of the GTP biomass before and after solvent extraction at severe GTP processing conditions [ $\log(R_0) = 5.03, 5.20$ ] was similar. Furthermore, after 12 h of hydrolysis, the saccharification rate decreased for the solvent extracted GTP biomass, resulting in a lower ultimate digestibility compared to that of GTP biomass before delignification. As demonstrated previously, over 60% delignification was obtained for high GTP severities after solvent extraction, leaving less than a half of the original lignin remaining in the solvent extracted biomass. Zhu et al. (2008), suggested that delignification over 50% could potentially cause biomass pores to collapse, resulting in decreased enzyme accessibility to cellulose. If the residual lignin bears surface chemistry favored by protein adsorption, or sterically hinders the attachment of cellulase enzymes or CMBs (Rollin et al., 2011) from redeposition or rearrangement, enzymatic saccharification could also be impeded even after delignification. The results demonstrate that in this study, but not always the case, delignification was not helpful in improving biomass saccharification.

### 3.6. Effect of alkaline extraction on GTP biomass saccharification

Glucuronoxylan is the major hemicellulose in hardwood, which normally assembles along the cellulose microfibrils interacting with cellulose through various intermolecular forces. In addition to lignin, xylan is viewed as a major barrier, blocking cellulase enzyme accessibility and limiting the initial hydrolysis (Li et al., 2013). Recent studies have demonstrated that removal of hemicellulose can enhance enzymatic hydrolysis by cellulases (Varnai et al., 2010). To explore this, an alkali extraction of 4% NaOH was performed to isolate polymeric xylan from the GTP pretreated biomass. At the highest GTP severity condition ( $\log(R_0) = 5.20$ ), 56% of the initial xylan was removed during the alkali extraction. The residual alkali-extracted biomass, with only 21% of initial xylan, was enzymatically hydrolyzed and results were compared with the digestibility of biomass before alkaline extraction (Fig. 1d).

Interestingly, a significant increase in the initial hydrolysis rate was observed for the alkali extracted GTP biomass compared to that for the water and solvent extracted GTP biomass (Fig. 1d). After the initial 2 h hydrolysis, the digestibility was 28% for the alkali extracted biomass but only 9% and 8% for the water and solvent extracted biomass, respectively. Using the target cellulose to glucose conversion of 60% for comparison, alkaline extracted biomass reached this point in 12 h, but it took 48 h for the water extracted GTP biomass. Thus, the extensive removal of xylan during the alkaline extraction process enables the conversion of the biomass substrate to one which is more susceptible to cellulase enzyme attack, which greatly increases the hydrolysis rate. Ultimately, 78% of cellulose digestibility was observed after 72 h of hydrolysis. Compared to the 68% digestibility of water extracted biomass, this number is not that impressive since biomass digestibility was already enhanced simply by GTP pretreatment. However, a significant increase in hydrolysis rate could be both a time-saving economic benefit, if the biofuel industry were further developed to include xylan isolated as polymeric byproduct.

Comparing the different glucose yields from the various starting materials, taking into account glucose release at various pretreatment and extraction stages, there is an increase in glucose yield as the severity of the pretreatment process going from 3.4% for the unprocessed control, to 34.3% for GTPSG4, and increasing to 60.9% and 70.2% for GTPSG8 and GTPSG9, respectively. The glucose yield decreased with solvent extraction for GTPSG8 and GTPSG9 to 50.0% and 53.6%, respectively, primarily due to hindered hydrolysis (Fig. 1c). The total glucose yield increased subsequently with alkali extraction to 77.7% for GTPSG9. Overall, the yield data shows that higher temperatures and longer processing times provides the

most disruption to the cell wall and access to the cellulose for hydrolysis. For GTPSG8 and GTPSG9 there were only four minute difference in processing (Table 1) with nearly identical composition (Table 2); however the additional processing time yielded approximately 10% increase in glucose. From this data it appears the GTP conditions are not fully optimized for glucose recovery and further processing limits need to be established.

#### 4. Conclusions

A new pretreatment method that involved heating and shearing biomass in the presence of glycerol, up to 240 °C, was utilized to probe the impact of structure and composition on enzyme catalyzed cellulose digestibility. Disruption of the cell wall without a noticeable change in chemical composition, besides the minor side chain hemicelluloses, significantly impacted the enzymatic conversion of cellulose into glucose. Furthermore, higher crystallinity induced from pretreatment had no negative impact on conversion rates. The removal of polymeric xylan from the GTP processed biomass through alkaline extraction was the dominant factor in increasing hydrolysis rate of the pretreated biomass.

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