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1 **Combination of enzymatic hydrolysis and ethanol organosolv**
2 **pretreatments : effect on lignin structures, delignification yields**
3 **and cellulose-to-glucose conversion**
4

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16 83 68 44 98
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18
19 *Abstract*

20
21 Enzymatic pre-hydrolysis using the industrial enzymatic cocktail Cellulyve® was assessed as
22 a first step in a pretreatment process of *Miscanthus* biomass involving an aqueous-ethanol
23 organosolv treatment. ¹³C and ³¹P Nuclear Magnetic Resonance and Size Exclusion
24 Chromatography were used to analyze the cellulose and lignin before and after treatment. It
25 was demonstrated that despite a very low impact on the fibre structure (observed by Scanning
26 Electron Microscopy) and composition (in terms of sugars and polyphenolics content), the
27 enzymatic pre-treatment disrupted the lignocellulosic matrix in a considerable extend. This
28 weakening permitted to enhance the removal of lignin during the organosolv pulping and the
29 hydrolysability of the residual cellulosic pulp for the production of monomeric glucose. Using
30 this combined treatment, a delignification yield of 93% and an enzymatic cellulose-to-glucose
31 conversion of 75% were obtained.

32 Keywords : Enzyme, miscanthus, pretreatment, bioethanol
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1. Introduction

Utilization of lignocellulosic feedstock for fuel and chemical production requires the development of pretreatment technologies necessary to break up lignin structures, to separate the three biopolymers (lignin, cellulose and hemicelluloses) and to enhance enzyme digestibility of cellulose (FitzPatrick, 2010 ; Brodeur, 2011). Different kinds of physical and/or chemical pretreatments have been proposed for the conversion of wood and agricultural wastes. Among these methods, the ethanol organosolv process is promising since it allows clean fractionation of the biomass. This technology has already been developed at an industrial scale (Alcell® process) and recently has been evaluated and optimized for delignification of softwood, hardwood and crops (Pan et al., 2006, 2007 ; Brosse et al., 2009). One of the major goals of optimizing a pretreatment process is the full recovery of the feedstock through optimum utilization of all lignocellulosic components ; however, conditions known to promote lignin depolymerisation also cause degradation of hemicellulose sugars into furfural, hydroxymethyl furfural (HMF) and carboxylic acids. These degradation products inhibit downstream processing (Thomsen et al., 2009). To circumvent this drawback, two-stage pretreatments were described. In the 1st step hemicelluloses is hydrolysed and in the second step the solid material from the first step is treated at higher severity. This approach can result in higher sugar yields than one-step pretreatment. Thus, fungal (Munos et al., 2007) or dilute sulphuric acid prehydrolysis (Brosse et al, 2009; Patel and Varshney, 1989) before an organosolv treatment demonstrated a favorable effect on delignification. In previous studies, autohydrolysis of *Miscanthus* straw was assessed as a first step of a pretreatment process involving an aqueous-ethanol organosolv treatment (El Hage et al., 2010a). During autohydrolysis, acetic acid is produced from xylans and enables the hydrolysis and the

1 dissolution of a great part of hemicellulose. It also permits the cleavage of lignin-carbohydrate
2 bonds (Garrote et al., 2001, 2002 ; Lee et al, 2009). After autohydrolysis, delignification
3 reactions are accelerated and the solubility of lignin in a subsequent extraction with aqueous
4 organic solvent is increased. The autohydrolysis process also impacts lignin structure (El
5 Hage et al., 2010a). During autohydrolysis lignin homolytical fragmentation should promote
6 repolymerisation reactions which negatively affect the subsequent organosolv delignification
7 step. Furthermore, autohydrolysis conditions lead to a loss of polysaccharides through
8 dehydration and degradation reactions and generate weak acids, furfural and 5-
9 hydroxyfurfural which negatively impact microorganisms involved in the fermentation step
10 (Palmqvist & Hahn-Hagerdal, 2000).

11 Enzymatic hydrolysis is now usually employed in many industrial processes. In cellulosic
12 ethanol industry and in most studies on the conversion of biomass to fuel, enzymes are
13 usually added at the end of the process to complete the conversion of cellulose to glucose
14 (Katzen & Schell, 2006). In the development of sustainable processes, the implementation
15 methods reducing the amount of chemicals and energy consumption are of interest and thus, it
16 may be appropriate to carry out pre-treatments enzymatically.

17 In the present study, pretreatment of lignocellulosic biomass was carried out involving a
18 biological prehydrolysis step using an industrial enzymatic cocktail mainly composed of
19 cellulase and xylosidase activities and an ethanol organosolv treatment performed at low
20 severity. Enzymatic prehydrolysis was compared to autohydrolysis, a hydrothermal treatment
21 previously studied in terms of delignification yields and cellulose-to-glucose conversion. The
22 impact of the processes on the cellulose and lignin structures was investigated. The purpose of
23 this 2-steps process is to propose an optimized method for the separation of lignocellulosic
24 components from lignocellulosic feedstock.

25

1 **2. Materials and Methods**

2 *Miscanthus* was harvested in the spring 2009 at Courcelles-Chaussy (France). The dried
3 *Miscanthus* biomass was milled to a particle size of 1–3 mm using a Wiley mill and stored at
4 5 °C. All chemical reagents used in this study were purchased from Sigma Aldrich and VWR
5 (France) and used as received. Dried matter contents were determined using a moisture
6 balance, KERN MRS 120-3 Infra-red moisture analyser (drying at 105°C to constant weight).
7 The effective dry matter content of raw *Miscanthus* biomass was 96 %.

8

9 **2.1. Enzymatic activities**

10 Cellulyve®, a commercial *Trichoderma reesei* cellulase (E.C. 3.2.1.4) preparation was kindly
11 provided by Lyven (Colombelles, France). The cellulase activities were quantified following
12 IUPAC recommended procedures (T.K. Ghose, 1987). Endo-glucanase (E.C. 3.2.1.4), 1,4- β -
13 cellobiosidase (E.C. 3.2.1.91) and β -glucosidase (E.C. 3.2.1.21) activities were respectively
14 measured on carboxymethylcellulose (CMC, Sigma C8758, 10 IU/ml), filter paper (Whatman
15 3001-917, 4 IFPU/ml) and cellobiose (Serva, 16400, 7 IU/ml) as substrates. After hydrolysis
16 of polysaccharides, reducing sugar content was determined by the Nelson-Somogyi method
17 (Somogyi, 1952). Hemicellulase activities were assayed in a reaction mixture containing 900
18 μ L of the appropriate substrate (1 g/L) and 100 μ L of diluted enzymatic preparation both
19 prepared in citrate buffer (0.05M, pH 4.8). The hydrolysis was followed at 50°C as long as
20 necessary to measure the kinetic (linear part of curve) at 405 nm. α -arabinosidase (E.C.
21 3.2.1.99), β -xylosidase (E.C. 3.2.1.37) and β -galactosidase (E.C. 3.2.1.23) activities were
22 determined using *p*-nitrophenyl- α -L-arabinofuranoside (Sigma, 122H5031), *p*-nitrophenyl- β -
23 D-xylopyranoside (Sigma 125K4054) and *p*-nitrophenyl- β -D-galactofuranoside (Sigma
24 92H5003) substrates respectively. Activities were calculated following the release of free *p*-
25 nitrophenol in the reaction mixture per minute and were quantified at 10 IU/ml and 4740

1 IU/ml for α -arabinosidase and β -xylosidase respectively; no activity was detected for β -
2 galactosidase activity. No pectinolytic activity (E.C. 3.1.1.11, E.C. 3.2.1.15) was detected in
3 the preparation.

