Grant agreement no: KBBE-2009-3-244362

LIGNODECO PROJECT

Optimized pre-treatment of fast growing woody and nonwoody Brazilian crops by detailed characterization of chemical changes produced in the lignin-carbohydrate matrix

Second Periodic Report

1-Jul-2011 to 31-Dec-2012

Grant agreement no: KBBE-2009-3-244362

Project acronym: LIGNODECO

Project title: Optimized pre-treatment of fast growing woody and nonwoody Brazilian crops by detailed characterization of chemical changes produced in the lignin-carbohydrate matrix

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*Use the font **Times New Roman**. Font size in text is **12 points**. Page numbering is header - right, no numbering on the front page. Line spacing single, 0 point before and after paragraph.*

For those who have been active in research, the appropriate length would be 5–10 pages. For the institutes/companies that have not worked, please report only the appropriate items e.g. plans.

1. SUMMARY OF THE WORK

The primary objective of the **LIGNODECO** project is the development of optimized/new pre-treatment technologies for deconstruction of high productivity and fast-growing Brazilian selected eucalypt clones and nonwoody lignocellulosic feedstock aimed at production of special pulps, biofuels and bio-materials, taking advantage from the use of modern analytical techniques, enabling in-depth identification of the changes produced in the main plant polymers and other minor constituents. The work plan encompasses seven work packages (WP1-WP7) broken down into twenty tasks, which resulted in twenty three deliverables. In WP1 the collection/selection of feedstock for deconstruction studies were dealt with. The optimization of pre-treatments for woody and nonwoody materials was largely covered in WP2. Work package three (WP3) involves the in depth physical/chemical characterization of the original and pre-treated materials using advanced analytical tools. The tie in between pre-treatment and industrial use of lignocellulosics is being studied under WP4. The demonstration studies are included in WP5 and the management and dissemination activities in WP6 and WP7, respectively.
This second periodic report presents developments on WP1, WP2, WP3, WP4 and WP5 that were under UFV, CTP, VTT, CIB/IRNAS and NOVOZYMES responsibility, namely: ***General characterisation of the lignocellulosic feedstocks (Task 1.2); Chemical deconstruction by alkaline processing(Task 2.1); Chemical deconstruction by solvent process (Task 2.2); Enzymatic deconstruction using hydrolases and oxidoreductases (Task 2.3); Fibre morphology and strength (Task 3.1); Polysaccharide analyses (Task 3.2); Analysis of lignin and minor components (Task 3.3); Analysis of black liquors and other side streams (Task 3.4); Pulp characteristics and papermaking evaluation (Task 4.1); Biofuel potential of pre-treated materials and residues (Task 4.2); Upgrading xylans as paper grade pulp additives (Task 4.3);VTT organosolv demonstrations (Task 5.1); CTP enzymatic pre-treatment and bleaching pilot plant trials (Task 5.2); Suzano pulp beatability pilot trials (Task 5.3).***The main findings on the work accomplished during the last sixth month period were: (1) the new Eucalyptus globulus sample batch aimed at the demonstration studies showed desirable characteristics for pulp and bioproducts production; (2) the new Eucalyptus globulus was evaluated in the kraft and Soda-AQ process and the results were good in both processes; (3) with the results obtained, it was concluded that this new one Eucalyptus globulus is good to continue the research and finalize the Lignodeco project; (4) the LGF cooking was successfully demonstrated at pilot scale, resulting in LGF pulp with good glucose release and high ethanol yield (~90% of the theoretical); (5) in pilot scale better delignification efficiency and lower residual lignin content was reached than in any of the LGF laboratory experiments; (7)cooking process (Soda-AQ or kraft) and kappa number of unbleached pulp (20 or 15) did not seem to have

impact on mean area weighted length, mean fibre curl index, broken fibre content, fine content of the bleached pulps; (8) vessel content was lower after Soda-AQ cooking than after kraft cooking and a higher delignification of unbleached pulp (reduction of kappa number 20 to 15) induced (i) a reduction of mean fibre width, the hydrogen bonding potential and (ii) the increase of fibrillation of the bleached fibres and (iii) reduction of vessel content in the bleached pulp; (9) Soda-AQ process seems to be the best process, due to reduction of vessel content in the pulp which could reduce speckles and picking problems during the printing of the paper; (10) the black liquor studies demonstrate that different pulping conditions can result in various changes in the composition and structure of the main black liquor compounds; (11) in the case of the pulps intended for bioethanol and biogas production, it seems that the soda-O2 process is more efficient than soda-AQ process for depolymerization of eucalypt wood and elephant grass lignins; (12) no clear correlation between pulp hydrolysability and cellulose crystallinity of raw material was detected; (13) the alkaline extraction improves LGF pulp hydrolysability because of enhanced of lignin and xylan removal and in the most cases, the hydrolysis time of 48h is required for the maximum sugar release; (14) significantly higher ethanol yield was obtained after ball milling than pressafiner treatment when no additional enzymatic treatments were performed; (15) Cel45A and Cel7b endoglucanase families demonstrated the ability to improve resultant handsheet physical strength properties, the latter enzyme treatment without sacrificing tear strength; (16) for unconventional pulps the high redox-potential laccase from Polyporus pinsitus showed synergistic behaviour when combined with violuric acid, reaching a brightness increase of 16 units for the kappa 20 pulp; (17) the data continuously favours the use of this unconventional sulphur-free pulp, especially at kappa 20, with regards to enzymatic strengthening and enzymatic bleaching; (18) the ideal conditions for xylans extraction are: 15 minutes, 15% of consistency and 400 kg/odt of NaOH; (19) grass xylan deposition on wood pulp was successful, increasing the xylan content from 14.4 to 17.3%; (20) the deposited xylans are stable across bleaching and beating; (21) bleaching yield gains of about 3% due to xylans deposition were achieved; (22) pulps with improved strength properties and beatability were obtained; (23) the removal of the xylans from pulp using alkali treatment reduced significantly the chlorine demand in the bleaching sequence DPD at 90% ISO brightness; (24) the xylan depleted pulps derived from the xylan extraction treatment showed potential for production of special tissue grade papers with improved drainability, bulk, softness and water absorption capacity, and with acceptable tensile and tear strength; (25) The LGF organosolv cooking was successfully demonstrated also at pilot scale, producing well hydrolysable biomass with high ethanol yield; (26) alkaline demonstration trial at pilot scale validated results obtained at laboratory; (27) The enzymatic deconstructions using oxido-reductases or hydrolases performed in laboratory were also validated at pilot scale.

Some interesting conclusions were obtained and they were summarized above but details can be found in the individual reports of each partner that are shown in the **Appendixes A-F**. The LIGNODECO 36th month meeting took place in Madrid - Spain in the date of December 12, 2012, with 17 participants (see photo).

Picture taken in Centro de Investigaciones Bioógicas (CIB) during Lignodeco's sixth technical meeting (December 12, 2012).

2. PROJECT OBJECTIVES FOR THE PERIOD

The LIGNODECO project objectives predicted for the thirty sixth months period were: (1) characterization of new Eucalyptus globulus for demonstration studies; (2) optimization of physical-chemical and enzymatic pre-treatments adapted to rapid-growth feedstocks of interest for the Brazilian lignocellulose sector by organosolv pulping, together with use of hydrolases and oxidoreductases as deconstruction biocatalysts; (3) developmentes on demonstrations studies. The objectives were achieved.

