ETHANOL ORGANOSOLV PRETREATMENT OF *TYPHA CAPENSIS* FOR BIOETHANOL PRODUCTION AND CO-PRODUCTS

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Typha capensis (TC), a highly prolific, invasive grass found in many parts of the world, is a common pest that grows in waterways, but it may be a good lignocellulosic substrate for bioethanol production. Sulfuric acid-catalyzed ethanol organosolv pretreatment was used to investigate the possibility of harnessing the benefits of both fermentable sugars and lignin by reacting at varying defined severity levels. It was observed that TC polysaccharides were particularly susceptible to hydrolysis, which was associated with the formation of a large amount of pseudo-lignin due to the degradation of sugars. Pseudo-lignin had a negative impact on enzymatic hydrolysis. At optimal conditions, the process enabled the fractionation of TC into glucan-rich solid fractions with enhanced digestibility, recovery of organosolv lignin, and easily hydrolysable hemicellulose sugars in the liquid stream of pretreatment analytes. About 68.33% of the glucan in the raw TC was recovered, and 85.23% fermentable sugars from water-soluble and enzyme-hydrolyzed pulp were attained. Up to 67% of the initial lignin in TC was extracted as ethanol organosolv lignin (EOL).

Keywords: Typha capensis; Ethanol organosolv; Pretreatment; Lignin; Fermentable

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INTRODUCTION

There is a rapidly increasing trend in energy demand occasioned by changes in human lifestyle and rapid population increases. Because of the non-renewable nature of fossil fuels, increasing greenhouse gas emissions, and the risks associated with price fluctuation in international markets, it is apparent that the current global dependence on fossil fuels is not sustainable. The search for renewable alternatives is therefore imperative and has been a focus of considerable research. Biomass has the potential to be a renewable energy source that is highly productive, carbon neutral, easy to store and transport, and can be directly converted to transport fuels such as ethanol (Dwivedi *et al*. 2009). Current production of bioethanol relies on ethanol from starch and sugars from corn, sugar cane, and cassava. However, the cost of making ethanol from these crops is high, as these feedstocks are expensive (Pan *et al*. 2004). In addition, there has been considerable debate about their sustainability, especially as their use may undermine food security. Lignocellulosic biomasses offer great prospects as non-food, renewable alternatives that are widespread and abundant. Their annual production has been estimated to be 1×10^{10} tonnes worldwide (Sanchez and Cardona 2008).

The most widely investigated cellulosics as prospective feedstocks for bioethanol production are dedicated crops grown on arable lands, wastes from forest resources, and agricultural residues. However, the use of arable land for fuel crops will also affect food pricing and economics. In addition, forest and agricultural residues have alternative applications, such as pulp and paper, animal feed, house construction, direct combustion for cooking and heating, *etc*. This highlights the need to explore non-food lignocellulosics such as *Typha* grass, which grows on marginal lands, for bioethanol production.

Typha capensis (TC) is a fast growing and highly prolific invasive grass found in many parts of the world as a common pest that grows in wetlands (Voigt and Porter 2007). TC has special features that enable it to cope with both extremes of flood and drought (Voigt and Porter 2007). TC is abundantly available in most wetlands, and production can be sustained with this renewable source in places where they are locally available. Because it is inedible and not used as animal feed, there is no conflict with food security. The dry matter density of *Typha* varies with soil type, water depth, and availability of nutrients. Yield estimates for TC range from 6 to 20 tonnes of dry matter per hectare (Elbersen 2005). This lignocellulosic residue is thus being investigated for its susceptibility to hydrolysis for ethanol production.

The three main components of cellulosic biomass are cellulose, hemicelluloses, and lignin. Its native form, with three dimensional cross-links of a lignin network and strong hydrogen bonds within the polymer matrix, make hydrolysis of the cellulose within lignocellulosic biomass difficult (Xie and Zhao 2011). Hence, pretreatment is required to break the barriers and render the cellulose and hemicelluloses accessible for enzymatic hydrolysis to create fermentable sugars. Several pretreatment processes have been developed to enhance enzyme accessibility, among which are mechanical comminution, pyrolysis, microwave irradiation, steam explosion, sulfuric acid, sulfur dioxide, ionic liquids, organosolv, ammonia, aqueous lime, sodium hydroxide, and wet oxidation. Kumar *et al.* (2009) and Alvira *et al*. (2009) provided detailed reviews on this topic. Previous work on conversion of members of the *Typha* genus to bioethanol applied alkali pretreatment used NaOH (Zhang *et al.* 2010a), hot water pretreatment (Zhang *et al*. 2010b), and supercritical fluid extraction to convert *Typha* to liquid products using low boiling point organic solvents (methanol, ethanol, and acetone) catalyzed by NaOH and $ZnCl_2$ (Kucuk *et al*. 2005). Most of these methods focused on converting cellulose into fermentable sugars for ethanol production, while other valuable byproducts like lignin were not given due consideration. In light of the high cost of pretreatment and the need for the efficient utilization of available resources in a sustainable and environmentally friendly way, the most promising strategy is to integrate ethanol production into a biorefinery scheme in which lignin, hemicellulose, and extractive components of the lignocellulosic biomass are converted into valuable co-products (Pan *et al.* 2007). One of the investigated pretreatment methods that fits this model is ethanol organosolv, in which a high-quality lignin fraction with the possibility for use in several industrial applications is produced, in addition to a cellulosic substrate able to be easily enzymatically digested into fermentable sugars (Brosse *et al.* 2011). In order to compare the effect of this process using a combination of ethanol and water under acid catalyzed conditions, experiments were carried out under different defined severity factor levels in this study. The Severity factor (R_o) describes (Equation 1) the severity of pretreatment as a function of pretreatment time (t, min), temperature (T, ^oC) with a reference temperature T_{ref} of 100 ^oC (Montane *et al.* 1998):

$$\text{Log}(R_o) = \text{Log}[\text{texp}(T\text{-}T_{ref})/14.7] \quad (1)$$

When the effect of pH is considered, the term combined severity (CS) is used and defined (Equation 2) as:

$$CS = \text{Log}R_o - \text{pH} \quad (2)$$

The organosolv pretreatment process involves extraction of lignin from lignocellulosics. Pretreatment in a temperature range of 100 to 250 °C has been attempted with a large number of organic or aqueous-organic solvents, either catalyzed or un-catalyzed (Zhao *et al.* 2007). The most widely used alcohols for organosolv pretreatment are lower molecular weight aliphatic alcohols, with normal primary alcohols as better agents than secondary or tertiary alcohols for delignification (Zhao *et al.* 2009). During high temperature operations (185 to 210 °C), organic acids are released from the biomass, which catalyzes the rupturing of the lignin-carbohydrate complex (Duff and Murray 1996).

