DILUTE SULPHURIC ACID AND ETHANOL ORGANOSOLV PRETREATMENT OF *Miscanthus x Giganteus*

NICOLAS BROSSE, ROLAND EL HAGE, POULOMI SANNIGRAHI* and ARTHUR RAGAUSKAS*

Laboratoire d'Etude et de Recherche sur le Materiau Bois, Faculté des Sciences et Techniques, Nancy-Université, Bld. des Aiguillettes, F-54500 Vandoeuvre-lès-Nancy, France
**School of Chemistry and Biochemistry, Georgia Institute of Technology, Atlanta, GA 30332, USA*

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Three processes for the pretreatment of *Miscanthus x Giganteus* were compared, namely, dilute sulphuric acid treatment, an ethanol organosolv treatment and a two-step protocol involving a presoaking step prior to the ethanol organosolv treatment. The pretreatment assays were evaluated and compared on the basis of their Combined Severity factors. It was shown that the organosolv processes permitted an efficient removal of both lignin and hemicelluloses from the solid residue. A presoaking step prior to an organosolv process performed at low severity permitted to enhance the removal of lignin and hemicelluloses and the recovery of hemicellulose sugars.

Keywords: bioethanol, *Miscanthus x Giganteus*, pretreatment, presoaking, extracts

INTRODUCTION

Lignocellulosic biomass is mainly composed of cellulose, hemicelluloses and lignin. Cellulose can be enzymatically hydrolyzed to its monomeric constituents (glucose units) and then fermented to ethanol for the production of biofuels. Since lignocellulosic biomass is naturally recalcitrant to enzymatic hydrolysis, pretreatment is essential for improving its enzyme digestibility and also for obtaining solubilized sugars. The pretreatment process continues to be one of the most expensive steps, and improvements to pretreatment will have significant benefits for subsequent enzymatic hydrolysis and fermentation.[1-3] Various pretreatment methods acting to decrease the recalcitrance of lignicellulosic biomass to enzymatic deconstruction have been developed.[4-9] Dilute sulphuric acid-based chemical pretreatment[7,8] is one of the most widely used, but the ethanol organosolv process also appears to be very promising. Indeed, recently, Pan *et al*.[9,10] have successfully developed this pretreatment technology for poplar and pine, producing substrates with very good enzymatic digestibility. Pretreatments can be evaluated and compared on the basis of the severity correlation (Ro), which describes the severity of the pretreatment as a function of treatment time and temperature.[11] When the pretreatment is performed under acidic conditions, the effect of pH can be taken into consideration by Combined Severity,[12] defined as:

$$\text{Combined Severity (CS)} = \text{Log(Ro)} - \text{pH}$$

There exists a great variety of lignocellulosic feedstocks, which can be potentially used for ethanol production. Among them, special mention is to be made of *Miscanthus x Giganteus*, a perennial grass, which presents some valuable advantages: simple cultivation and harvesting, good yield (>20 tons of dry matter per hectare in France or Germany), non-invasive character.[13] Moreover, it is a rhizomatous C4 grass species, with a high carbon dioxide fixation rate. *Miscanthus* could be an interesting raw material for industrial bioconversion processes since it is rich in carbohydrates, which constitute approximately 75% of the dry matter content. Nevertheless, while the literature related to the cultivation of MxG is

well documented, studies on its chemical valorization are not as extensive. So far, only a few papers have been devoted to the pretreatment and enzymatic hydrolysis of *Miscanthus*. These include ammonia fiber expansion,[14] one-step extrusion/NaOH[15] and acetosolv[16] pretreatment. Recently, we described[17] an aqueous-ethanol organosolv treatment for the conversion of *Miscanthus x Giganteus* (MxG), permitting an efficient fractionation of the raw material into a cellulose rich residue, an ethanol organosolv lignin fraction and a water soluble fraction containing mainly hemicellulose sugars.