3. WORK PROGRESS AND ACHIEVEMENTS DURING THE PERIOD

3.1 Work Progress

Provide a concise overview of the progress of your work (results and discussion). Report according to WPs and Tasks

- *Highlight clearly significant results.*
- *Explain the reasons for possible deviations from Annex1 and their impact*

- *as well as corrective actions taken / suggested*

3.1.1 Progress on WP1

Task 1.2 General characterisation of the lignocellulosic feedstocks

A general characterization of the new *Eucalyptus globulus* sample batch intended to the demonstration work was accomplished. This characterization was necessary since the original sample provided by ENCE-Spain was totally consumed in the optimization part of the work and a new sample (large volume) was required for the demonstration work. The large batch of the new sample was collected and evaluated for the same traits as the previous samples. The goal here was proving that the sample to be used for the demonstration studies had similar characteristics when compared to the one used for the optimization study.

Biomass density: Density is a very important factor affecting biomass utilization since it influences harvesting, transportation and processing costs. In this regard, the new *E. globulus* showed a slightly higher density (591 kg/m^3) than the European *E. globulus* (532 kg/m^3). A high density means more weight of biomass charge per unit volume of the equipment used for biomass deconstruction, which improves pulp mill productivity. However, the differences between the two materials were not so significant to the point of causing significant impact in the demonstration studies.

Biomass Inorganic Composition: The most significant inorganic chemical traits of the various biomass types are presented in Table 1. The total inorganics measured on the new sample by complete biomass combustion (ash content) was 0.25%; this value was slightly lower than that of the previous sample used in the optimization study (0.31%). The low ash content is advantageous since minerals are very detrimental for most industrial utilization of biomass because of corrosion and deposits in equipment, reduced biomass heating value and decreased mill throughput. In general the amount of inorganics present in both eucalypt wood samples were very low and quite acceptable for most industrial applications.

Table 1: Inorganic composition of *Eucalyptus globulus* samples used in the optimization and demonstration studies

Composition		*E. globulus (optimization studies)*	*E. globulus (demonstration studies)*
Ash	Ash, %	0.31	0.25
Metals	Cu, mg/Kg	2.5	1.5
	Fe, mg/Kg	13.3	6.6
	Ca, mg/Kg	668.5	307
	Mn, mg/Kg	43.4	28.9
	Mg, mg/Kg	227.1	175

Biomass Organic Composition: The basic organic composition of the *E. globulus* samples used in the optimization and demonstration studies is presented in the Table 2. Extractives

are quite troublesome since they cause many difficulties in operating the industrial facilities, causing unexpected lost time in the operation for cleaning of equipment and instruments due to their stickiness and tackiness. There were no significant differences between the extractive contents of the *E. globulus* samples used for the optimization and demonstration studies, regardless of the extraction methods, i.e., acetone extraction or total extraction (ethanol/toluene (1:2) → ethanol → hot water solvent system).

In order to measure the biomass cell wall components, it is relevant to remove all extractives present in the material. The standard procedure for removing all extractives from biomass is through of extraction using ethanol/toluene(1:2) → ethanol → hot water solvent system to prepare the so-called extractive free wood. Table 2 shows the chemical composition of the extractive free E. globulus wood samples. The main biomass components that find application for production of biofuel and bio-materials are the cellulose, hemicelluloses and lignin fractions. The new E. globulus wood batch showed slightly lower lignin content that the one used for the optimization studies. On the other hand, the new batch presented lignin of lower S/G ratio in relation to the previous sample, but still quite high. As a consequence of its lower lignin content, the new sample presented higher glucans content than the previous sample. In general, the two E. globulus samples presented similar hemicellulose sugars (mannans, galactans and arabinans), uronic acids and acetyl groups content. An overall assessment indicate that that the *E. globulus* samples used for optimization and demonstration studies are similar and no big surprises shall be expected in the demonstration studies.

Table 2: Chemical characteristics of *Eucalyptus globulus* samples used in the optimization and demonstration studies

Sample	Acetone Extractive	Total Extractive	Sugar Composition, %				Acid Soluble Lignin, %	Klason Lignin, %	Total Lignin, %	Lignin S/G ratio	Acetyl groups, %	Uronic Acids, %
			Glucan	Xylans	Mannans	Arabinans						
Optimization studies	1.2	2.5	46.6	13.6	1.4	0.2	4.8	23.8	28.6	4.0	2.6	3.0
Demosntration studies	2.3	3.0	53.0	13.7	0.9	0.3	2.5	22.7	25.2	3.4	2.0	2.8

Conclusions

The new *Eucalyptus globulus* sample batch aimed at the demonstration studies showed desirable characteristics for pulp and bioproducts production, namely: low lignin content, acceptable S/G ratio, low metal content, high density and high carbohydrate content. There were no large differences between the main wood traits when comparing this sample with the one used in the optimization studies.

3.1.2 Progress on WP2 - Optimised pretreatments for woody and non woody materials

Task 2.1 Chemical deconstruction by alkaline processing

Due to preservation problems with the *Eucalyptus globulus*, it was necessary to get more wood chips to proceed with the research at the Lignodeco. UFV have arranged a new sample material for us to do the trials.

Wood chips samples of *Eucalyptus globulus* were evaluated on different types of cooking processes: Kraft and Soda-AQ (soda anthraquinone). The wood chips were delivered at Suzano Mucuri mill by the Pulp and Paper Laboratory of Universidade Federal de Viçosa (UFV). New delignification curves were done in order to generate data to the partners, so they can continue with the research. As all the work were done before, we focus on the development of new delignification curves for the already chosen processes, Kraft and Soda-AQ, also in the range defined as appropriated in each case. No need to redo all the study in all processes.

A brief comparison for the two *Eucalyptus globulus* material was done also, the main characteristics and the pros and cons of each wood sample. The preparation of chips for cooking was performed identically for all types of cooking simulated. Chips were classified according to standard SCAN-CM 40: 94 . The chip quantity classified between 4 to 6 mm thicknesses was packed in bags made of polyethylene (that prevents moisture exchange with the environment) in the amount established for each cooking and then stored.

Cooking trials were held in a CRS digester (CPS 5010 Recycle Digester System), with 2 individual reactors of 10 liters each, equipped with a forced liquor circulation system and electrically heated with temperature and pressure control. The digester is coupled with a cooling system (Coil System with residual liquor, involved with water at room temperature), to ensure the cooling of the liquor after the cooking simulation.

Several cooking experiments were performed for each sample, using different active alkaline charge expressed as NaOH, to establish the delignification curves for each sample. At this case, as we didn't have enough samples to perform the trials and all the repetitions required for the development of the mathematical model, we based on the previous results with the *Eucalyptus globulus*, and then developed a new delignification curve with these samples. This strategy was proven to be a good one at the end, when the results were obtained and analyzed.

Alkaline content, yield and reject content were estimated for each wood using the previous equations for retrieval of kappa numbers 15, 20, 35, 50 and 70. For the kraft process, we just perform the trials for the kappa numbers 15 and 20, which were elected the most important for this process, as we are analyzing the kraft process with high yield, to achieve a good quality pulp for paper production. The conditions of the Soda-AQ cooking are: Anthraquinone (AQ): 0,05% wood based, Liquor/Wood Ratio = 4/1, Temperature of the cooking = maximum of 170°C, Time at the temperature = 90 minutes, Time at the temperature = 50 minutes. For the Kraft Cooking, the fixed conditions were Sulfidity (S): 25%, Liquor/Wood Ratio = 4/1, Temperature of the cooking = maximum of 170°C, Time at the temperature = 90 minutes, Time at the temperature = 50 minutes.

Table 2: Main results of the Soda-AQ cooking trials. Kappa number between 15 and 70.