4

5 **2.2. Hydrothermal processing / Autohydrolysis**

6 *Miscanthus* samples 1-3 mm particle size (20 g dried matter, raw material) were loaded into a
7 0.6-L stainless steel pressure Parr reactor with a Parr 4842 temperature controller (Parr
8 Instrument Company, Moline, IL) and were supplemented with an appropriate amount of
9 deionized water to make a final solid to liquid ratio of 1/ 9, taking into account the moisture
10 content of the sample. The mixture was heated at 150°C with continuous stirring for 8 h (time
11 zero was set when the preset temperature was reached with heating rate of 5 K / min). At the
12 end of each reaction, the reactor was cooled and the liquid phase was recovered by filtration
13 through. Solid residue was washed with 70°C water (3 x 50 ml) and a portion of solid residue
14 and liquid were stored in a freezer at -5°C before analysis.

15

16 **2.3. Enzymatic prehydrolysis step**

17 Ten g of *Miscanthus* biomass (dry weight) were loaded into an Erlenmeyer flask containing
18 100 mL of deionized water. Five % (w/w based on dry mass of *Miscanthus*) of Cellulyve®
19 were added. The flask was placed in an incubator at 50 ° C and shaken at 140 rpm. After each
20 reaction, the mixture was centrifuged at 2000 x g during 5 min and the solids were washed
21 three times (3 x 100 mL) with deionized water. A few mL of the aqueous phase were kept at -
22 5 ° C for subsequent chemical analysis.

23

24 **2.4. Organosolv treatment**

1 Twenty-five grams (dry weight) of native or autohydrolysed *Miscanthus* biomass was milled
2 (200 μm) and mixed with 80 % aqueous ethanol (EtOH / H₂O : 8/2 v/v) and 0.5 % or 1% w/w
3 (based on raw material dried mass) sulfuric acid as a catalyst. The mixture was heated at
4 150°C or 170°C for 30 min or 60 min. The solid-to-liquid ratio used was 1 : 8. Treatments
5 were carried out in a 0.6 L stainless steel pressure Parr reactor with a Parr 4842 temperature
6 controller (Parr Instrument Company, Moline, IL). The reaction mixture was heated at a rate
7 of 5 K/min with continuous stirring. At the end of the treatment, the pretreated *Miscanthus*
8 biomass was washed with 60°C ethanol-water (8 : 2, 3 \times 50 mL) and air-dried overnight. The
9 washes were combined, and three volumes of water were added to precipitate the ethanol
10 organosolv lignin (EOL), which was collected by centrifugation at 2000 x g during 5 min and
11 then air-dried. A portion of each the solid residue and the liquid was separated and stored in a
12 freezer at -5°C before analysis.

13

14 **2.5. Cellulose extraction**

15 Holocellulose was isolated from the untreated *Miscanthus* particles, autohydrolysis and
16 enzyme hydrolysis residues by repeatedly treating with a mixture of acetic acid and sodium
17 chlorite until the sample had a very low Klason lignin content (< 1%). The holocellulose
18 sample (1 g) was further treated with 50 mL of NaOH, 17.5% during 30 min at 25 ° C. Fifty
19 mL of deionized water was added and the resulting slurry was stirred at 25° C for 30 min. The
20 resulting cellulose was filtered, washed successively with 50 ml of 1% acetic acid in water
21 and with 100 mL of deionized water and then dried in an oven at 40 ° C overnight.

22

23 **2.6. Enzymatic hydrolysis (cellulose-to-glucose conversion)**

24 Enzymatic hydrolysis of raw and pretreated *Miscanthus* biomass was performed using
25 Celluclast® 1.5 L (E.C. 3.2.1.4) from *Trichoderma reesei* supplemented with β -glucosidase

1 preparation (Novozym 188®, E.C. 3.2.1.21) from *Aspergillus niger* at a loading of 40 IFPU
2 and 80 IU per g cellulose respectively. Both enzyme preparations were purchased from
3 Novozymes (Bagsværd, Denmark). Enzymatic hydrolyses were performed at 2 % (cellulose
4 w/v) in 100 mL of 50 mM acetate buffer, pH 4.8. The reaction mixtures were incubated at
5 50°C in a rotary shaker at 170 rpm, sampled periodically and centrifuged (2000 x g) to
6 remove the insoluble materials for analysis. Glucose contents of the aqueous phase were
7 quantified using high-performance anion-exchange chromatography with pulsed
8 amperometric detection (HPAEC PAD) (section 2-7). Hydrolysis data are averages from
9 duplicate experiments.

10

11 **2.7. Analytical Procedures**

12 A KERN MRS 120-3 Infra-red moisture analyser (drying at 105°C to constant weight) was
13 used for moisture determination.

14 Ash content was determined (by calcination at 525°C for 5 h).

15 Extractables were determined by extracting untreated *Miscanthus* biomass (50 g) with
16 dichloromethane (DCM, 1L), using a Soxhlet apparatus (volume = 2L) for 6 h (about 36
17 extraction cycles).

18 Carbohydrate and lignin contents were measured on extractive-free material, ground to pass a
19 40-mesh screen, according to the laboratory analytical procedure (LAP) provided by the
20 National Renewable Energy Laboratory (NREL). Samples were hydrolyzed with 72 %
21 sulfuric acid for 1 h and autoclaved after being diluted to 3 % sulfuric acid through the
22 addition of water. The autoclaved samples were filtered, and the dried residue was weighed to
23 give the Klason lignin content. Monosaccharide contents in the filtrate were quantified using
24 high-performance anion-exchange chromatography with pulsed amperometric detection
25 (HPAEC PAD). The acid-soluble lignin content was determined from absorbance at 205 nm

1 according to Lin and Dence 1992. In brief, the lignin content in the filtrate was calculated
2 from the following expression of Beer's law : Lignin (g/L) = Absorbance /110. Furan
3 contents were estimated in the water-soluble fraction from the absorbance at 284 and 305 nm
4 according to Martinez et al. (2000).