Formation of pseudo-lignin, an aromatic material that yields a positive Klason lignin value, has been reported during dilute acid pretreatment of lignocellulosic materials (Hu *et al*. 2012; Li *et al*. 2007). Researchers have explained the phenomenon to be due to repolymerization of polysaccharide degradation products, like furfural and hydroxymethyl furfural, and/or polymerization with lignin to form a lignin-like material called pseudo-lignin. In a recent study by Hu *et al*. (2012) in which dilute acid pretreatment of hybrid poplar was reported, a similar trend was observed where acid-insoluble lignin (pseudo-lignin) increased with an increase in pretreatment severity. On the mechanisms of pseudo-lignin formation, Hu *et al*. (2012) suggested that 1,2,4-benzenetriol (BTO) and 3,8-dihydroxy-2-methylchromone (DMC) are the key inter-mediates for pseudo-lignin formation during dilute acid pretreatment. They suggested that the reaction involves poly-condensation and/or a polymerization reaction, because BTO can rapidly react with HMF and/or furfural via electrophilic substitution to produce a three-dimensional polymer under acid catalysis. Thus, pseudo lignin is not only lignin but includes all acid-insoluble compounds such as ash and degraded sugars.

In the current research, sulfuric acid-catalyzed ethanol/water organosolv pretreat-ment was investigated to explore the optimum conditions for the recovery of lignin and substrates and to minimize the formation of pseudo-lignin. The enhanced hydrolysis of *Typha capensis* was assessed using the enzyme celluclast from *Trichoderma reesei*, complemented by cellobiase from *Aspergillus niger*. Lignin was recovered as a fine precipitate by flashing the pulping liquor to atmospheric pressure, followed by rapid dilution with water (Pan *et al.* 2004) and was characterized by using solution-state ^{13}C nuclear magnetic resonance (NMR) analysis and gel permeation chromatography (GPC).

EXPERIMENTAL

Materials

Typha capensis samples were obtained from canals in the campus of the Asian Institute of Technology (AIT). The National Renewable Energy Laboratory (NREL) protocol (Hames *et al.* 2008) was used for sample preparation. Cut samples were dried at 105 °C to determine the initial moisture content. Samples used for further analysis were

prepared by drying to 6% moisture content at 45 °C and milled to ≤ 2 mm using a Polymix Hammer Mill PX-MFC 90D at maximum 6000 rpm. The hammer mill, equipped with three sets of hammers, was operated at 4500 rpm, and 2 mm screens were used. Dried and milled samples were packed in sealed polyethylene bags and kept at room temperature until further use.

Enzymes used include cellulase (celluclast) produced by *Trichoderma reesei* (product number C2730) and cellobiase liquid from *Aspergillus niger* (Novozymes 188 C6105), supplied by Sigma Aldrich. Other chemicals used include absolute ethanol, sulfuric acid, sodium hydroxide, potassium sodium tartrate, 2-hydroxy-3,5-dinitrobenzoic acid, acetic acid glacial, and ready-to-use glucose oxidase/peroxidase (bioMérieux product number 61270).

Methods

Sulfuric acid-catalyzed ethanol/water organosolv pretreatment

An aqueous ethanol/water solution in a 65:35 (v/v) ratio was prepared and used as diluent to prepare different concentrations (0.2 to 1.5% w/w) of sulfuric acid. 6.3 g dried mass of *Typha capensis* was treated with different sets of acid solvents to obtain a total solids ratio of 11% (w/v). Treatments were carried out in a 1.0 L Parr reactor equipped with a Parr 4842 temperature controller with a temperature increase rate of about 5 °C per minute, reaching a pressure in the range of 18 to 28 bar, depending on the acid concentration and desired reaction temperature. Cooling was aided by decoupling the heating jacket and immersing the reaction vessel in cool water. Under these conditions, the vessel cooled down to about 40 °C within 5 to 10 minutes. Pretreated TC was washed three times using 12 mL of the ethanol/water solution (4:1 proportion, v/v) at about 60 °C, and then separated by filtration under reduced pressure using a Buchner funnel (Brosse *et al.* 2009). The precipitate was oven dried overnight at 40 °C and preserved in sampling tins until further analysis. The filtrates were combined and distilled water was added until the volume reached 300 mL. An aliquot (1 mL) from this was preserved for sugar analysis, while the remaining portion was centrifuged at 2000 rpm for 20 minutes to precipitate the ethanol organosolv lignin (EOL). The recovered EOL was further washed with distilled water and oven dried overnight at 40 °C and the dried samples were preserved for further analysis.

Enzymatic hydrolysis

Preliminary analyses were conducted to determine the activity of the enzymes using procedures outlined by Ghose (1987). Cellulase activity (FPU) was found to be 77 U/mL, while cellobiase activity (CBU) was 317.24 U/mL.

Consequently, to establish the optimal combinations of the two enzymes, tests were performed using different combinations of enzyme loadings (5 to 50 FPU and 30 to 90 CBU) for incubation periods of up to 72 hours. The optimal loading combination was found to be 35 FPU of cellulase and 70 CBU of cellobiase per gram of substrate. Reducing sugar yields increased with time; however, there was minimal increase of RSY after 46 hours, and therefore 46 hours was taken as the optimal trade-off incubation residence time for enzymes hydrolysis. These enzyme levels were used to hydrolyse pretreated TC in 40 mL Erlenmeyer flasks in pH 5 acetic acid buffer at 2% solids content (w/v) and incubated at 50 °C in an air-bath shaking incubator at 150 rpm. In order to prevent microorganism growth during hydrolysis, sodium azide was added to the slurry at 0.3% loading (w/v). About 0.2 mL samples were drawn periodically, diluted in 1 mL

distilled water, and placed in a 1.5 mL Eppendorf safe-lock tube. In order to arrest enzymatic activity, the samples in the Eppendorf tubes were placed in boiling water at 97 °C for 5 minutes, cooled, and kept in a refrigerator at -4 °C until further analysis.