	Alkali Charge	kappa	Screened Yield	Rejects Content	Viscosity	pH	Residual Active Alkali	Brightness	Solids Content	Organic	Inorganic
	%		%	%	dm³/kg		g/L	ISO	%	%	%
NaOH+AQ	25,0	15,0	50,2	0,1	962	13,7	18,0	30,6	10,7	50,3	49,7
	19,0	21,2	51,8	1,7	1083	12,7	11,2	29,8	10,5	56,2	43,8
	15,0	29,9	49,8	7,1	1105	12,4	7,1	22,7	10,4	58,5	41,5
	14,5	36,5	46,4	12,4	1130	12,4	6,8	20,9	10,1	59,0	41,0
	13,5	44,7	44,0	16,5	1148	12,1	5,9	18,1	9,3	60,1	39,9
	13,0	57,5	33,4	29,9	1170	11,7	4,8	16,4	9,2	62,4	37,6
	12,0	73,1	26,9	42,0	1228	11,2	3,1	13,1	8,0	61,0	39,0

The major advantage of using the non-sulfur processes is the simplification of gas handling and black liquor recovery in a mill operating as a biorrefinery. The results of the pulping are good. The beneficial effect of AQ (protection against peeling reactions) was quite significant at the lowest kappa target. At this time of the project was not possible to generate a good mathematical model for this cooking trial. With few points in the curve, the model was not reliable enough. To do such analysis would be necessary to have more wood sample to generate data. Anyway, it was enough material to give some guidance to the partners to proceed with the research, as planned. The main objective was accomplished.

Kraft Process: Also were performed with the new *Eucalyptus globulus* wood chips cooking trials for the kraft process. For those we prefer to focus on the lower kappa number, between kappa numbers 15 and 20. The main reason for that is that the kraft process is the best choice to produce pulp to paper purposes, so, the lower kappa number will gives us a higher screened yield.

Table 3: Main results of the Soda-AQ cooking trials. Kappa number objective were between 15 and 20.

	Alkali Charge	kappa	Screened Yield	Rejects Content	Viscosity	pH	Residual Active Alkali	Brightness	Solids Content	Organic	Inorganic
	%		%	%	dm³/kg		g/L	ISO	%	%	%
Kraft	22,0	14,9	53,1	0,2	1077,0	13,1	15,1	31,4	12,4	50,5	49,5
	18,0	17,3	53,2	1,1	1083,0	12,8	13,0	26,7	12,1	57,5	42,5
	16,0	20,2	52,5	3,5	1112,0	11,5	7,4	26,3	10,4	59,2	40,8

Comparing the Soda-AQ process with the kraft process for the same wood material, we can observe that the kraft pulping delivers a higher screened yield, and a lower alkali charge is necessary to reach the same kappa level. The Soda-AQ process gives also a good screened yield, with similar results to the kraft. Cooking trial for both Globulus at the Soda-AQ process, comparing screened yield results and reject content for each kappa number (Figure 1).

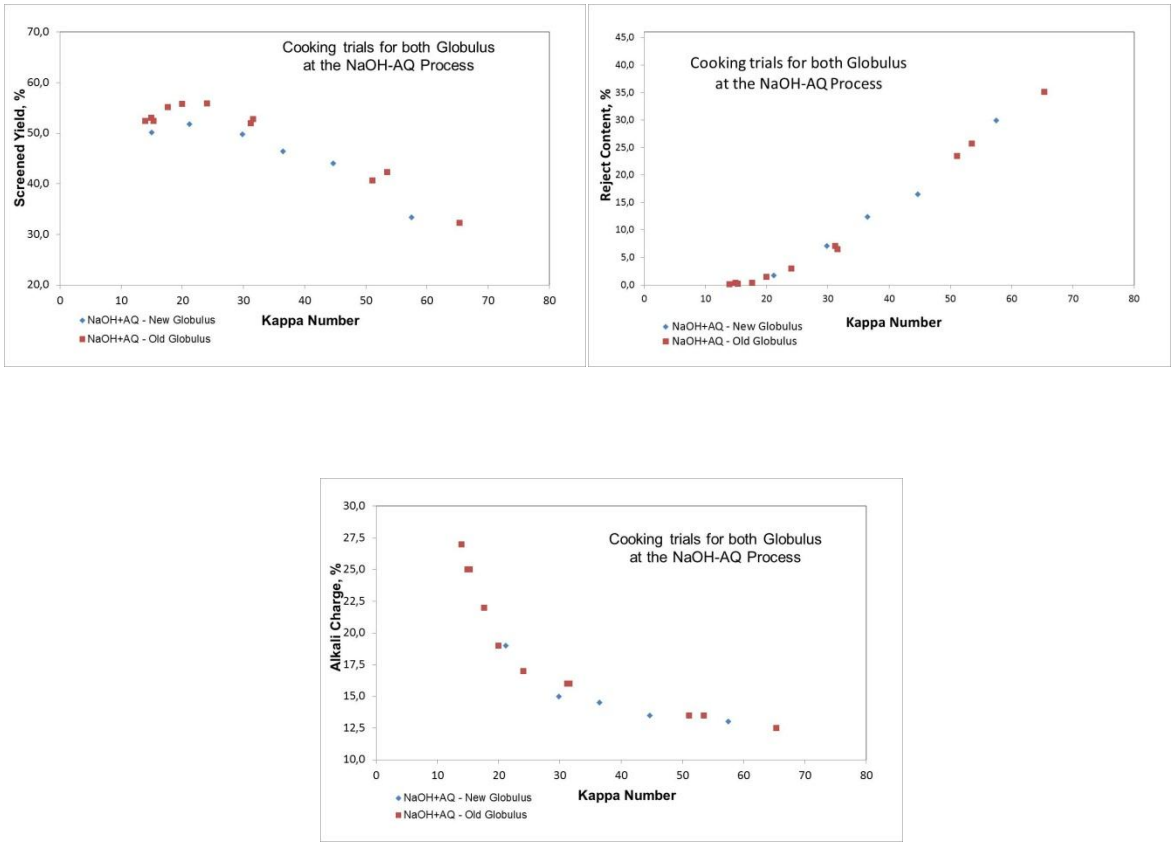


Figure 1. Cooking trial for both Globulus at the Soda-AQ process, comparing alkali charge for each kappa number.

The results for the previous wood material (Figure 1), can be observed that the new material delivers a slightly lower screened yield, and a higher alkali charge is necessary to reach the same kappa level. The results of rejects content are similar. In general, both materials are good, the differences are small, and with more chips available maybe the optimization of these results could be done to reach the same results of the previous material.

Production of NaOH + O_2 samples for the partners; Analysis of the pulp produced and its characterization

Pulp was cooked in the NaOH+O_2 process, at kappa number 35. It was utilized 7,0% of O_2 in each cooking trial, added in 3 different times, to guarantee excess of oxygen. The time of addiction was after 50 minutes, after 70 minutes and after 110 minutes. The temperature curve applied was: 90 min to reach the temperature of 170°C and more 50 min at the temperature of 170°C. Pulp was sent to the partner without screening, as done before, and as agreed. The CRS equipment was used to perform this trial. The capacity of the digester is 10 liters each. The digester has forced liquor circulation system and electrically heated with temperature and pressure control. A cooling system completes the equipment.

Table 4. Results for the trials of the NaOH+O_2 process

New globulus	Alkaline Charge	Kappa Number	Yield, %	pH	Residual Active Alkali, g/L
NaOH+O_2	30,0	36,1	51,1	12,1	10,1
	30,0	37,4	51,9	12,0	9,1
	30,0	33,9	52,4	12,5	12,0

This new wood material, which is a *globulus* from Brazil, contains more lignin and extractives. This explains that this wood material requires more alkaline charge to reach the same kappa number.

Task 2.2 Chemical deconstruction by solvent process

Experimental

The optimal cooking time was screened for a triplet hybrid of *E. grandis x [E. globulus x urophylla] (G1xUGL)* and *Suzano* clone, aiming to produce hydrolysable pulp with sufficient delignification and defibration without compromising cellulose yield. The cooking experiments were carried out under 3 bar pressure in 2L oil heated Juccheim reactor, using 3.5% phosphinic acid charge at 130 °C and water content of 15%. The alkaline extraction of LGF pulps was performed in constant conditions (1M NaOH, 2.5% consistency, room temperature, 24h with continuous agitation). For comparison of raw materials, the same conditions with optimized cooking time of 20h were used.