5 To determine the composition of the water-soluble fractions recovered after autohydrolysis, a
6 post hydrolysis was performed because of the presence of sugars in oligomeric forms. An
7 aliquot (10.00 mL) of the solution was freeze-dried, dissolved in deionized water (10.00 mL),
8 and the post hydrolysis of oligosaccharides was accomplished by addition of 72 % H₂SO₄ (to
9 obtain a 3% sulfuric acid solution (348 μL). Regarding the liquid phase recovered after
10 enzymatic treatments, production of monomeric sugars was observed (no increase in the sugar
11 concentrations after post hydrolysis) and direct chromatographic analysis was performed. The
12 monosaccharide contents were quantified using a high-performance anion-exchange
13 chromatography with pulsed amperometric detection.

14

15 **2.8. High-performance anion-exchange chromatography**

16

17 Separation and quantification of neutral sugars were performed using a Dionex ICS-3000
18 system consisting of a SP gradient pump, an AS autosampler, an ED electrochemical detector
19 with a gold working electrode, an Ag/AgCl reference electrode and Chromeleon version 6.8
20 (Dionex Corp., USA). A Carbopac PA1 (4 x 250 mm, Dionex) column with a guard column
21 (4 x 50 mm, Dionex) was used as a stationary phase using isocratic conditions with 1 mM
22 sodium hydroxide as eluent. Eluents were prepared by dilution of a 46-48% NaOH solution
23 (PA S/4930/05 Fisher Scientific) in ultrapure water. All eluents were degassed before use by
24 flushing with helium for 20 min; subsequently they were kept under constant helium pressure
25 (eluent degassing module, Dionex). After each run, the column was washed for 10 min with
26 200 mM NaOH and reequilibrated for 15 min with the starting conditions. Samples were

1 injected through a 25- μ L full loop and separations were performed at 25 °C at a rate of 1
2 mL/min. The pulse sequence for pulsed amperometric detection consisted of a potential of
3 +100mV (0–200ms), +100mV integration (200–400 ms), -2000mV (410–420 ms), +600mV
4 (430ms), and -100mV (440–500ms).

5

6 **2.9. NMR and SEC analysis**

7 All NMR experiments were performed on a Bruker Avance-300 spectrometer operating at a
8 frequency of 100.59 MHz for ^{13}C . Organosolv lignin (100 mg) were dissolved in DMSO d6
9 (0.50 mL). ^{13}C NMR spectra were acquired at 50 °C in order to reduce viscosity. Quantitative
10 NMR spectra were acquired using an inverse-gated decoupling (Waltz-16) pulse sequence to
11 avoid Nuclear Overhauser Effect (NOE) effects. Ten thousand scans were collected with a
12 pulse delay of 12 s. ^{13}C data were processed offline using XWinNMR processing software.
13 ^{31}P NMR spectra were acquired after derivatising 25 mg lignin with 2-chloro-4,4,5,5-
14 tetramethyl-1,1,3,2-dioxaphospholane (TMDP)(Granata and Argyropoulos, 1995).

15 The number average molecular weights (M_n) and weight (M_w) of lignin and cellulose were
16 determined after derivatization by size exclusion chromatography with a Dionex Ultimate-
17 3000 HPLC system consisting of an autosampler and a UV detector and using tetrahydrofuran
18 as eluent. Standard polystyrene samples were used to construct a calibration curve. Data were
19 collected and analysed with Chromeleon software version 6.8 (Dionex Corp., USA).

20 Cellulose and lignin samples were analysed after tricarbanilation (Foston and Ragauskas,
21 2010) and after acetylation respectively (El Hage et al., 2009).

22

23 **3. Results and discussion**

24

25 **3.1. Prehydrolysis**

1
2 The experimental conditions used for the autohydrolysis and for enzymatic prehydrolysis of
3 *Miscanthus* biomass were selected considering previous results (El Hage et al., 2010a) and
4 preliminary studies not reported here. The compositions of *Miscanthus* biomass and solid
5 residues after autohydrolysis and enzymatic hydrolysis are given in Table 1. The untreated
6 *Miscanthus* biomass consisted of ~49% glucans, ~22% xylans, ~2.5% of other sugars and
7 ~25% lignins. As expected, the data concerning glucans and lignins in the solid residues show
8 that the autohydrolysis induced some degradation of cellulose and lignin whereas
9 hemicellulose polysaccharides (mainly xylans) were depolymerized; 63% of the original
10 xylans were removed from the pulp and solubilized. In contrast, after enzymatic hydrolysis,
11 little impact of the treatment was observed on the composition of the resulting pulp. The
12 removal of 9% in weight of xylans and of glucans was observed demonstrating the xylosidase
13 and cellulase activities of the enzymatic cocktail. The soluble lignin and furans contents of the
14 two hydrolytic treatment solutions are given in Table 1. The mild conditions used for the
15 bioprocess produced lower content of soluble lignin and furans suggesting a very low
16 degradation of lignin and sugars.

17 Figures 1A and 1B give the sugars concentrations in the liquid phase as a function of the
18 duration of autohydrolysis and enzymatic hydrolysis respectively. The xylose concentrations
19 were higher than those of other sugars and reach a maximum at ~ 8 h (Figure 1A). As
20 previously described, a further increase in the residence time led to a decrease in the recovery
21 of xylose, owing to degradation reactions (El Hage et al., 2010a). In contrast to the
22 hemicellulose components, glucans essentially remained in the solid phase and the
23 solubilisation of ~ 4 % of glucans was observed after 15 h of treatment. The sugar recovery
24 during enzymatic hydrolysis displayed a totally different pattern (Figure 1B). A very good
25 selectivity of hydrolysis toward glucans and xylans was observed with formation of

1 monomeric sugars. Very low arabinose, galactose and mannose contents were detected in the
2 liquid phase. The concentrations of xylose and cellulose increased to reach a maximum after
3 10 h.

4 Using SEM, the microscopic surfaces of untreated and pretreated pulp fibers were compared.
5 The examination showed differences between fibres. Untreated pulp fibres and enzymatically
6 hydrolysed fibres had an intact and smooth surface and well-defined folds whereas
7 autohydrolysed material exhibited smaller fibres with a disrupted surface with small tears and
8 holes (Supplementary materials). This observation could be attributed to a preferential
9 removal of hemicelluloses during the autohydrolysis step and/or cellulose chain scissions
10 within the fibril structure.

11 Gel Permeation Chromatography (GPC) has been shown to be a valuable tool in monitoring
12 the degradation of cellulose (Barth and Regnier, 1980, Foston and Ragauskas, 2010). Thus,
13 the question as to whether the autohydrolysis and the enzymatic treatment caused some
14 cellulose depolymerization was addressed by investigating the GPC elution curves of
15 cellulose samples isolated from the solid residue recovered after treatment and modified with
16 phenylisocyanate before analysis. The cellulose fractions after autohydrolysis had a weight
17 average ($M_w = 518 \times 10^3$) and a number average ($M_n = 108 \times 10^3$) molecular weight
18 significantly lower than those of cellulose after enzymatic pretreatment ($M_w = 640 \times 10^3$ and
19 $M_n = 183 \times 10^3$). In addition, the value of polydispersity was higher after hydrolysis than
20 after an enzymatic treatment (M_w/M_n 4.81 and 3.51, respectively). These results show that
21 the autohydrolysis conditions reduced the cellulose molecular mass by chain scissions within
22 the fibril structure. This result is in accordance with the microscopic study and with previous
23 studies which proposed spatially localized hydrolysis in the amorphous regions during acidic
24 hydrolysis of cellulose (Foston and Ragauskas, 2010). The data of native cellulose showed
25 that enzymatic hydrolysis did not significantly change the cellulose molecular mass

1 distributions. This result is in agreement with those from previous studies which suggested
2 that the cellulases work ablatively on cellulose microfibril surfaces, consuming the less
3 ordered surface layers of cellulose (Park et al., 2010, Hall et al., 2010).