In the present study, dilute sulphuric acid and ethanol organosolv treatments of *Miscanthus x Giganteus* were investigated. The effect of a presoaking step prior to the organosolv treatment was also evaluated. The experiments involved utilization of the Combined Severity factors and the different pretreatments have been compared on the basis of lignin loss, carbohydrate hydrolysis and recovery, as well as production of furans.

EXPERIMENTAL

The raw *Miscanthus x Giganteus* was harvested in the spring of 2008, at Trier (Germany), and air-dried; the dried *Miscanthus* straw was sent to the USA by air mail, milled to a particle size of 1-3 mm using a Wiley mill, and stored at -5°C during the study. All chemical reagents used were purchased from VWR International and applied as received.

Ethanol organosolv treatment

25 g (oven dry matter) of *Miscanthus* was treated with aqueous ethanol and sulfuric acid as a catalyst (see Table 3 for the pretreatment conditions), following the method discussed by Pan *et al*.[9,10] (schematically presented in Fig. 1). The solid-to-liquid ratio applied was 1:8. The pretreatments were carried out in a 1.0 L glass-lined Parr pressure reactor with a Parr 4842 temperature controller (Parr Instrument Company, Moline, IL). The reaction mixture was heated at a rate of ~3 °C/min, with continuous stirring. Pressure was increased to 15-20 bars, as a function of temperature and ethanol concentration. The pretreated *Miscanthus* was washed with 60 °C ethanol water (8:2, 3 x 50.00 mL) and then air-dried overnight. The washings were combined and 3 volumes of water were added to precipitate the Ethanol Organosolv Lignin (EOL), which was collected by centrifugation and air-dried. A portion of the solid residue and of the liquid was separated and stored in a freezer at -5 °C before analysis.

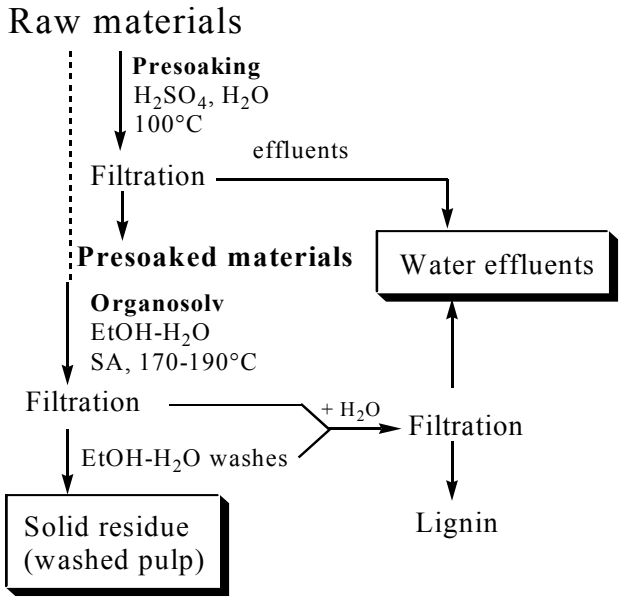

Figure 1: Schematic presentation of the ethanol organosolv treatment and the two-step presoaking and ethanol organosolv pretreatment

Presoaking

55 g (dry weight, dry matter content – about 90%) of *Miscanthus*, 500.00 mL of water and 40.00 mL of sulphuric acid 2 M (concentration of H_2SO_4 = 0.15 mol·L^{-1}) were mixed in a flask and heated to reflux for 17 h. At the end of the reaction, the residue was filtered and air-dried. A portion of the solid residue and an aliquot of the

liquid were separated and stored in a freezer at -5 ^{o}C before analysis.

Dilute sulfuric acid treatment

Miscanthus was soaked overnight with dilute H_2SO_4 (0.9 or 1.2%, w/w based on the dry matter content, solid-to-liquid ratio of 1:8) and treated at 170 to 190 °C for 2 to 10 min (Table 3). The pretreatments were carried out in the 1.0 L Parr pressure reactor described previously. The pretreated *Miscanthus* was washed thoroughly with water and air-dried.