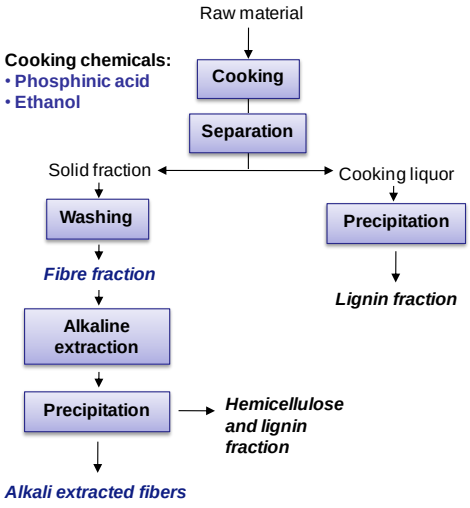


Figure 2. Two-stage fractionation procedure based on LGF organosolv cooking and alkaline extraction of fibre fraction.

The delignification efficiency of LGF cooking and the following alkaline extraction was evaluated according to the total lignin content of the unscreened pulps after acetone

extraction (NREL/TP-510-42618). The pulp total carbohydrate content was determined using total lignin and acetone extractives contents: Carbohydrate, % = 100% - total lignin, % - extractives, %. Pulp carbohydrate composition was determined after acid hydrolysis using ion exchange chromatography (AEC-PAD), and the polysaccharide composition was calculated according to Janson (1970) using constants of birch. As unscreened pulps were used, relatively large deviation in the chemical compositions of the samples was detected, making comparison of the samples rather difficult especially when alkaline extraction was not performed.

Optimisation of cooking time

Pulp yield, and especially cellulose yield, is one of the most important factors affecting how efficiently the original raw material can be converted to bioethanol. As expected, higher pulp yields could be obtained with shorter cooking times with both clones. To be able to evaluate the cellulose yields available for the enzymatic hydrolysis and fermentation and thus the degree of wood utilization, the pulp chemical compositions were normalized to pulp yields (Figure 3). With shorter cooking times the delignification became less efficient but the carbohydrates were better preserved as expected. After alkaline extraction the differences were minor, and lignin content still remained relatively low with cooking time of 16 h. With the alkaline extracted *G1xUGL* pulps, the lignin content started to increase steadily when cooking time was shorter than 20h (not shown, reported earlier). However, no clear gain in polysaccharide yield was obtained when cooking time was reduced below 16-20h. The shortest cooking time of 10h preserved especially xylan in LGF pulps of *Suzano* clone (Figure 4), but for *G1xUGL* the effect of cooking time on xylan content was not so clear. After alkaline extraction the differences in cellulose or xylan content were in both clones minor. No clear indication on optimal cooking time for maximal cellulose yield could be identified either.

Based on chemical composition, and especially lignin content, sufficient deconstruction of Eucalyptus clones should be reached with cooking time of 16-20h. This is well in accordance with the hydrolysis results obtained in WP4.

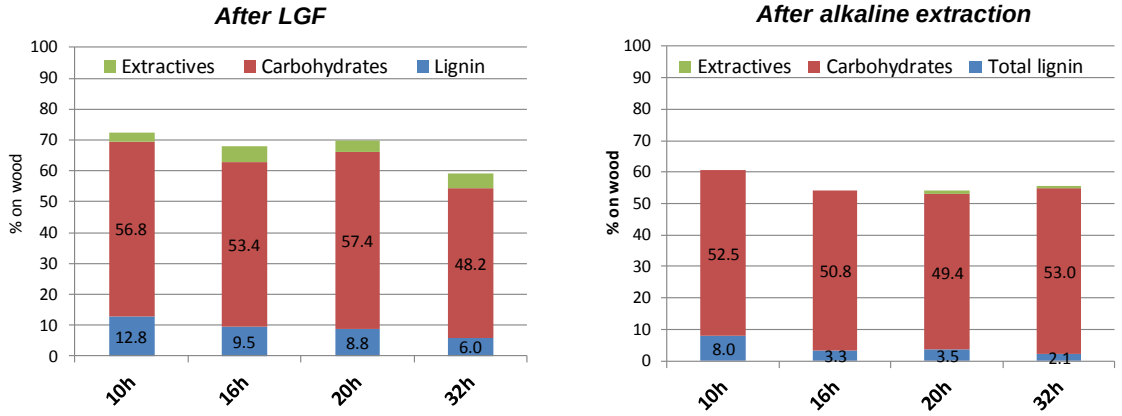


Figure 3. Chemical compositions after LGF organosolv cooking and the following alkaline extraction (scaled to pulp total yield) for Suzano's eucalyptus clone.

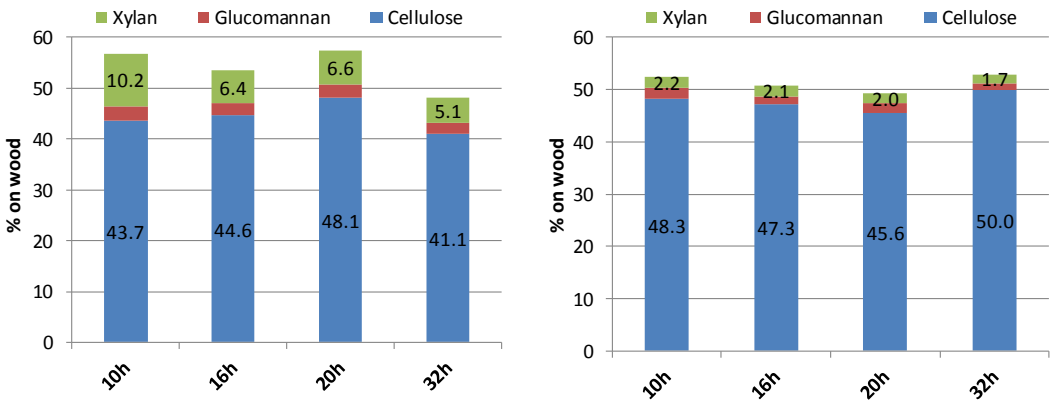


Figure 4. Polysaccharide contents after LGF organosolv cooking and the following alkaline extraction (scaled to the pulp total yields) for the Suzano's clone, describing the recovery of original wood raw material as polysaccharides. (after LGF=left, after alkaline extraction=right)

Comparison of feedstock

To rank the raw materials in respect of bioethanol production potential, the available feedstocks were cooked in the optimised conditions (130°C, 3.5% H_3PO_2, 15% water content) using cooking time of 20h.

After 20 h cooking and alkaline extraction, the DGxU2 clone gave the best yield (figure 5), whereas the lowest lignin contents were reached for *Suzano*, *G1xUGL* and *U1xU2* clones (Figure 6). These seem to cook easier than the other clones. After LGF cooking and alkaline extraction, the *E. globulus* provided the lowest lignin content as assumed based on the low initial wood lignin content. For some reason, the delignification was not effective for the elephant grass (*EG1*). The polysaccharide yield remained highest in *DGxU2* and *IP* clones, and the pulps with highest total polysaccharide content also had the highest cellulose yields (Figure 7). In elephant grass (EG1) pulp, the xylan content remained higher compared to the eucalyptus pulps even after alkaline extraction, resulting in lowest cellulose yield.

According to the chemical composition, the *DGxU2* clone seemed best for the bioethanol production. This is well in accordance with the pulp hydrolysability determined at WP4. In overall, the differences in pulp compositions were relatively small and obscured by the inaccuracy of unscreened pulp results.