Analytical Methods

Reducing sugar, glucose, and lignin tests

Reducing sugar yields from the hydrolyzates were determined using the 3,5-dinitrosalicyclic acid method by Miller (1959), with a slight modification as described by Nigam and Ayyani (2007). Glucose was estimated using ready-to-use (RTU) glucose oxidase/peroxidase from bioMérieux, using the test protocol recommended by the company.

Klason lignin (KL) in untreated raw and treated TC samples was determined using a two-step acid hydrolysis method based on the National Renewable Energy Laboratory (NREL) protocol (Sluiter *et al.* 2008) with a slight modification. Aliquots of the hydrolyzates from this determination were preserved for sugar analysis using HPLC, as described below.

For all pretreated TC samples under different severity conditions, pseudo-lignin (ΨL) contents were calculated. This calculation is based on mass balance of the lignin component with reference to initial mass of lignin (^{0}KL) contained in the raw TC before pretreatment. The sum of KL, EOL and ASL should equal to ^{0}KL, but in all the assays these sums were above ^{0}KL. The excesses were designated as pseudo-lignin. These were determined using the relation,

$$\Psi L = KL + EOL + ASL - {}^0KL \quad (3)$$

where KL = acid-insoluble lignin, ASL= acid-soluble lignin, EOL = lignin recovered in the pretreated pulp as ethanol organosolv lignin, and ^{0}KL = acid-insoluble lignin in the original raw TC (18.13). All values are given in g/100 g of raw TC.

Values of reducing sugar yields (RSY) were calculated in g/100 g of pretreated substrate using the relation,

$$RSY = (R1*V/SL)*100 \quad (4)$$

where *R*1 is the reducing sugar yield concentration in mg/mL, *V* is the volume of liquid in the aqueous hydrolysate solution in mL, and SL is the weight of pretreated substrates in the hydrolysate in mg on a dry basis. One hundred is the conversion factor to obtain results in g/100 g.

Acid-insoluble lignin (AIL), or KL, was calculated based on the relation,

$$\%AIL = [(W_{cair}-W_c)/W_s]*100 \quad (5)$$

where W_{cair} is the dry weight of crucible and acid insoluble residue, W_c is the oven-dried weight of the empty crucible, and W_s is the oven-dried weight of the sample. All values were measured in grams.

The acid-soluble lignin content was measured using UV-visible absorbance at 320 nm, and values were calculated based on the relation given below.

$$\%ASL = [(UV_{abs} * Volume_{filtrate} * dilution)/(\epsilon * ODW_{sample})]*100 \quad (6)$$

Here, ODW_{sample} is the oven dry weight of sample, UV_{abs} is the absorbance of the filtrate sample at 320 nm, and ϵ is the absorptivity of biomass at a specific wavelength. TC have a close similarity to the morphology and chemical composition to corn, so a value of 30 L/g *cm for 320 nm corresponding to the absorptivity for corn stover from the list provided in the protocol (Sluiter *et al*. 2008) was assumed for TC.

Measurement of monosaccharides and uronic acids using HP AEC-PAD

A Dionex ICS-3000 HPLC system was used to separate and quantify neutral sugar and uronic acids. The system consisted of a SP gradient pump, an AS auto-sampler, an ED electrochemical detector with gold working electrode, an Ag/AgCl reference electrode, and Chromeleon software version 6.8 (Dionex Corp, USA). The stationary phase used was a Carbopac PA20 (3 x 150 mm), Dionex column with a guard column (3 x 50 mm, Dionex), while the mobile phase was water, 250 mM NaOAc, and 1 M NaOH/20 mM NaOH. Monomers and uronic acids were separated using isocratic conditions and linear gradient elution. All eluents were degassed before use by flushing with helium for 20 minutes with a 250 mM NaOH solution and re-equilibrated for 10 minutes in the starting conditions. Samples were injected through 25 L full loop at a discharge rate of 0.4 mL/min and separations effected at 35 °C column temperature. The pulse sequence for pulsed amperometric detection consisted of potential +100 mV (0 to 200 ms), +100 mV integration (200 to 400 ms), -2000 mV (410 to 420 ms) +600 mV (430 ms), and -100 mV (440 to 500 ms).

Characterization of ethanol organosolv lignin (EOL)

Solution-state ^{13}C nuclear magnetic resonance (NMR) analysis was carried out for EOL samples after removing impurities by pentane extraction using a Soxhlet apparatus. NMR experiments were conducted on a Bruker Avance 400 spectrometer, operating at a frequency of 100.59 MHz. One hundred mg of EOL were dissolved in 0.50 mL of slightly heated DMSO-d6. The spectra for ^{13}C were acquired at 50 °C using an inverse gated decoupling pulse sequence. Several scans for each sample were collected with a pulse delay of 12 s (Robert 1992).

Gel Permeation Chromatography (GPC) was used to determine the number average (M_n) and weight average molecular weight (M_w) of extracted lignin after acetylation to allow its dissolution in tetrahydrofuran (THF). Twenty mg of lignin was dissolved in 1 mL of acetic anhydride/pyridine mixture in a 1:1 proportion and stirred for 24 h at room temperature. Twenty-five mL of ethanol was added to the reaction mixture, kept for 30 minutes, and then evaporated using a rotary evaporator. Subsequently, ethanol was added and evaporated 4 times to ensure complete removal of acetic acid and pyridine from the sample. This was followed by dissolving acetylated lignin in 2 mL chloroform and adding the mixture dropwise to 100 mL diethyl ether and then centrifuging. Dimethyl ether was then used to wash the precipitate 3 times, which was then dried under vacuum at 40 °C for 24 h. GPC lignin analysis was performed using a Dionex UltiMate 3000 HPLC system consisting of an auto-sampler and a UV detector, with THF as eluent. The derivatized lignin was dissolved in THF (1 mg/mL), and the solution was filtered through a 0.45 µm filter. Twenty µL of the filtered solution was then injected into the HPLC system, and concentrations were measured using a UV detector at 280 nm. Standard polystyrene samples were used to construct a calibration curve. Data were stored and analyzed using Chromeleon software version 6.8 (Dionex Corp, USA).