Combined Severity (CS) determination

The organosolv and dilute sulphuric acid treatments were evaluated with the severity correlation, which describes the severity of the pretreatment as a function of treatment time (min) and temperature (°C), where T_{ref} = 100 °C:

$$\text{Log(Ro)} = \text{Log}\,(t \exp(T - \text{Tref})/14.7$$

The effect of pH was taken into consideration by Combined Severity:

$$\text{Combined Severity (CS)} = \text{Log(Ro)} - \text{pH}$$

As a first very practical approximation, the pH of the liquor can be employed as a measure of the hydrogen ion concentration for sulphuric acid ethanol-water solutions.[12]

Analytical procedures

The oven-dry weights were determined on an Infra-red moisture analyser (Mettler HR73).

The untreated *Miscanthus* (5 g) was extracted with dichoromethane (DCM), using a Soxhlet apparatus, for 4 h. The solvent was dried with a rotary evaporator and the residue weighed to obtain the DCM extractive content. An aliquot of the extractive was collected during concentration, for GC-MS characterization. The sample was concentrated under a stream of nitrogen, at room temperature and then derivatized with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA). The prepared sample was GC-MS analyzed with splitless injection, on a Hewlett-Packard 5890 II GC equipped with a Hewlett-Packard 5971A mass selective detector. A 0.25 mm x 60 m DB-5 fused silica capillary column with a 25 μm coating stationary phase was used for chromatographic separations. The GC conditions were as follows: initial temperature – 150 °C; initial time – 5 min; rate – 15 °C/min; final temperature – 280 °C; final time – 25 min; inject port temperature – 250 °C. The mass detector was operated under the following conditions: EI model; 70 eV; filament on delay time, 8 min; mass scan range – 45-650 m.u. Quantification of the individual components was based on the total ion peak area. The GC response factor of each individual compound was assumed to be the same for all calculations. The carbohydrate and lignin contents were measured on an extractive-free (Soxhlet extracted with dichloromethane overnight) material, ground to pass a 40 mesh screen, according to the laboratory analytical procedure (LAP) provided by the National Renewable Energy Laboratory (NREL). The samples were hydrolyzed with 72% sulphuric acid for 1 h and then autoclaved after dilution to 3% sulphuric acid, with addition of water. The autoclaved samples were filtered and the dried residue was weighed to give the Klason lignin content. The monosaccharide contents in the filtrate were quantified[18] by the HPAEC-PAD procedure. The acid soluble lignin content was determined from the absorbance at 205 nm, according to Lin and Dence.[19] For determining the composition of the water soluble fractions, an aliquot (10.00 mL) was freeze-dried and re-dissolved in DI water (10.00 mL) and the monosaccharide contents were quantified by HPAEC-PAD, both before and after hydrolysis, to determine the amount of oligomers. The latter was accomplished by the addition of 72% sulphuric acid to obtain a 3% sulphuric acid solution (348 μL). The furan contents were estimated in the first effluent (obtained through the sampling valve) and in the water soluble fraction from the absorbance values at 284 and 305 nm, according to Martinez *et al*.[20]

RESULTS AND DISCUSSION

Table 1 lists the *MxG* compositions used in the study. Sugars accounted for more than 70% of the whole plant, similarly to other major sources of lignocellulosic biomass, indicating that *MxG* is a potentially useful biomass resource for the production of biofuels. The compositions of other *Miscanthus* sources, previously reported[21-24] in the literature, are also presented for the sake of comparison. The raw material used in the study has a comparable amount of Klason lignin but a substantially higher proportion of xylose than the previously reported ones. The results of GC-MS analysis and characterization of the DCM extracts from the raw material used are presented in Table 2. The yield of DCM extractives from *MxG* was 1% of the dry mass, which is quite similar to the values reported for other herbaceous crops.[25-29] Very recently, Villaverde *et al*.[25] described the chemical composition of the lipophilic extracts of the bark and core of the *Miscanthus x Giganteus* stalk. The extracts are mainly composed of sterols and fatty acids (with a high octacosanoic acid content). Compared to the composition described by Villaverde *et al*.,[25] lower

amounts of aromatic compounds and a comparable amount of long-chain aliphatic alcohols (mainly octacosanol) and of sitosterol and stigmasterol were now recorded. This high sterol content of the extractive fraction may be a potentially valuable by-product during the conversion of biomass to bioethanol.