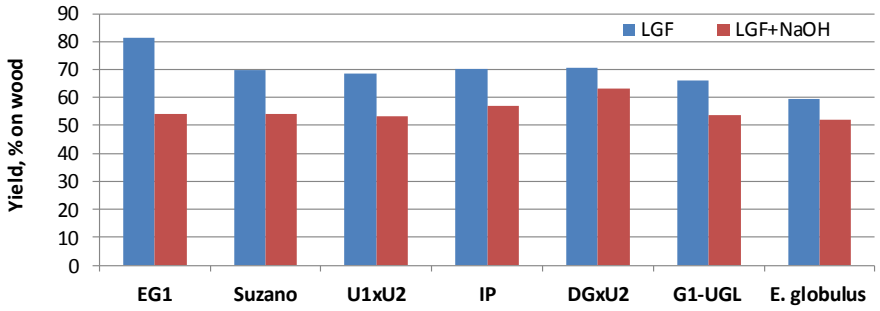


Figure 5. Pulp total yields (unscreened) after LGF cooking and the following alkaline extraction for selected raw materials.

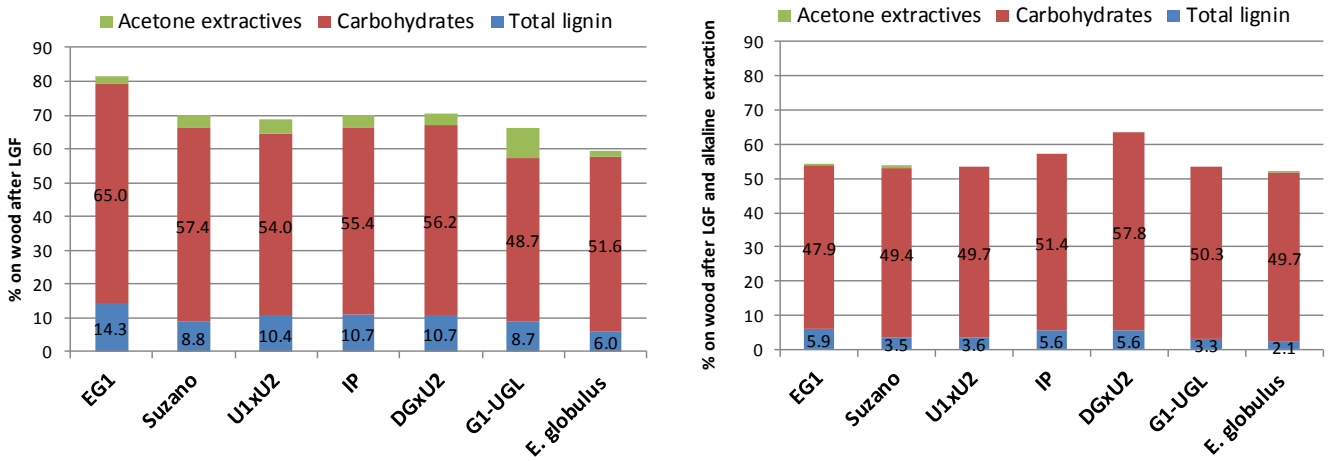


Figure 6. Chemical compositions after 20h LGF cooking (left) and alkaline extraction (right). The results scaled to pulp total yield. (* Ash content not taken into account, which probably has largest effect on EG1).

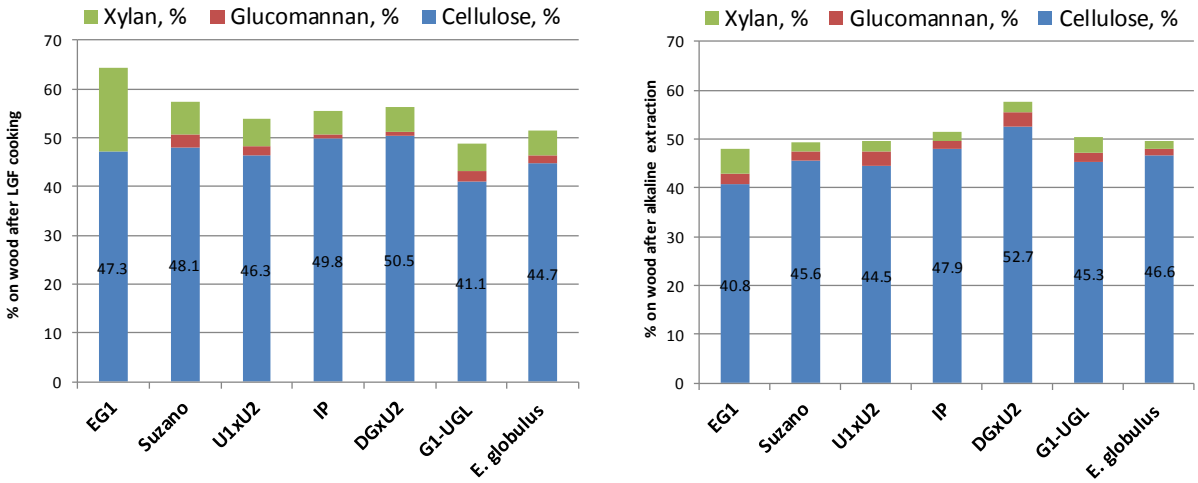


Figure 7. Polysaccharide contents after 20h LGF cooking (left) and alkaline extraction (left). Results scaled to the pulp total yields.

Task 2.3. Enzymatic deconstruction using hydrolases and oxidoreductases

Lignocellulosic samples. Air-dried Elephant grass (*P. purpureum* and eucalypt (*E. globulus*) wood were grounded in a Retsch cutting mill to pass through a 100-mesh screen and then finely ball-milled in a Retsch S100 centrifugal ball at 400 rpm using agate jar and balls. Samples of *P. purpureum* pith and cortex manually separated were milled using a knife mill (Janke& Kunkel, Analysemühle) and subsequently ball-milled in an agate container in a Retsch S100 centrifugal ball mill.

Fungal laccases and mediators. Two fungal laccase preparations, obtained from the basidiomycetes *Trametes villosa* (*TvL*) and *Myceliophthora thermophila* (*MtL*), were provided by Novozymes (Bagsvaerd, Denmark) for this study. Laccase activity was measured as initial velocity during oxidation of 5 mM ABTS from Roche to its cation radical (ε_{436} 29300 $M^{-1}\cdot cm^{-1}$) in 0.1 M sodium acetate (pH 5) at 24ºC. The mediators used were HBT and methyl syringate (MeS) for *TvL* and *MtL*, respectively.

Laccase-mediator treatments. The enzymatic treatments were performed using finely divided (ball-milled) woody (eucalypt) and nonwoody (elephant grass) samples. The high redox potential *TvL* was used in the presence of HBT as mediator, while the low redox potential *MtL* was used with MeS as mediator. Several laccase doses (10, 25 and 50 U/g) were assayed, together with several mediator doses (1-3%). The treatments were carried out in 200-mL pressurized bioreactors (Labomat, Mathis) placed in a thermostatic shaker at 170 $rev\cdot min^{-1}$ and 50 ºC, using 2 g (dry weight) of lignocellulosic samples at 6% consistency (w:w) in 50 mM sodium tartrate (pH 4) as a buffer under oxygen atmosphere (2 bars) for 24 h. In a subsequent step, samples at 6% consistency (w:w) were submitted to a peroxide-reinforced alkaline extraction (Ep) using 1% (w:w) NaOH and 3% (w:w) H_2O_2 (both referred to pulp dry weight) at 80 ºC for 90 min, followed by water washing. Cycles of four successive enzyme-extraction treatments were applied. Controls including laccase without mediator were also performed.