Determination of furan

The formation of inhibitory products mainly containing furfural and 5-hydroxy-methyl furfural (HMF) after the pretreatment reaction stage was monitored as total furans in mg/L, as estimated by applying the method developed by Martine *et al*. (2000). Aliquots of the filtrates were diluted 1000-fold, and absorbance was measured at 284 nm and 320 nm. The formula developed by Martinez *et al*. (2000) was used to estimate the total furans in mg/L.

$$F_t = (A_{284} - A_{320} - 0.056)/0.127 \quad (7)$$

where F_t = total furans (furfural + HMF) in mg/L. A_{284} and A_{320} are the absorbances of each sample taken at 284 nm and 320 nm, respectively.

Statistical analysis

All experiments in this study were carried out in 2 or 3 replications, and analytical errors were calculated based on these replications. The reported results are the averages of the replications. The standard curves used for reducing sugar and glucose yield calculations had coefficient of determination (R^2) values of 0.9903 and 0.9874, while values for the standard curves of cellulase and cellobiase activity determination and characterization were 0.9816 and 0.9276, respectively.

The maximum error for Klason lignin and carbohydrate measurement of treated TC as calculated from replicate samples was 10.75% (standard deviation). Sugar yield data following enzymatic hydrolysis of treated samples were reported as the reducing sugar yield. There was a maximum error of 8.9% and that of glucose yield was 15.72% (standard deviation, respectively).

RESULTS AND DISCUSSION

The composition of the *Typha* grass samples used in this study was initially determined in terms of cellulose, hemicelluloses, and lignin. The values were compared to those of other lignocellulosic residues that are being considered for ethanol production by other researchers (Table 1). Also, based on a two-stage exhaustive extraction, TC is composed of about 21.31% extractives, consisting of both water and alcohol soluble components. These are low molecular weight organic substances such as resin (terpenes, lignans, and other aromatics), fats, waxes, fatty acids and alcohols, terpentines, tannins, and flavonoids, (Sluiter *et al.* 2008). Other components include ash (about 4%) and proteins (not quantified).

There was a difference in cellulose level of up to 10%, even within the *Typha* grass data. This is because the composition depends on the soil and water conditions, climate, maturity of the grass, *etc*. Broadly, however, the concentrations of the three components were within the range expected. The bonding and interactions between these components are usually different in the different residues; hence, they require different types of treatment to make the cellulose components accessible to hydrolysis and retain the quality of the lignin that can be separated. These parameters were studied for different levels of severity using the organosolv pretreatment method.

Table 1. Comparison of Composition of Other Lignocellulosic Biomasses with *Typha capensis*

Lignocellulosic material	Cellulose	Hemicellulose	Klason lignin
Typha capensis[a]	36.25	16.35	18.13
Typha (cattails)[b]	32.3	18.9	20.7
Typha (cattails)[c]	34.3	11.6	26.4
Typha (reed)[d]	47.6	15.8	21.9
Performer switchgrass[e]	31.99	21.5	21.37
Switchgrass[f]	32.0	21.5	21.4
Wheat straw[g]	36.3	21.1	25.5
Wheat straw[h]	39.8	27.3	22.6

NB: Composition in % of initial biomass, dry basis. [a]This study, [b]Zhang *et al*. 2010a, [c]Zhang *et al*. 2010b, [d]Kucuk *et al.* 2005, [e]Xu *et al*. 2010, [f]Xu *et al*. 2011, [g]Kootstra *et al*. 2009, [h]Kristensen *et al.* 2008

Chemical Composition of Organosolv-pretreated TC

Previous ethanol organosolv pretreatment studies examined how four parameters (temperature, reaction time, sulfuric acid concentration, and ethanol/water ratio) influenced the recovery of ethanol organosolv lignin (EOL) and the composition of solid and liquid fractions of the materials (Brosse *et al*. 2009; Pan *et al*. 2004). The results indicated that the temperature level and the sulfuric acid concentration significantly affected the pretreatment outcome, in terms of carbohydrates released and EOL recovered. The test conditions that yielded optimum values for TC were in the range of 170 °C to 190 °C and 0.5% to 1.2% w/w sulfuric acid concentrations, with 65% ethanol and 60 min reaction time. The selection of reaction conditions in the present study was therefore guided by these previous studies. Table 2 gives the details of the experimental conditions investigated and the results observed. The solid and water-soluble components were calculated as g/100 g of dry raw material (TC), taking into consideration the mass loss, and therefore represent the overall benefits of the process.

As seen in Table 2, raw TC is rich in glucan, xylan, and lignin, comprising 36.25, 11.49, and 19.06% of the dry biomass, respectively. Following pretreatment, almost all hemicellulose sugars (mainly xylans) were completely removed in all assays under the conditions studied. Residual hemicellulose sugars in the recovered pulp were found to be inversely proportional to the severity of the treatment, with a value of zero indicating 100% removal occurring at the maximum severity (assay R8). The maximum value of residual xylan in the solid fraction was 1.49 g/100 g of dry raw TC, corresponding to 13% of the total xylan in the biomass for assay R1, which had the least CS. This testifies to the fact that hemicelluloses are rather easily removed from the natural biomass and open to hydrolysis. In the case of *Typha*, the removal of hemicellulose sugars is much easier under mild severity conditions, compared to other biomasses, like *Miscanthus*, for example (Brosse *et al.* 2009). Almost all hemicelluloses were removed for assays R2-8 and well recovered in water-soluble (WS) fractions.