4 5 **3.2. Ethanol organosolv treatment**

6 Results, including the composition of the pulps in terms of sugars, Klason lignin, amount of
7 organosolv lignin, content of furans and soluble lignin in the liquid phases are given in the
8 Supplementary Materials. Starting with raw *Miscanthus* biomass, the organosolv treatment
9 performed at low severity treatment (O1 and O2, Table 2) did not act as an effective
10 solubilizer of lignin but by increasing the severity (higher reaction time, higher sulfuric acid
11 concentration), a larger part of lignin can be extracted (O3 and O4). As previously
12 demonstrated starting from empty palm fruit bunch (Chun Sheng et al., 2011), a critical
13 concentration of hydronium ions (1% w/w) seems to be required to obtain an efficient
14 hydrolysis and a subsequent solubilization of lignin fragments during an ethanol organosolv
15 pretreatment.

16 Starting with autohydrolysed material (A+O), a larger part of lignin can be extracted during
17 organosolv treatment than especially when using low sulfuric acid concentrations (A+O1 and
18 A+O2). As previously described, after autohydrolysis, organosolv pulping of miscanthus is
19 accelerated and the solubility of lignin in organic solvent is increased (El Hage et al., 2010).

20 When the ethanol organosolv treatment was performed starting with solid materials recovered
21 after enzymatic hydrolysis, better results were obtained in terms of delignification. In these
22 conditions, the critical parameter was the temperature of the organosolv treatment and the best
23 results were observed at 170°C. Thus pulps with very low lignin contents (< 2 %) were
24 isolated even using low severity conditions for the organosolv step (E + O1, T = 170°C).

25

1 **3.3. Organosolv lignins analysis**

2 Three organosolv lignin fractions isolated from the raw miscanthus (O2) after the two-steps
3 processes (A + O2 and E + O2) using the same organosolv conditions (t = 60 min, sulfuric
4 acid concentration = 0.5 %, T = 150°C) were analyzed and compared in order to obtain a
5 more complete picture of the lignin changes occurring during the prehydrolysis steps. The
6 results are given in the Table 3.

7

8 3.3.1. Molecular weight

9 The weight average (Mw) and number average (Mn) molecular weight of the three organosolv
10 lignin fractions were computed from their chromatograms. After enzymatic hydrolysis (E +
11 O2), Mw and Mn of the organosolv lignins were clearly lower than those of the other
12 fractions (O2 and A + O2). These results indicated that enzymatic prehydrolysis facilitated
13 extensive cleavage of some inter-unit bonds in lignin during the organosolv treatment,
14 producing smaller fragments. Moreover, a good correlation was observed between the
15 molecular mass of lignin and the lignin organosolv extractability (see Figure 2).

16

17 3.3.2. ¹³C NMR

18 The quantitative ¹³C NMR spectra of the three organosolv lignin fractions are given in
19 Supplementary Materials. Based on previous data (El Hage et al., 2009, 2010b), Table 3 gives
20 the signal assignments in the spectra and the quantification of the signals. The integral values
21 for the structural moieties are reported per aryl group. The aromatic region of the ¹³C NMR
22 spectra can be divided into three regions of interest: the protonated aromatics (δ 123–106
23 ppm), condensed aromatics (δ 140–123 ppm) and oxygenated aromatics (δ 154–140 ppm).
24 Since the oxygenated aromatic and condensed aromatic regions have some degrees of overlap,
25 calculation of the degree of condensation is less precise when using these regions ; however, a

1 comparison of the integration values of these three regions can provide some information on
2 the degree of condensation of the lignin fractions. As can be seen in Table 3, the lignin
3 recovered after an autohydrolysis step (A + O2) exhibited a higher number of condensed
4 aromatic carbons and a lower number of protonated aromatic carbons per aromatic ring. This
5 observation translates to an increase in the degree of condensation during hydrothermal
6 treatment.

7 The aliphatic region (89-61 ppm) of the ^{13}C NMR spectra displays signals arising from the
8 aliphatic C-O on the lignin propyl side chain. $\text{C}\alpha$, $\text{C}\beta$, and $\text{C}\gamma$ can be identified in the regions
9 73-71, 89-78, and 64-61 ppm, respectively (Table 3). O2 and A + O2 displayed a strong
10 decrease of aliphatic C-O with respectively 0.8 and 1.1 C per aryl group compared to 2.6 C
11 per aryl for E + O2. This observation suggested that starting from the biotreated pulp, the
12 deconstruction of lignin during the organosolv pulping occurred through β -O-4 solvolytic
13 cleavages under mild conditions without further dehydration reactions or formation of
14 Hibbert's ketones.

15

16 3.3.3. ^{31}P NMR

17 Data from the quantitative ^{31}P NMR spectra of the three lignin samples, obtained following
18 their derivatization with TMDP, are presented in Table 3. The concentration of each hydroxyl
19 functional group (in $\text{mmol}\cdot\text{g}^{-1}$) was calculated on the basis of the hydroxyl content of the
20 internal standard cyclohexanol and its integrated peak area (Granata and Argyropoulos, 1995).
21 Lower concentration of aliphatic OH groups and higher concentration of phenolic OH groups
22 were observed for A + O2. This observation could be rationalized by an extensive
23 depolymerization of lignin during the autohydrolysis step with β -O-4 scissions producing

1 phenolic OH groups and subsequent dehydration reactions through acid-catalysed elimination
2 reactions.

3

4 **3.4. Cellulose-to-Glucose conversion**

5 The cellulose-to-glucose conversion after 72 h given in Fig.3 and 4 shows the conversion
6 yields at different time points during the experiments starting from 6 pulps : raw material
7 (RM), prehydrolysed materials (A and E) and organosolv pulps (O2, A + O2, E + O2).

8 Raw *Miscanthus* (RM) and prehydrolysed materials (A and E) showed a low to very low
9 cellulose-to-glucose conversion. By means of the organosolv treatment, cellulose became
10 more amenable to enzymatic hydrolysis in the order: $O < A + O < E + O$. A very good
11 correlation can be established between Klason lignin content of the cellulosic pulps and their
12 hydrolysability by enzymes. Thus, without prehydrolysis and using low severity organosolv
13 conditions, most of the lignin remained in the resulting pulp which continued to be very
14 resistant to enzymatic hydrolysis. Using a two-steps process enzymatic prehydrolysis and
15 subsequent organosolv treatment, very low lignin content in the pulps were associated to good
16 conversion yields, even using mild organosolv conditions (e.g., E + O1).