Hydrolytic enzymes. The multienzyme preparation from *Humicola insolens* (Ultraflo), as well as Cellulase NS22086 and Celluclast preparations, and NZ188 were kindly provided by Novozymes. The A-type feruloyl esterase from *Aspergillus niger* (AnFaeA) was heterologously expressed in *Pichia pastoris* as previously described (Juge et al, 2001). The recombinant C-type feruloyl esterase from *Talaromyces stipitatus* was a kind gift from Biocatalysts Ltd.

a) Elephant grass and eucalypt wood delignification with the high-redox potential TvL and HBT

The lignin contents (as Klason lignin) of Elephant grass and eucalypt samples after the laccase-mediator (*TvL*-HBT) treatments were determined and compared with their respective controls (Table 5). The lignin content in both lignocellulosic samples decreased after the enzymatic treatments concomitantly with increasing laccase doses. In Elephant grass, the lignin content decreased about 11, 22 and 32% after the laccase-mediator treatments using enzyme doses of 10, 25 and 50 U/g, respectively. The reduction in lignin

content of eucalypt wood samples was more pronounced, attaining 32, 34 and 48% lignin decrease using laccase doses of 10, 25 and 50 U/g, respectively. The treatments with laccase alone (without mediator) scarcely decreased the lignin content (<5%) in both materials. In addition to assess the lignin content decrease, further insight into the lignin structure modification by the enzymatic treatments was achieved by 2D NMR analyses of the lignocellulosic samples as described below.

Table 5. Lignin content of Elephant grass and eucalypt samples treated with three doses of laccase (from *T. villosa*) and HBT (2.5%) in a sequence including 4 enzymatic treatments, each of them followed by an alkaline peroxide extraction (using 3% H_2O_2 in 1% NaOH), compared with a control without enzyme and a treatment with laccase alone.

	Elephant grass	Eucalypt
Control	21.1	18.0
Laccase (10 U/g)-HBT	18.8	12.2
Laccase (25 U/g)-HBT	16.4	11.9
Laccase (50 U/g)-HBT	14.3	9.4
Laccase (50 U/g)	20.7	17.5

The enzymatic modification of elephant grass lignin was revealed by 2D NMR, obtained at the gel state in DMSO-d_6. Fig. 8 includes the expanded aliphatic oxygenated (δ_C/δ_H 50-110/2.5-5.5) and aromatic (δ_C/δ_H 100-150/5.7-8.3) regions of the spectra. The main lignin substructures identified are shown in Fig. 9, and the different lignin cross-signals assigned on the spectra are listed in Table 6. A semiquantitative analysis of similar NMR signals in the different regions of the HSQC spectra can be performed, as shown in Table 6 for the aromatic region of the Elephant grass samples.

The aliphatic oxygenated region of the spectrum of control Elephant grass (Fig. 8A) showed signals of both lignin and carbohydrates (X) that mainly correspond to xylan since crystalline cellulose is nearly "silent" under solution NMR conditions. In this region, cross-signals of methoxyls and side-chains in β-*O*-4' lignin substructures (A), including C_γ-H_γ, C_β-H_β and C_α-H_α correlations (A_γ, A_β and A_α, respectively) were observed. The former widely overlap with other C_γ-H_γ correlations in lignin (and related correlations in other lignocellulose constituents). The C_β-H_β correlations gave two different cross-signals corresponding to β-*O*-4' substructures where the second aromatic units is a G unit or an S units ($A_{\beta(S)}$ and $A_{\beta(G)}$, respectively). The main cross-signals in the aromatic region of the HSQC spectrum of control Elephant grass (Fig. 8D) corresponded to the benzenic rings of the different lignin units, including signals from guaiacyl (G) and syringyl (S) units. The S-lignin units showed a prominent cross-signal for the $C_{2,6}$-$H_{2,6}$ correlation ($S_{2,6}$), while the G-lignin units showed different correlations for C_2-H_2 (G_2), C_5-H_5 (G_5) and C_6-H_6 (G_6). Signals corresponding to $C_{2,6}$-$H_{2,6}$ correlations in C_α-oxidized S-lignin units ($S'_{2,6}$) were hardly observed. On the other hand, signals of *p*-coumaric acid (PCA) structures were prominent in this region including cross-signals corresponding to the $C_{2,6}$-$H_{2,6}$ ($PCA_{2,6}$) and $C_{3,5}$-$H_{3,5}$ ($PCA_{3,5}$) aromatic correlations, and olefinic signals for the correlations of the unsaturated C_α-H_α (PCA_α) and C_β-H_β (PCA_β) of the *p*-coumarate unit.

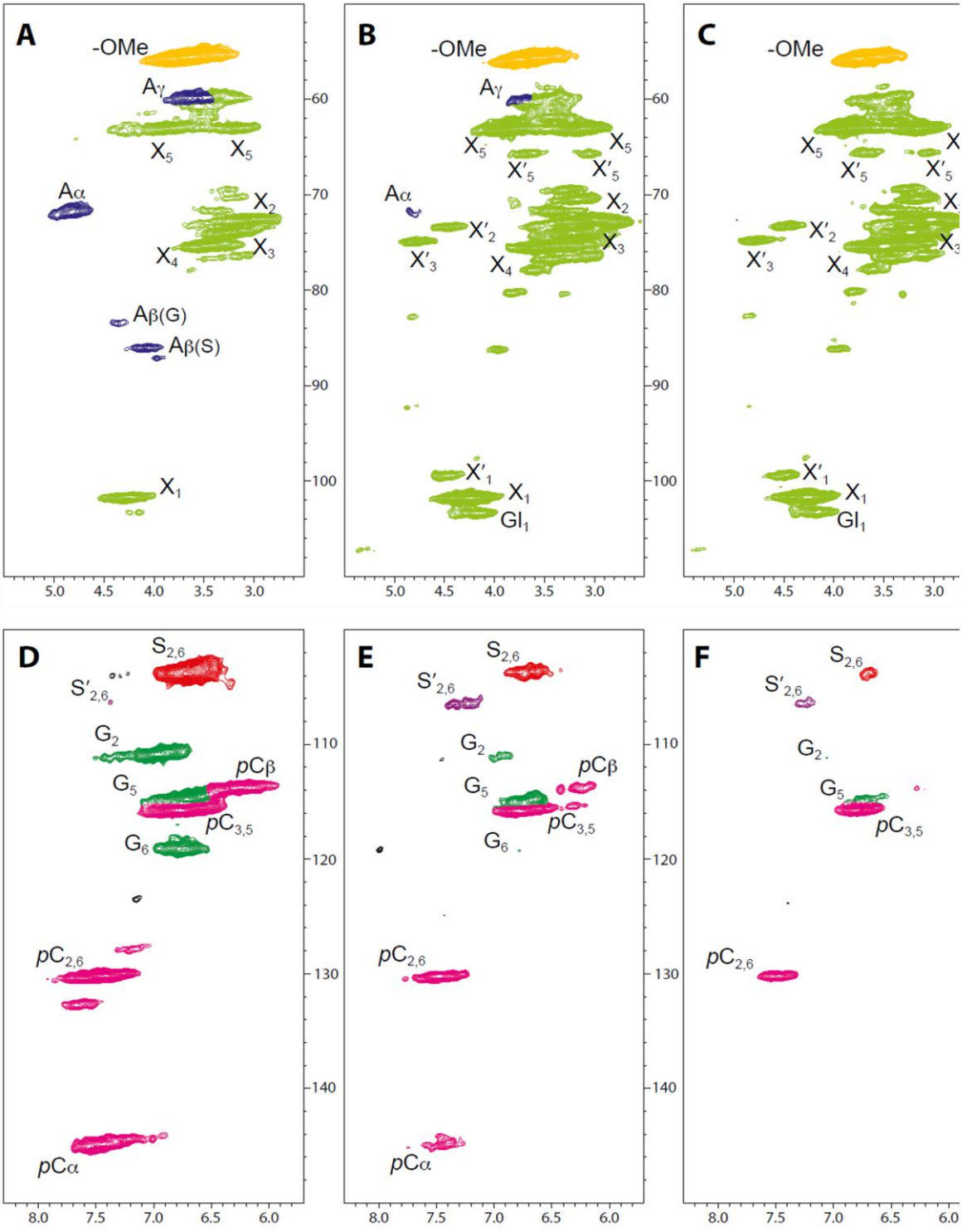


Figure 8. Expanded aliphatic oxygenated (δ_H-δ_C, 2.5-5.5 and 50-110 ppm; top) and aromatic (δ_H-δ_C, 5.7-8.3 and 100-150 ppm; bottom) regions of the HSQC NMR spectra of Elephant grass treated with and high doses of *T. villosa* laccase in the presence of HBT: A and D) Control without enzyme; B and E) 10 U/g enzyme; and C and F) 50 U/g enzyme. See Table. 9 cross-signal assignment, Fig. 10 for the main lignin structures identified, and Table 7 for quantification of these lignin structures.