Table 2 shows how CS (a combination of reaction time, SA concentration, pH, and temperature) affects the response parameters with varying trends. While pseudo-lignin and furan formation and mass loss increased with increasing CS continually, RS and Glc yields increased from minimum CS to climax values of 78.12 and 60.89 g/100 g,

Table 2. TC Pretreatment Experimental Conditions Investigated and Effects on Composition of TC, Recovery of Lignin, and Enzymatic Hydrolysis

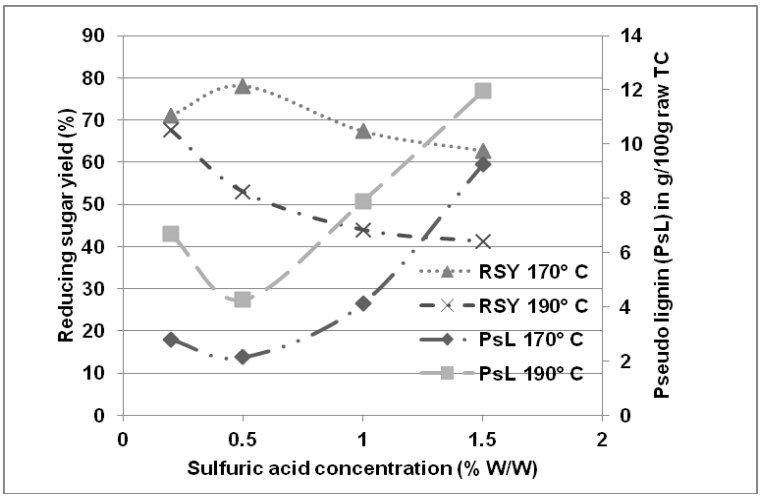
	ASSAY	TC	R1	R2	R3	R4	R5	R6	R7	R8
Con	T		170	190	170	190	170	190	170	190
	SA		0.2	0.2	0.5	0.5	1	1	1.5	1.5
	CS		0.76	0.87	1.36	1.47	1.86	1.97	2.14	2.25
	SL		11	11	11	11	11	11	11	11
	ML (%)		39.63	40.35	48.12	44.72	50.69	61.72	60.58	62.10
[a]Solids	KL	18.13	9.27	15.13	8.03	16.30	12.25	16.84	20.38	20.54
	ASL	0.93	0.31	0.34	0.22	0.29	0.32	0.29	0.32	0.33
	ΨL[c]		2.79	6.68	2.14	4.26	4.13	7.90	9.24	11.96
	Rha	0.22	0.02	0.00	0.01	0.00	0.00	0.00	0.00	0.00
	Ara	2.79	0.08	0.00	0.05	0.00	0.01	0.00	0.00	0.00
	Gal	1.64	0.08	0.00	0.06	0.00	0.01	0.00	0.00	0.00
	Glc	36.25	24.31	19.76	24.77	18.16	17.66	11.38	13.13	10.46
	Xyl	11.49	1.49	0.05	0.47	0.04	0.04	0.00	0.00	0.00
	Man	0.21	0.02	0.04	0.15	0.03	0.06	0.00	0.01	0.00
	GalUA	0.88	0.02	0.00	0.01	0.00	0.00	0.00	0.00	0.00
	GlcUA	1.26	0.11	0.02	0.05	0.01	0.01	0.00	0.01	0.00
[a]WS	EOL		11.34	9.34	12.03	5.80	9.69	8.89	6.66	9.22
	Furans		0.35	1.07	0.32	1.16	0.42	1.07	0.77	1.66
	Ara		1.47	1.03	1.03	0.80	1.54	0.38	0.70	0.82
	Gal		0.57	0.96	0.69	0.90	0.85	0.42	0.61	0.47
	Glc		0.22	1.91	0.23	1.65	0.17	2.60	1.09	2.98
	Xyl		5.32	5.97	5.25	5.85	4.39	4.26	2.24	3.75
[b]EH	RSY	35.47	71.10	67.54	78.12	53.01	67.35	44.01	62.70	41.24
	Glc		53.06	48.99	60.89	42.91	51.73	41.20	48.15	38.54

Con = pretreatment conditions; T = temperature in °C; SA = sulfuric acid concentration (% w/w); SL = total solid on dry basis (w/v%); Solids = solid fraction; WS = water-soluble fraction, ML = mass loss; KL = Klason lignin; ASL = acid-soluble lignin; EOL = ethanol organosolv lignin recovered; ΨL[c] = pseudolignin; EH = after enzymatic hydrolysis; RSY = reducing sugar yield; Glc = glucose; Rha = rhamnose; Ara = arabinose; Gal= galactose; Xyl = xylose; Man = mannose; GalUA = galacto-uronoic acid; GlcUA = glucuronoic acid; Furans = furfural + hydroxymethyl furfural. [a]Data of components in g per 100 g of dry raw material (TC); [b]data of components in % of pretreated, enzymatically hydrolyzed dry substrates

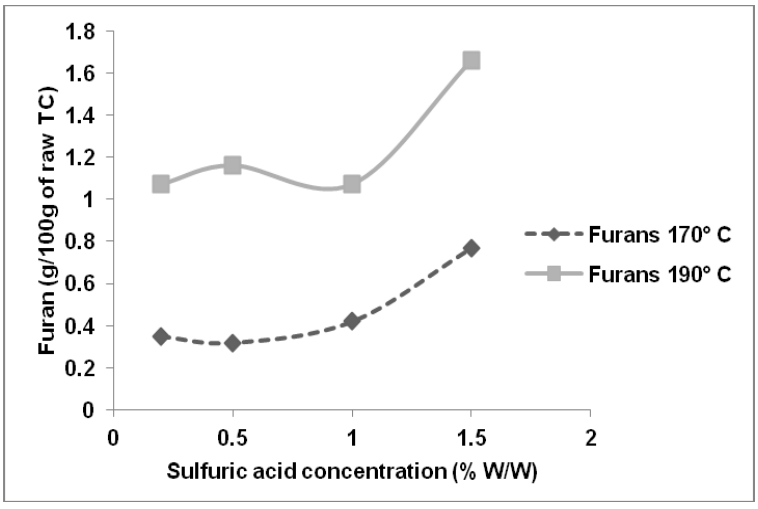
respectively at CS (1.36); thereafter RS and Glc yields declined to minimum values of 41.24 and 38.54 g/100g in that order at maximum CS (2.25). These trends correlate with the residual Glc in the solid substrates after pretreatment, where minimum values were observed at maximum severity. Unusually high losses were observed for both cellulose and hemicelluloses sugars in TC pretreatment compared to *Miscanthus* and lodgepole pine treated under similar conditions (Brosse *et al.* 2009; Pan *et al.* 2007). The phenomenon of thermal decomposition of carbohydrates in the presence of acidic conditions leading to the formation of hydroxymethylfurfural and furfural derived from hexoses and pentoses respectively; and later repolymerization and/or polymerization of these products with lignin to form pseudo lignin are well known (Wenzl 1970; Hu *et al.*