17

18 **3.5. Discussion**

19 The two steps processes involving a prior fractionation of the hemicelluloses have a dual aim:
20 not only a better hemicelluloses sugars recovery but also the optimisation of the subsequent
21 aqueous-ethanol delignification step. Based on the results presented above and on previously
22 described studies (El Hage et al., 2010a), the lignin deconstruction during the hydrothermal
23 treatment increases its extractability during the organosolv treatment through the cleavage of
24 lignin-carbohydrate bonds and the formation of small fragments of lignin. In the same time
25 this lignin deconstruction is associated with re-polymerization reactions through formation of

1 new C-C linkages (β - β , β -1 and β -5). These condensation reactions result in negative effects
2 on lignin properties and impair the degree of delignification. As a consequence, a fraction of
3 the re-polymerized lignin in the biomass is difficult to extract, even using severe conditions
4 (Lora and Wayman 1978, Leschinsky et al., 2008).

5 The utilization of an industrial enzymatic cocktail (with cellulase and xylosidase activities)
6 for the prehydrolysis of *Miscanthus* biomass, despite a very low impact on the fibre structure
7 and composition, disrupted enough of the remaining polymers to enhance the removal of
8 lignin during the organosolv step to a large extent. The main reason explaining the enhanced
9 delignification could be the weakening of the lignin-holocellulose complex in mild conditions
10 facilitating depolymerisation of lignin (through the cleavage of aryl-ether bonds) during the
11 ethanolysis and leading to the formation of small, easy removable fragments. As a result, very
12 mild conditions were required for the organosolv delignification step to obtain a good
13 solubilisation of the disrupted lignin leading to cellulosic pulps with very low lignin content.
14 Table 2 lists the organosolv conditions for *Miscanthus* pretreatment optimized for one- and
15 two-steps processes, the lignin contents of the residual pulps, the furans content in the liquid
16 phase and the combined severity parameters of each organosolv treatment. Combined severity
17 correlation has been described to be an efficient tool to evaluate and compare pretreatments as
18 a function of the reaction time, the temperature and the pH of the medium (Chun Sheng, 2011
19 ; Brosse et al., 2009, 2010). It clearly appears that when starting from a biotreated material, a
20 lower process severity was required for better lignin removal from the pulp. Interestingly, the
21 mild organosolv conditions used starting from biotreated material clearly produce a lower
22 concentration of furans (degradation products). Nevertheless, in order to make this process
23 cost effective, further studies are required to optimize the enzymatic step and to reduce the
24 amount of enzyme cocktail during pretreatment.

25

1 **4. Conclusion**

2 Enzymatic pre-hydrolysis was assessed as a first step in a pretreatment process of *Miscanthus*
3 biomass involving an aqueous-ethanol organosolv treatment. Despite a very low impact on
4 fibre structure and composition, the enzymatic pre-treatment disrupted enough of the
5 lignocellulosic matrix to enhance removal of lignin and hydrolysability of the cellulosic pulp
6 for the production of monomeric glucose. The purpose of this process is to facilitate the
7 separation of lignocellulosic components during the delignification step ; however further
8 evaluations are required to determine the specific action of the enzymatic step and the
9 effective global energy input in the whole process.

10

11 **Appendix. Supplementary data**

12 Supplementary data associated with this article can be found, in the online version

13

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20 **References**

- 21 Barth H, Regnier F., 1980. High-performance gel permeation chromatography of water-
22 soluble cellulose. J Chromatogr 192:275-93.
- 23 Brodeur, G., Yau, E., Badal, K., Collier, J. Ramachandran, K.B., Ramakrishnan, S., 2011.
24 Chemical and Physicochemical Pretreatment of Lignocellulosic Biomass: A Review
25 SAGE-Hindawi Access to Research Enzyme Research, Article ID 787532, 17 pages
- 26 Brosse, N., Sannigrahi, P., Ragauskas, A., 2009. Pretreatment of *Miscanthus x giganteus*
27 using the ethanol organosolv process for ethanol Production. Ind. Eng. Chem. Res. 48,
28 8328–8334.

- 1 Brosse, N., El Hage, R., Sannigrahi, P., Ragauskas, A., 2010. Dilute Sulphuric Acid and
2 Ethanol Organosolv Pretreatment of Miscanthus x Giganteus. Cellulose Chemistry and
3 Technology. 44 (1-3), 71-78.
- 4 Chun Sheng G., Hui Teng T., Keat Teong L., Brosse N, 2011. Evaluation and optimization of
5 organosolv pretreatment using combined severity factors and Response surface
6 methodology, Biomass Bioener, in press
- 7 El Hage, R., Brosse, N., Chrusciel, L., Sanchez, C., Sannigrahi, P., Ragauskas, A., 2009.
8 Characterization of milled wood lignin and ethanol organosolv lignin from Miscanthus.
9 Polym. Degrad. Stab. 94, 1632–1638.
- 10 El Hage, R., Chrusciel, L., Desharnais, L., Brosse, N., 2010a. Effect of Autohydrolysis of
11 Miscanthus x giganteus on Lignin Structure and Organosolv Delignification. Bioresour.
12 Technol., 101, 9321-9329.
- 13 El Hage, R., Brosse, N., Sannigrahi, P., Ragauskas, A., 2010b. Effect of the Severity of the
14 Ethanol Organosolv Process on the Chemical Structure of Ethanol Organosolv Miscanthus
15 Lignins, Polymer Degrad. Stab., 95, 997-1003.
- 16 FitzPatrick, M., Champagne, P., Cunningham M.F., Whitney, R.A., 2010. A biorefinery
17 processing perspective: Treatment of lignocellulosic materials for the production of value-
18 added products Bioresource Technology 101, 8915–8922.
- 19 Foston, M., Ragauskas, A.J., 2010. Changes in lignocellulosic supramolecular and
20 ultrastructure during dilute acid pretreatment of Populus and switchgrass Biomass and bio
21 energy 34, 1885- 1895
- 22 Garrote, G., Dominguez, H., Parajo, J.C., 2001. Generation of xylose solutions from
23 eucalyptus globulus wood by autohydrolysis–posthydrolysis processes: posthydrolysis
24 kinetics. Bioresour. Technol. 79 (2), 155–164.
- 25 Garrote, G., Dominguez, H., Parajo, J.C., 2002. Autohydrolysis of corncob: study of non-
26 isothermal operation for xylooligosaccharide production. Journal of Food Engineering 52,
27 211–218.
- 28 Ghose, T.K., 1987. Measurement of cellulase activities. Pure & Appl. Chem. 59(2), 257-268.
- 29 Granata, A., Argyropoulos, D.S., 1995. 2-chloro-4,4,5,5-tetramethyl-1,3,2-
30 dioxaphospholane, a reagent for the accurate determination of the uncondensed and
31 condensed phenolic moieties in lignins. J Agric Food Chem. 43, 1538–1544.
- 32 Hall, M., Bansal, P., Lee, J.H., Realff, M.J., and Bommarius A.S., 2010. Cellulose
33 crystallinity – a key predictor of the enzymatic hydrolysis rate. FEBS Journal, 277, 1571-
34 1582.
- 35 Katzen, R., Schell, D.J., 2006. [Lignocellulosic feedstock biorefinery: history and plant
36 development for biomass hydrolysis.](#) Biorefineries-Industrial Processes and
37 Products, 1, 129-138.
- 38 Lee, J.M., Shi, J., Venditti, R.A., Jameel, H., 2009. Autohydrolysis pretreatment of Coastal
39 Bermuda grass for increased enzyme hydrolysis. Bioresource Technology 100, 6434–
40 6441.
- 41 Leschinsky, M., Zuckerstatter, G., Weber, H.K., Patt, R., Sixta, H., 2008. Effect of
42 autohydrolysis of Eucalyptus globulus wood on lignin structure. Part 1 : Comparison of
43 different lignin fractions formed during water prehydrolysis. Holzforschung. 62, 645-652
- 44 Lin, S.Y., Dence C.X., 1992. “Methods in Lignin Chemistry”, Springer Series in Wood
45 Science, Springer-Verlag Berlin, Heidelberg.
- 46 Lora, J.H., Wayman, L., 1978. Delignification of hardwoods by autohydrolysis and
47 extraction. 61, 47-50.
- 48 Martinez, A., Rodriguez, M.E., York, S.W., Preston, J.F., Ingram, L.O., 2000. Use of UV
49 absorbance to monitor furans in dilute acid hydrolysates of biomass. Biotechnol. Prog.
50 16(4), 637-641.