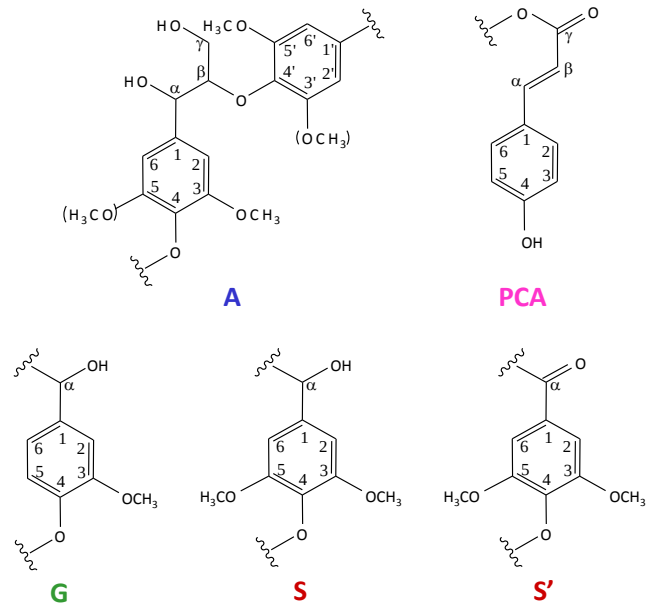


Figure 9. Main lignin and cinnamic structures identified in the Elephant grass and eucalypt samples analyzed by HSQC NMR: A) β-*O*-4' lignin substructures (including a second S or G unit); PCA) *p*-coumarate units; G) guaiacyl units; S) syringyl units; and S') oxidized S units.

Table 6. Lignin units (G, S and α-oxidized S) and *p*-coumaric acid (PCA) contents in the HSQC spectra of the Elephant grass and eucalypt wood treated with three doses of *TvL* and HBT (in a sequence including four enzymatic treatments and four alkaline peroxide extractions) compared with a control without enzyme and a treatment with laccase alone.

	G (%)	S (%)	S_{ox} (%)	PCA (%)
Elephant grass				
Control	30	38	0	33
Laccase-HBT (10 U/g)	20	26	14	40
Laccase-HBT (25 U/g)	17	29	9	45
Laccase-HBT (50 U/g)	0	29	21	51
Laccase alone (50 U/g)	26	35	0	40
Eucalypt				
Control	23	77	0	0
Laccase-HBT (10 U/g)	0	56	44	0
Laccase-HBT (25 U/g)	0	41	59	0
Laccase-HBT (50 U/g)	0	40	60	0
Laccase alone (50 U/g)	9	91	0	0

The HSQC spectra of the Elephant grass samples after the enzymatic treatments with different laccase doses showed important differences compared to the control ones (Fig. 8). The methoxyl cross-signal and most signals of side-chains in β-*O*-4' lignin substructures

(A) present in the aliphatic oxygenated region of the spectrum strongly decreased after laccase-mediator treatment (Figs. 8 B and C). Likewise, signals of S lignin units present in the aromatic region of the spectrum also strongly decreased after the laccase-mediator treatment (Figs. 8E and F) with respect to the control, and the cross-signal of oxidized S-lignin units ($S'_{2,6}$) increased concomitantly. The decrease in G units occurred to a greater extent than S ones and almost disappeared after the enzymatic treatments using higher laccase doses. Indeed, the Elephant grass lignin which has a similar proportion of S and G units with an S/G ratio around 1.2 in control samples, became an S lignin after the enzymatic treatments (Table 6). On the other hand, signals corresponding to the aromatic ring of *p*-coumarate structures ($PCA_{2,6}$ and $PCA_{2,5}$) remain in the spectrum at higher laccase doses, although with lower intensities with respect to the carbohydrate signals. The relative content of the different lignin units and *p*-coumaric acid structures, as molar percentages of total aromatic units, are shown in Table 2.3.3, revealing a preferential removal of lignin with respect to *p*-coumaric acid.

A visual inspection of the above spectra revealed the general decrease of lignin signals after the laccase-mediator treatment, and the relative increase of signals assigned to carbohydrates (mainly corresponding to xylan) as mentioned above. The intensities of the aromatic (from lignin and *p*-coumaric acid) and aliphatic (from carbohydrate, etc) cross-signals in the NMR spectra of the Elephant grass samples treated with the different laccase doses (10, 15 and 50 U/g) in the presence of HBT and with laccase (50 U/g) alone, compared with the corresponding control, are shown in Fig. 10A. Although the intensities of aromatic and aliphatic cross-signals cannot be compared (even on a semiquantitative basis) due to their very different $^1J_{CH}$ coupling values, the above figure provides a qualitative picture on the composition changes produced by the different enzymatic treatments. The general tendency at increasing enzyme doses is a decrease of lignin carbon and an increase of polysaccharide carbon, in agreement with the chemical analyses. In particular, a decrease of the aromatic carbon in lignin G and S units and *p*-coumaric acid, and the aliphatic carbon in lignin side-chains and methoxyls (that also include contributions from hemicelluloses), was observed. In addition, the increase of oxidized S units (relatively moderate in the case of treated Elephant grass) and the strong increase of acylated xylan were observed. In the case of laccase alone, the tendency was the same but the changes observed were relatively minor.