Three pretreatment methods have been evaluated for the conversion of *Miscanthus x Giganteus* to ethanol: dilute sulphuric acid pretreatment (SA), ethanol organosolv pretreatment (organosolv) and a two-step procedure involving a dilute acid presoaking step and aqueous-ethanol organosolv treatment (PS + organosolv). Due to the high xylose content of the raw material, these conditions were applied to optimize the recovery of hemicellulose sugars. The condition sets used in the present study, given in Table 3, were selected on the basis of the previously described results. The assays can be evaluated and compared with the Combined Severity factors given in Table 3, which describes the severity of the pretreatment as a function of treatment time (min), temperature (°C) and pH of the medium. The investigation covered a Combined Severity range of CS = 0.73-2.86.

The assays were analysed in terms of the Klason lignin content in the solid residue (delignification of pulp), sugar recovery in solid and liquid phases, and furan content of effluents, which is a measure of sugar degradation extent.

Figure 2 represents the effects of the Combined Severity parameter on pulp delignification. It appears that, for all CS values, the sulphuric acid treatments resulted in a very low delignification rate (<20%), whereas the ethanol-rich cooking liquors used in the organosolv processes act as an effective solubilizer of lignin.

Table 1
Composition of *Miscanthus*

Components, %	Present study	Ligero *et al.*[23]	Ye *et al.*[24]	de Vrije *et al.*[25]	Sørensen *et al.*[26]
Ash	2.0	0.4	0.7	2	5.9
Cellulose		65.4	72.5	38.2	40
Hemicellulose				24.3	18
Klason lignin	25.0	23.5	19.9	24.1	25
Xylans	33.8			19	25

Table 2
Evaluation of DCM extracts from *Miscanthus x Giganteus* by GC-MS

Compounds	mg/100g
Lignin	
Vanillin	9.07
Vanilic acid	2.09
p-Hydroxyl cinnamic acid	3.90
Ferulic acid	1.78
Fatty acids	
C14:COOH	5.50
C15:COOH	3.74
C16:COOH	69.85
9,12-octadecadienoic acid	27.40
Oleic acid	22.43
Linolenic acid	14.74
C18:COOH	12.47
C19:COOH	1.87
C20:COOH	12.88

C21:COOH	0.00
C22:COOH	9.75
C23:COOH	11.74
C24:COOH	12.21
C28:COOH	23.49
Resin acids	
Abietic acid	2.04
Alkanols	
C22:OH	0.80
C24:OH	3.69
C26:OH	7.99
C28:OH	71.65
Glycerides	
1-Monooleoylglycerol	5.22
Sterols	
Cholest-5-en-24-one	23.49
Stigmasterol	42.98
Sitosterol	77.30
Stigmast-4-en-3-one	19.95
Total	9.07

It appears from Figure 2 that the increase in the severity of the organosolv treatment performed without a presoaking step resulted in the reduction of the Klason lignin content of the pulp. On the other hand, starting from the presoaked material (PS + organosolv), high rates of delignification were observed even at low severity values. As previously proposed,[17] this observation can be rationalized by the removal of a part of hemicelluloses during the presoaking step, which disrupted enough of the remaining polymers to enhance the hydrolysis of lignin during the organosolv process. As a consequence, the partially disrupted lignin can be solubilised at low sulphuric acid concentration and/or high ethanol content (low Combined Severity). Thus, by this procedure, it is possible to remove up to 75% of the lignin in the raw material, at a low severity factor (CS ≈ 2).