1 Munoz, C., Mendonc, R., Baeza J., Berlin, A., Saddler, J., Freer, J., 2007. Bioethanol
2 production from bioorganosolv pulps of *Pinus radiata* and *Acacia dealbata* J Chem
3 Technol Biotechnol 82, 767–774.

4 Palmqvist, E., Hahn-Hagerdal, B., 2000. Fermentation of lignocellulosic hydrolysates. II:
5 inhibitors and mechanisms of inhibition. Bioresour. Technol. 75, 25-33.

6 Pan, X., Kadla, J.F., Ehara, K., Gilkes, N., Saddler, J.N., 2006. Organosolv ethanol lignin from hybrid
7 poplar as a radical scavenger: relationship between lignin structure extraction conditions
8 and antioxidant activity. J. Agric. Food Chem. 54, 5806–5813.

9 Pan, X.J., Xie, D., Yu, R.W., Lam, D., Saddler, J.N., 2007. Pretreatment of lodgepole pine
10 killed by mountain pine beetle using the ethanol organosolv process: Fractionation and
11 process optimization. Ind Eng Chem Res. 46(8), 2609–2617.

12 Park, S., Baker, J.O., Himmel, M.E., Parilla, P.A., Johnson, D.K., 2010. Biotechnol. Biofuels
13 3, 10.

14 Patel, D.P., Varshney, A.K., 1989. The effect of presoaking and prehydrolysis on the
15 organosolv delignification of bagasse. Indian J. Technol. 27(6) 285-288.

16 Söderstroem, J.; Pilcher, L. ; Galbe, M.; Zacchi, G., 2003. Two-step steam pretreatment of
17 softwood by dilute H₂SO₄ impregnation for ethanol production. Biomass and Bioenergy.
18 24, 475 – 486.

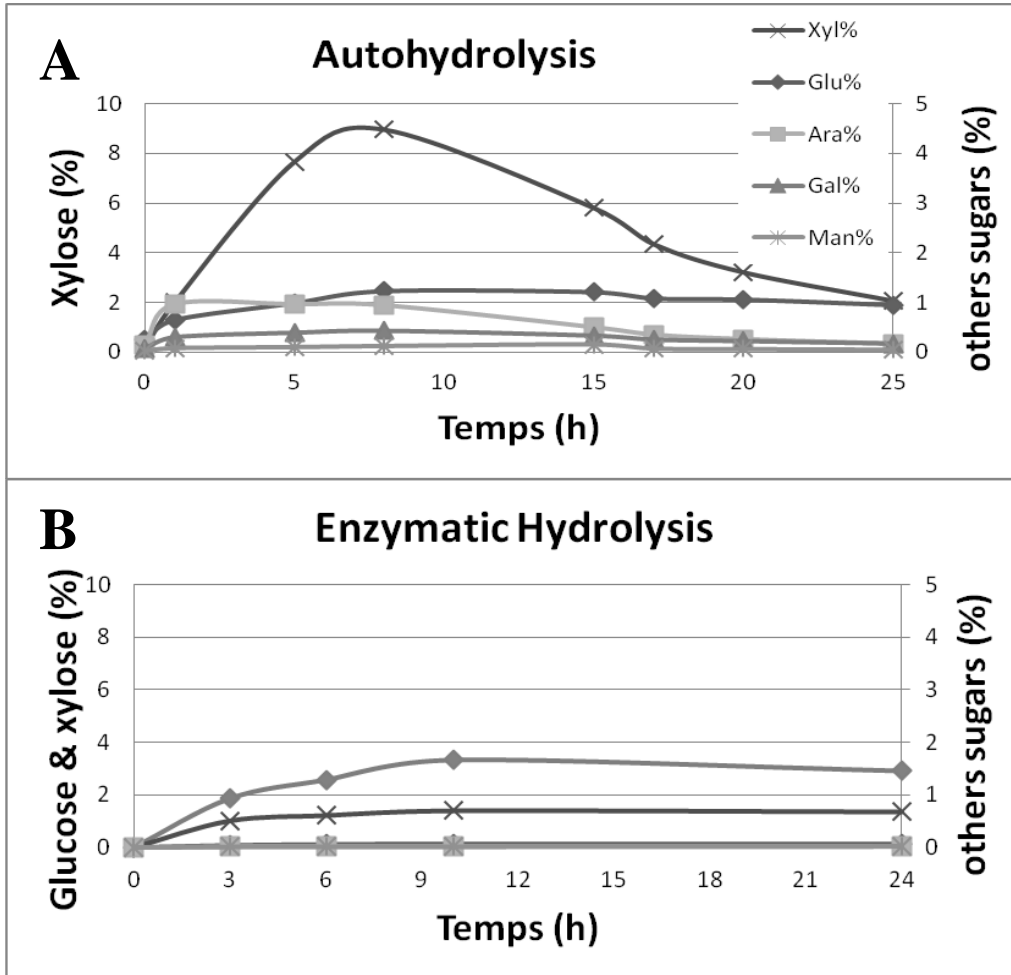
19 Somogyi, M., 1952. Notes on sugar determination. J. Biol. Chem. 195, 19–23.