2012). Both were observed in this study with increasing trend as CS increases and as both are detrimental to enzymatic hydrolysis, higher CS led to lower fermentable sugars recovery following enzymatic hydrolysis. In addition, another contributory factor for the losses could be that TC has low crystallinity compared to other feedstocks. Further study is required to ascertain this.

With the release of lignin into the liquid stream during pretreatment, (ASL + recovered EOL), residual acid-insoluble lignin (KL) content in the solid fraction was expected to decrease. However, it was observed for assays R4-R8 that KL in the treated TC had values far greater than the lignin content in the raw TC (Table 2). This observation can be rationalized by the formation of pseudo-lignin, resulting from the polymerization of polysaccharide degradation products (Hu *et al*. 2012; Li *et al*. 2007). Hu *et al*. (2012) suggested that high temperatures and the presence of acid and oxygen are crucial conditions for pseudo-lignin formation during dilute acid pretreatment.



(a)



(b)

Fig. 1. (a) Effects of temperature and sulfuric acid concentration on pseudo-lignin formation and impact on reducing sugar yield; (b) effects of temperature and sulfuric acid concentration on furans formation

The pseudo-lignin content in the solid residues was estimated using the equation provided in the Analytical Methods section (see Table 2). Moreover, during treatment under acidic conditions, the pentoses and hexoses that are formed may be further degraded to furans (furfural and hydroxymethylfurfural). The furans content recovered in the water-soluble fraction is also given in Table 2. Figures 1a and 1b show the variations of the pseudo-lignin content in the pulps, as well as the furans content in the liquid stream relative to the temperature and the catalyst concentration. As expected, an increase in temperature of the treatment and/or of the catalyst concentration resulted in a higher concentration of furans in the water effluent and a higher pseudo-lignin content in the solid residue.

For assay R1, glucans (mainly cellulose) largely remained in the solid fraction of the treated TC. However, it appears that increasing the temperature of the treatment and/or the catalyst concentration largely increased the hydrolysis of glucans released into the liquid stream, leading to less recovery of glucans in the solid fraction. At high temperatures and high acid concentrations, some of the monomers could be released, but they were degraded to furans and pseudo-lignin components (Fig. 1a, b).

Fermentable Sugar Yield

Enzymatic hydrolysis of pretreated TC showed that the digestibility of TC was enhanced, compared to untreated TC (Table 2). Figure 2 shows the trend in fermentable sugars and reveals that at 170 °C, sugar release increased with decreasing sulfuric acid concentration to a maximum value of 78.12 g and 60.89 g/100 g of RSY and Glc, respectively, at 0.5% w/w SA concentration. The trend was followed by a decline at 0.2% w/w SA, to a value of 71.10 g and 53.06 g/100 g of RSY and Glc, in that order. Conversely, concentrations of furans and pseudo-lignin were observed to be low in the assay corresponding to the maximum RSY (Table 2 and Fig. 1a, b). The trend at 190 °C continued to 0.2% w/w SA concentration, having maximum values of 67.54 and 48.99 g/100 g of RSY and Glc, respectively.

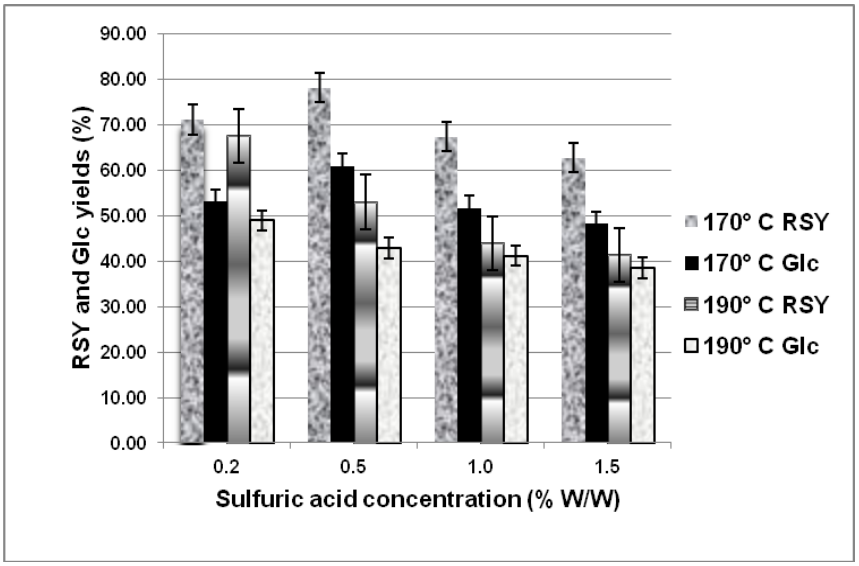


Fig. 2. Effects of temperature and sulfuric acid concentration on fermentable sugar (total reducing sugar (RS) and glucose (Glc)) yields under different ethanol-based (65%) organosolv pretreatment conditions

Assay 8, with the highest severity, revealed the highest lignin or pseudo-lignin content in the pretreated TC. There seemed to be a correlation between fermentable sugar yields and pseudo-lignin in the pretreated biomass, because a decreasing trend of RSY and Glc yields with increases in pseudo-lignin content was observed (Table 2 and Fig. 1a). Hu *et al*. (2012) have shown that pseudo-lignin is detrimental to enzymatic hydrolysis of pretreated biomass. The team observed pseudo-lignin/enzymes interaction, such that glucose yield declines as lignin and/or pseudo-lignin content increases. The mechanisms by which pseudo-lignin hinders effective enzymatic hydrolysis are similar to that of lignin, where lignin droplets tend to irreversibly bind to enzymes through hydrophobic interactions that cause a loss in enzyme activity (Donohoe *et al*. 2008). Pretreatment conditions therefore need to be optimized so as to remove or minimize the detrimental effect of pseudo-lignin on fermentable sugar release during enzymatic hydrolysis.