Table 3
Experimental conditions for different pretreatments evaluated for *Miscanthus*

Experiment	Pretreatment	T (°C)	t (min)	SA (%)	EtOH/H_20	CS
1	SA	170	2	0.9	0	0.73
2		180	2	0.9	0	1.03
3		180	5	1.2	0	1.43
4		190	10	0.9	0	2.03
5	Organosolv	170	60	0.5	0,65	1.75
6		170	60	0.9	0,75	1.99
7		170	60	0.9	0,65	2.07
8		180	60	1.2	0,65	2.16
9		170	60	0.9	0,5	2.26
10		170	60	1.2	0,65	2.27
11		180	60	0.9	0,65	2.36
12		170	80	1.2	0,65	2.39
13		190	60	1.2	0,65	2.86
14	PS + Organosolv	170	60	0.5	0,8	1.69
15		170	60	0.9	0,65	2.07
16		180	60	0.9	0,8	2.23
17		180	60	0.9	0,75	2.28
18		180	60	1.2	0,65	2.56

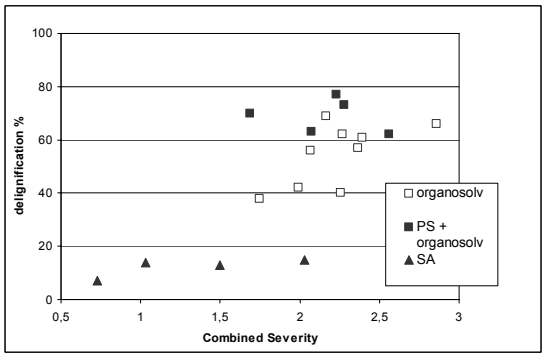

Figure 2: Delignification yield of the solid residue after the pretreatment step as a function of Combined Severity

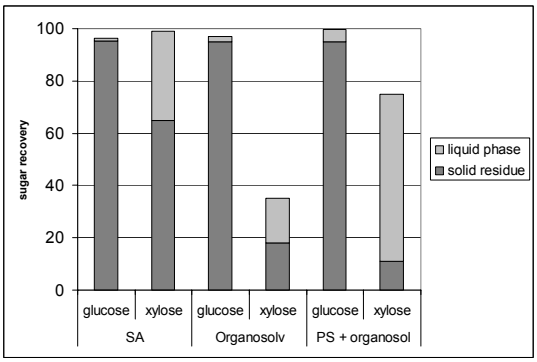

Figure 3: Recovery of glucose and xylose in the liquid and solid for experiments 2 (SA), 12 (organosolv) and 14 (PS + organosolv)

Figure 3 plots the recovery of glucose and xylose in the solid residue after the pretreatment and in the water washes, for 3 experiments (experiment 2: CS = 1.03, experiment 12: CS = 2.39, and 14: CS = 1.69; Table 3). The amounts of monomeric sugars of glucose and xylose of the water phase were determined after a post hydrolysis step using 3% H_2SO_4 (see Experimental). In the case of organosolv treatments, the composition of the liquid phases includes the filtrate and the water washes of the presoaking step and of the ethanol-water phase, after EOL precipitation in the organosolv step (Fig. 1).

In assay 2 (sulphuric acid treatment), almost all glucans and xylans were recovered: 95% of the glucans were in the solid fraction and only 35% of the xylans were hydrolysed and present in the aqueous effluent. Thus, the process does not allow an efficient removal of the hemicellulose sugars from *Miscanthus*.

In the case of the organosolv pretreatment (experiment 12), the conditions used allowed a very good recovery of glucans in the solid and a removal >80% of the xylans from the solid residue. However, under these conditions, an important loss of xylose was observed, suggesting a greater degradation of the sugars. Better results were obtained in experiment 14 (PS + organosolv), in which the conditions used allowed the removal of 90% of xylose from the starting material, coupled with an efficient recovery of glucans and xylans in the pulp and water effluents, respectively.

Figure 4 shows the xylans in oligomeric form, as percentage of the total xylans in the water effluents of the organosolv treatments, as a function of the severity of the pretreatment conditions.