20 Thomsen, M.H., Thygesen, A., Thomsen, A. B., 2009. Identification and characterization of
21 fermentation inhibitors formed during hydrothermal treatment and following SSF of
22 wheat straw. Applied Microbiol. Biotechnol. 83(3), 447-455.

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Figure 1 Composition of the hydrolysates (sugar contents) as a function of the residence time of autohydrolysis and enzymatic hydrolysis treatments.^a



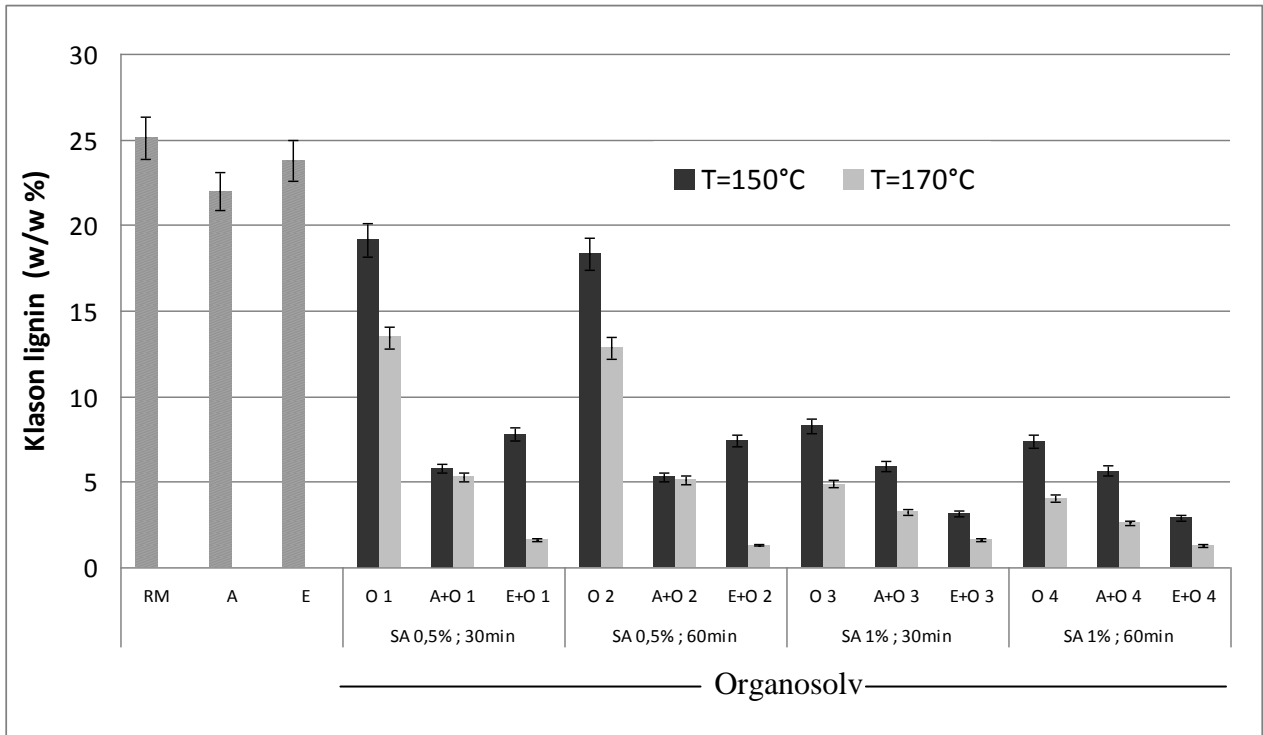
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^a All data are given as a percentage (w/w) with respect to initial weight of miscanthus.

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Figure 2 Klason lignin contents in the raw *Miscanthus* biomass and in pulps.

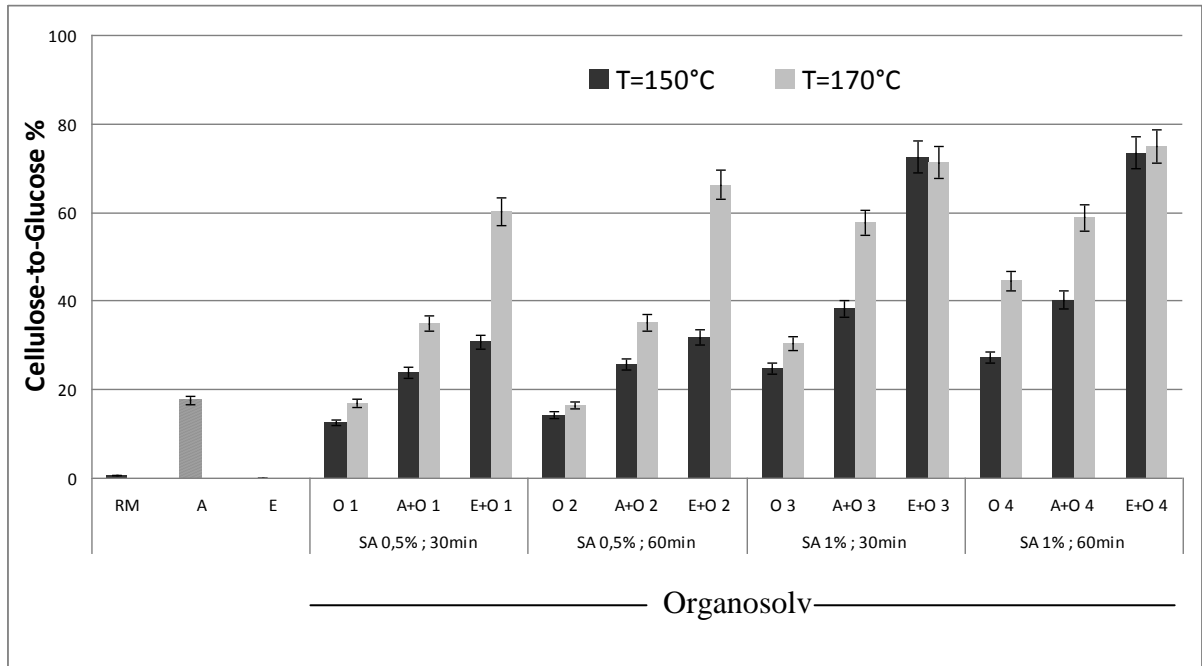


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RM = Raw Material ; A= autohydrolysis ; E = Enzymatic hydrolysis ; O = ethanol Organosolv treatment performed at 150°C and 170°C. Error bars represent standard deviations.

1 **Figure 3** Cellulose-to-Glucose conversion^a of pulps after organosolv treatments

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4 ^a Cellulose conversion to monomeric glucose, based on glucose content in the pulps.