Ethanol Organosolv Lignin (EOL) Recovery and Characterization

Figure 3 shows the effects of temperature and acid concentration on the recovered EOL. About 32% to a maximum of 67% of lignin in *Typha capensis* was recovered as ethanol organosolv lignin, with variations in values dependent on the assay conditions. Recovery of EOL at 170 °C reached a maximum value of 66.83% of the initial lignin in the biomass and occurred at 0.5% w/w SA concentration (assay R3). Similarly, total fermentable sugar, consisting of reducing sugar yields from the solid fraction that was enzymatically hydrolyzed and the water-soluble fraction, amounted to 85.32% and occurred in assay R3.

At 190 °C, no regular trend of EOL recovery was observed with a change in acid concentrations; a maximum value of 51.9% of the total initial lignin in the biomass was recovered at 0.2% w/w SA concentration. Thus, optimal conditions for the products sought, namely fermentable sugars and EOL, occurred at 170 °C with 0.5% SA concentration of ethanol/water (65/35 ratio) organosolv pretreatment of *Typha capensis*.

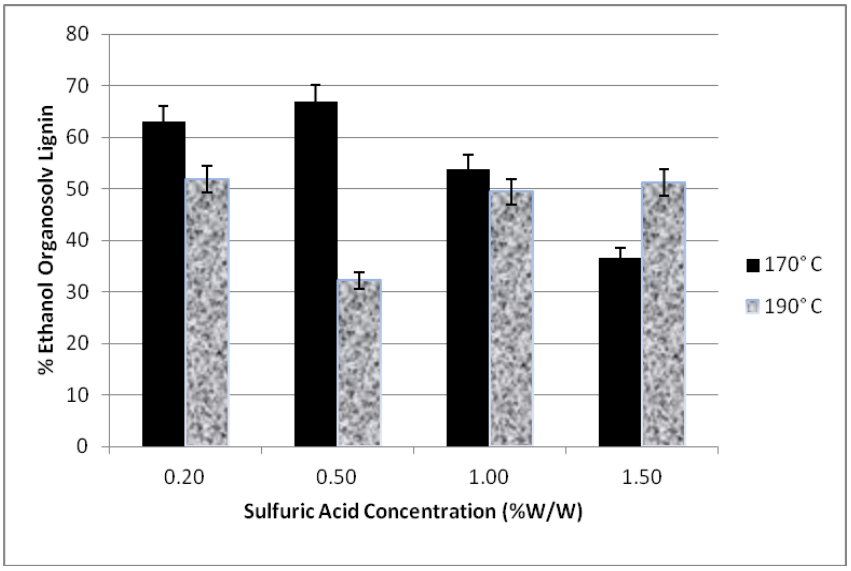


Fig. 3. Effects of temperature and acid concentration on EOL production in an organosolv pretreatment process using 65% ethanol

In order to have greater insight into the structural components of lignin recovered from TC, quantitative ^{13}C NMR spectroscopy experiments were conducted on the EOL recovered at optimum conditions (assay R3). The spectrum obtained is shown in Fig. 4, and previously assigned signals from the literature (Sanningrahi *et al.* 2008; Pu *et al*. 2006) were used to assign the observed resonance. Between 80 and 100 ppm, no signals were observed, indicating low sugar content in the extracted lignin. β-O-4 linkages, which constitute the main inter-monomeric linkage in lignin, can be usually detected by signals at 86.5 ppm, 73 ppm, and 61 ppm, which correspond to Cβ, Cα, and Cγ, respectively. As can be seen in Fig. 4, a lack of signals at 60 to 80 ppm is in accordance with an important scission of aryl-ether linkages. Thus, high lignin deconstruction was indicated, as beta-O-4 is largely affected. Signals observed at 171 to 173 ppm could be associated with carbon in carbonyl and carboxyl groups originating from aliphatic carboxyl and aliphatic esters (Yuan *et al*. 2011). The aromatic region is between 153 and 103 ppm, consisting of protonated aromatics (δ 123-106 ppm), condensed aromatics (δ 140-123 ppm), and oxygenated aromatics (δ 154-140) (El Hage *et al*. 2010). High peaks at 55.9 ppm are linked to the $–OCH_3$ groups in both syringyl and guaiacyl units.

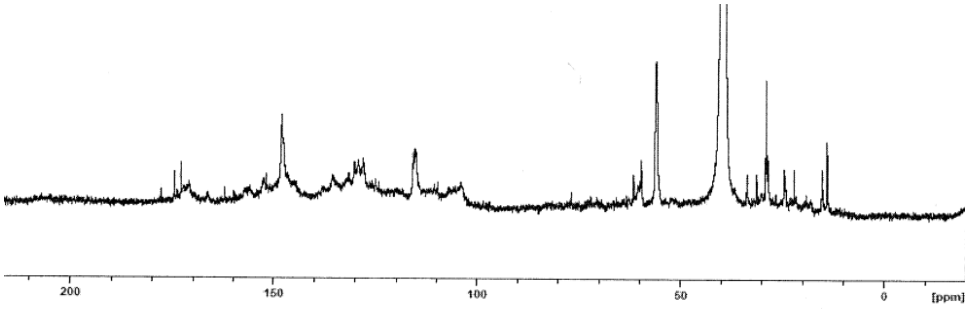


Fig. 4. ^{13}C NMR quantitative spectrum of EOL recovered from TC

Table 3 shows the weight average (M_w) and number average (M_n) molecular weights and the polydispersity index (M_w/M_n) of the EOL extracted from TC as revealed by gel permeation chromatography (GPC). This gives insight on the molecular weight distribution of the representative samples for two different conditions. Results in Table 3 reveal that both weight average (M_w) and number average (M_n) molecular weights were lower for higher severity of treatment.