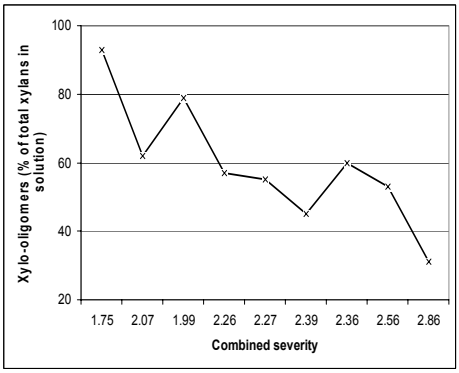

Figure 4: Percentage of xylose in oligomeric form in the hydrolysates of organosolv treatments as a function of Combined Severity

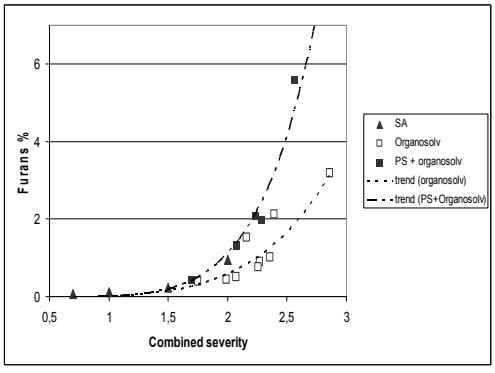

Figure 5: Concentration of furans in the liquid after the pretreatment step as a function of Combined Severity of the pretreatment

At low severity values, xylose is mainly recovered in the oligomeric form (>90%) while, with the increase in severity, a strong decrease of the oligomeric content (Fig. 4)

was noticed, to be explained by extensive hydrolysis of polysaccharides into monosaccharides, under more severe conditions (longer reaction time, higher temperature, lower pH). Thus, for an organosolv treatment performed at a CS of 2.86, 68% of xylans are recovered as simple sugars.

During pretreatment under acidic conditions, the pentoses and hexoses formed from hydrolysed hemicellulose and cellulose may be further degraded to furans (furfural and 5-hydroxymethylfurfural), together with other substances, such as acetic acid. These furans may cause inhibition in the fermentation step. In Figure 5, the furan content has been plotted against the severity parameters. An increase in CS resulted in a higher concentration of furans in the aqueous effluents, attesting the higher degradation of sugars under the conditions applied. In the case of organosolv treatments, it appeared that, at the same severity factor, presoaked *Miscanthus* gave higher furan content than the non-presoaked one. This can be explained by the fact that the presoaking step enhances the hydrolysis of the remaining polymers of the pulp (which facilitates delignification, as demonstrated above), while enhancing the degradation of sugars. Nevertheless, the two-step organosolv process (PS + organosolv) performed at a relatively low severity factor (<2) resulted in a very low production of furans.

CONCLUSIONS

The dichloromethane extractives fraction of *Miscanthus x Giganteus* was characterized by GC-MS and found to comprise mainly long-chain aliphatic alcohols and sterols. These compounds may be potentially valuable by-products during the utilization of this biomass resource in the production of ethanol and other biofuels. Three different processes for the pretreatment of *Miscanthus x Giganteus* were compared on the basis of their Combined Severity factors: the dilute sulphuric acid treatment, the ethanol organosolv treatment and a two-step protocol involving a presoaking step prior to an ethanol organosolv treatment. The results show that, compared to the sulphuric acid pretreatment, the organosolv protocol appears as optimum in terms of enhanced lignin and hemicellulose removal from *Miscanthus*, recovery of hemicellulose sugars in the pretreatment effluents and retention of cellulose in the pretreated *Miscanthus*. The addition of a dilute acid presoaking step prior to the organosolv step resulted in an enhanced lignin and hemicellulose removal, along with an enhanced sugar degradation into furans. However, this 2 step protocol, performed at low severity (CS ≈ 2), permitted the recovery of hemicellulose sugars in the pretreatment effluents at a good yield and a low production of furans.

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