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6 RM = Raw Material ; A= Autohydrolysis ; E = Enzymatic hydrolysis ; O = ethanol

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8 Organosolv treatment performed at 150°C and 170°C. Error bars represent standard

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10 deviations

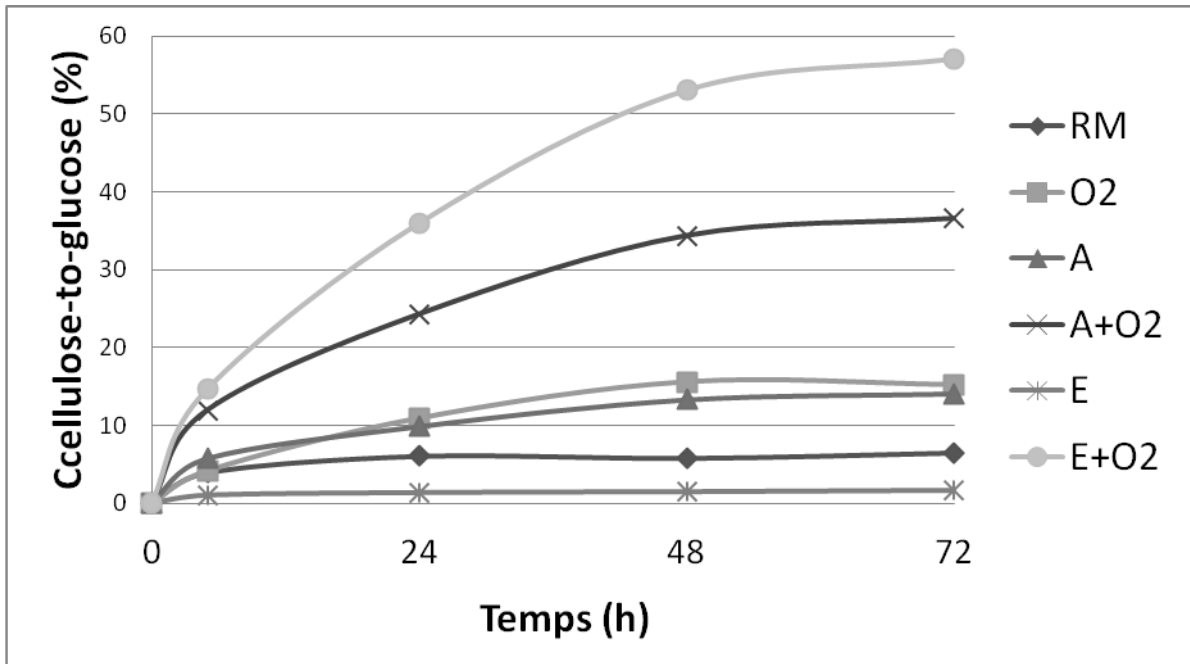
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1 **Figure 4** Cellulose-to-Glucose conversion (%)^a at different time points for 6 pulps^b
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^a Cellulose conversion to monomeric glucose, based on glucose content in the pulps.

^b RM = raw material ; O2 : organosolv pretreatment (experimental conditions see Figure 3) ;
 A = autohydrolysis ; E=enzymatic prehydrolysis

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Table 1 Composition^a of *Miscanthus* biomass, solid residue after autohydrolysis and enzymatic hydrolysis. Composition^a of soluble fractions after prehydrolysis.

^aAll data are yields of components(g) per 100g of oven-dried *Miscanthus*; values in parentheses correspond to

Prehydrolysis	Solid residues							Aqueous phase	
	Glc%	Xyl%	Ara%	Gal%	Man%	KL ^b %	SL ^c %	SL%	Furans%
Without	49,09 (0,6)	21,65 (0,5)	1,83 (0,03)	0,43 (0,01)	0,27 (0,01)	25,11 (0,35)	1,65 (0,83)	-	-
Autohydrolysis	48,09 (0,9)	8,13 (0,8)	0,19 (0,01)	0,07 (0,01)	0,11 (0,01)	22,01 (0,85)	0,65 (0,13)	2,53 (0,11)	1,52 (0,09)
Enzyme	45,5 (0,5)	19,73 (0,9)	1,69 (0,04)	0,33 (0,02)	0,19 (0,01)	23,19 (0,68)	0,85 (0,31)	1,26 (0,18)	0,02 (0,02)

6 the standard error established from three independent experiments ; ^b Klason lignin (including ashes) ; ^c soluble
7 lignin

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1 **Table 2.** Organosolv conditions with combined severity parameters for one- or two-steps
 2 processes and Klason lignin content of the pulps

Prehydrolysis	Organosolv conditions	Combined severity^a	Klason lignin^b	Furans^b
Without ^c	190°C, H ₂ SO ₄ 1.2%, 60min	2.77	5.1%	1.9%
Autohydrolysis	170°C, H ₂ SO ₄ 1 %, 60min	1.80	2.6 %	2.36%
Enzyme	170°C, H₂SO₄ 0.5%, 30min	1.46	1.3 %	0.6%

3 ^a Log(R₀)-pH, R₀ = t.exp(T-T_{ref})/14.7, T_{ref} = 100°C ; ^byields based on dry weight of raw miscanthus ; ^c Brosse et
 4 al, 2009.
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Table 3. Organosolv lignins characterization by GPC, by ^{13}C NMR and by ^{31}P NMR.,

			O2 ^a	A+O2 ^a	E+O2 ^c
GPC		Mw ^d	5715	5103	4281
		Mn ^e	1932	1496	1316
		Ip (Mw/Mn) ^f	2.96	3.41	3.25
<hr/>					
		ppm	NMR assignments		in number of carbon per aryl ^g
^{13}C NMR	155-142	Aromatic C-O	1.8	1.9	1.7
	142-124	Aromatic C-C	2.0	2.1	2.0
	124-102	Aromatic C-H	2.3	2.1	2.3
	89-78	C β in β -O-4, C α in β -5 et β - β	0.2	0.3	0.9
	73-71	C α in β -O-4	0.4	0.3	0.7
	64-61	C- γ	0.2	0.5	1.0
	56	OMe	1.2	1.0	1.1
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		ppm	NMR assignments ^h		in mmol/g ^{g,i}
^{31}P NMR	145.5-150.0	OH aliphatic	1.73	0.94	1.36
	144.9	OH cyclohexanol			
	136.5-144.5	Phénols	0.91	1.37	1.17
	141.8-143.5	OH syringyle	0.28	0.55	0.43
	138.7-140.1	OH guaiacyl	0.38	0.53	0.45
	137.2-138.1	OH p-hydroxyphenyle	0.25	0.29	0.29
	134.0-135.7	Carboxylic acid	0.02	0.07	0.05

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^a see Figure 3, O = organosolv lignin extracted from raw miscanthus ; ^b A= organosolv lignin extracted from residue after autohydrolysis ; ^c E = organosolv lignin extracted from residue after enzymatic hydrolysis ; ^d weight average molecular weight ; ^e number average molecular weight ; ^f polydispersity Mw/Mn ; ^g determined by integration ; ^h lignin samples phosphorylated with 2-chloro-4,4,5,5-tetramethyl-1,2,3-dioxaphospholane ; ⁱ determined by integration with cyclohexanol as an internal standard.