Table 3. Effect of Combined Severity (CS) Factor on Weight Average (M_w) and Number Average (M_n) Molecular Weight and Polydispersity Index of EOL

Assay	*CS*	M_w (Da)	M_n (Da)	M_w/M_n
EL1 (R3)	1.36	2740	2535	1.1
EL2 (R7)	2.14	2495	2155	1.15

Similar trends of decreasing M_w as severity is increased were previously observed for the extraction of *Miscanthus* organosolv lignin (El Hage *et al.* 2010). It was concluded that organosolv treatment extensively cleaved some inter-unit bonds in lignin, and that this degradation was a function of treatment temperature and acidity of the medium. In this study, EOL from TC behaved in a manner similar to that of *Miscanthus*; however, TC pretreatment produced lower molecular weights at lower severity, indicating that lignin in TC is more susceptible to organosolv deconstruction than *Miscanthus*.

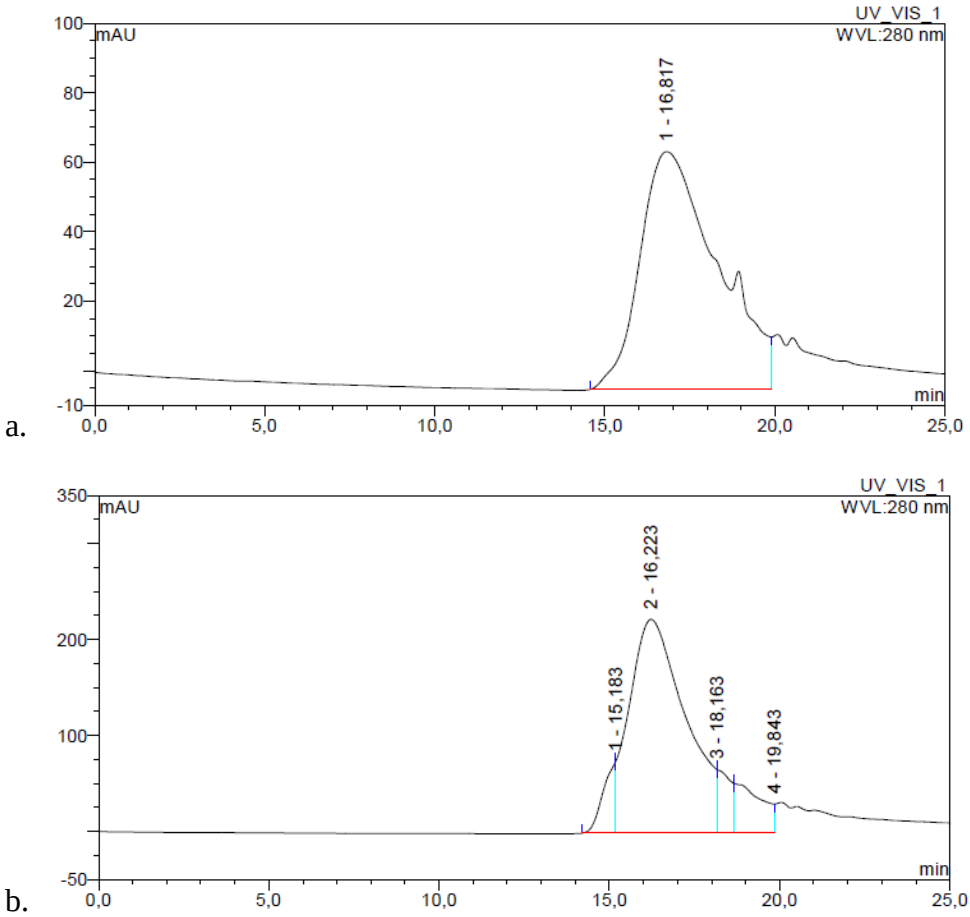


Fig. 5. Gel Permeation Chromatography Spectra of EOL extracted at CS: a. 1.36 and b. 2.14

The organosolv lignin extracted from TC exhibited relatively narrow molecular weight distribution, with a polydispersity index range of 1.1 to 1.15, compared to organosolv lignin extracted from fast growing poplar wood with a range of 1.63 to 1.9 (Yuan *et al.* 2010), or *Miscanthus* with a range of 1.4 to 2.6 (El Hage *et al*. 2010). This demonstrates the diversity in behavioral pattern among feedstocks.

CONCLUSIONS

1. Ethanol organosolv-based pretreatment of *Typha capensis* (TC) using sulfuric acid as a catalyst enabled separation of the main structural components of TC into solid pulp, resulting in increased digestibility to fermentable sugars, easily hydrolysable hemicellulose sugars, and separation of ethanol organosolv lignin (EOL) of good purity.
2. TC demonstrated the following favorable characteristics: it was easy to delignify even at low severity, it formed of low molecular mass EOL, and it contained easy-to-hydrolyze hemicelluloses and cellulose.
3. Formation of pseudo-lignin due to the degradation of sugars was observed, and since pseudo-lignin is detrimental to enzyme activity during hydrolysis, it is necessary to

carefully optimize the pretreatment conditions so as to remove or minimize this degradation product.

4. The low signal for carbohydrates and other impurities in the ^{13}C NMR sample of EOL implied that the recovered lignin is of high purity with potential for applications as biodegradable polymers, adhesives, and films.
5. The overall conclusion that can be drawn is that *Typha* grass, which grows uncontrolled, clogging waterways and ponds, has the potential to serve as a good substrate for bioethanol production and a source of pure lignin. The low severity conditions required to obtain pure streams of cellulose, hemicelluloses, and lignin of good quality can reduce the cost of pretreatment that is essential for the success of such biorefining processes.

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