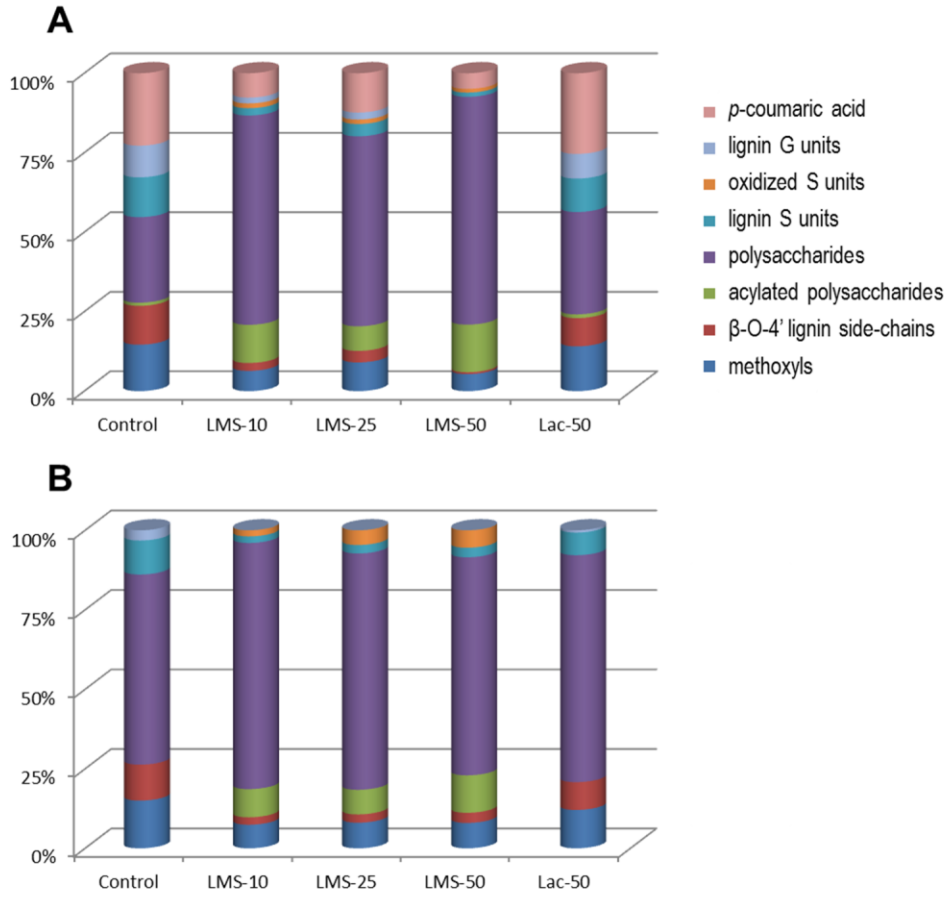


Figure 10. Changes in Elephant grass (**A**) and eucalypt (**B**) constituents during treatment with laccase-mediator using different enzyme doses (10, 25 and 50 U/g) as revealed by NMR, compared with a control without enzyme and the treatment with laccase alone (50 U/g).

The enzymatic modification of eucalypt lignin was also shown by 2D NMR obtained at the gel state. The detailed cross-signal assignments are shown in Fig. 11. The main lignin substructures identified are shown in Fig. 9 and the different lignin cross-signals assigned are listed in Table 7. As in the case of Elephant grass, Table 10 shows the semiquantitative analysis of the different NMR cross-signals in the aromatic region of the eucalypt samples. The aliphatic oxygenated region of the spectrum of control eucalypt sample (Fig. 11A) showed signals of both lignin and carbohydrates (X), the latter corresponding to xylan units, as in Elephant grass spectra. In addition to methoxyl cross-signals, signals of lignin side-chains were observed with lower intensities than found in Elephant grass, the latter corresponding to C_α-H_α correlations in β-*O*-4' alkyl-aryl ether substructures, and C_β-H_β correlations in β-*O*-4' alkyl-aryl ether substructures including a second S-units. The main

cross-signals in the aromatic region of the HSQC spectrum of control eucalypt wood (Fig. 11D) corresponded to the benzenic rings of the different lignin units, including the G and S correlations described above for the Elephant grass. The content in S units in eucalypt lignin is higher than in G units, as revealed by the prominent $S_{2,6}$ cross-signal, compared with G_2, G_5 and G_6 signals, with a S/G ratio around 3.3 (Table 10).

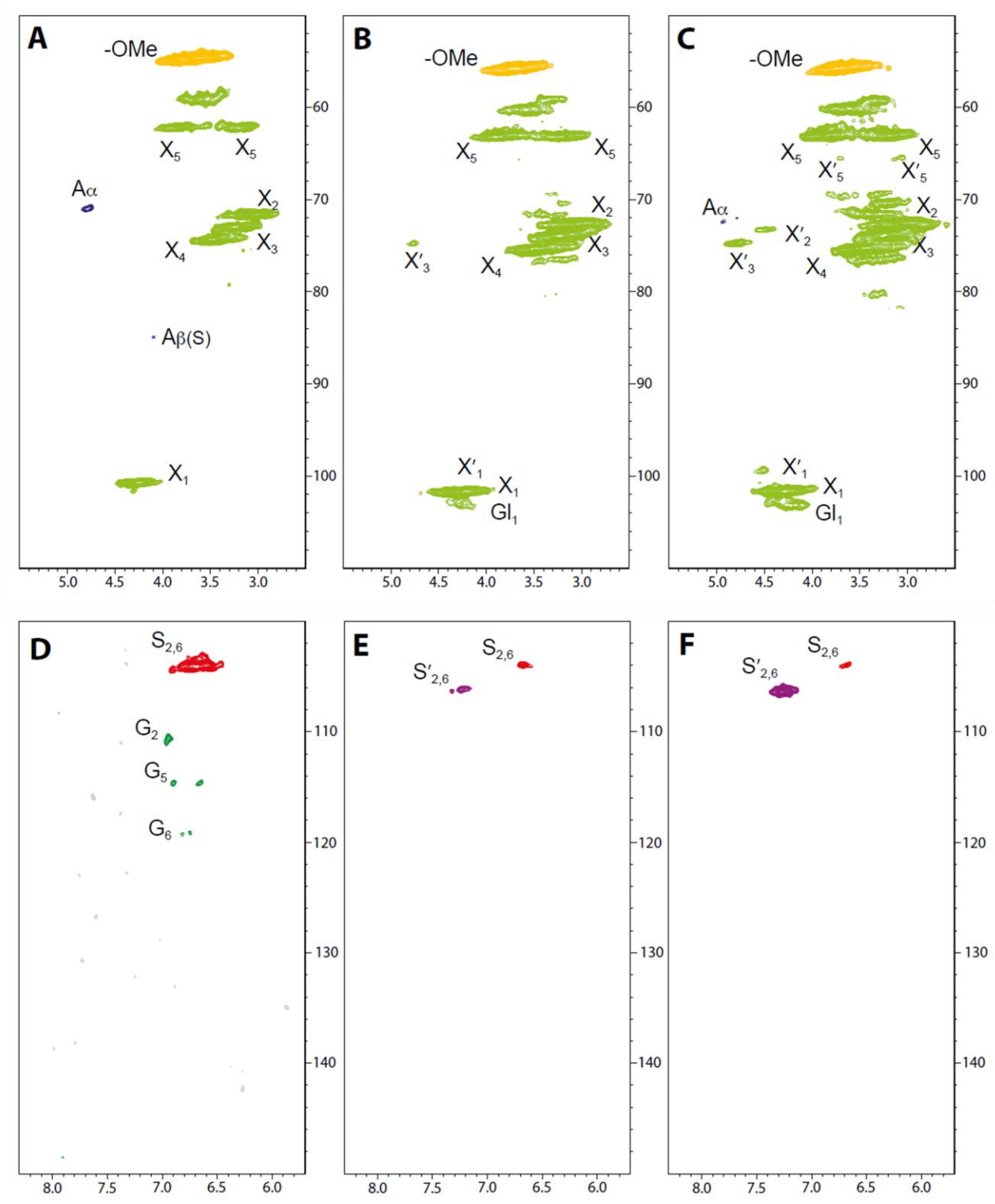


Figure 11. Expanded aliphatic oxygenated (δ_H/δ_C, 2.5-5.5/50-110; top) and aromatic (δ_H/δ_C, 5.7-8.3/100-150; bottom) regions of the HSQC spectra of eucalypt treated with *T. villosa* laccase in the presence of HBT: A and D) Control without enzyme; B and E) 10 U/g enzyme; and C and F) 50 U/g enzyme. See Table 9 for cross-signal assignment, Fig. 9 for the main lignin structures identified, and Table 6 for quantification of these lignin structures.

The HSQC spectra of the eucalypt samples treated with the laccase-mediator system showed important differences compared to the control ones (Fig. 11). The cross-signal of side-chains in β-*O*-4' lignin substructures (A_α) present in the aliphatic oxygenated region of the control spectrum completely disappeared even with the lower enzyme dose (Figs. 11B). Likewise, the G lignin signals, in the aromatic region of the spectrum, also completely disappeared with the lower enzyme dose (Fig. 11E), while the S lignin units were α-oxidized as revealed by the strong increase of the $S'_{2,6}$ cross-signal (to become the most prominent signal in this region) when the enzyme dose was increase, paralleling the decrease of the $S_{2,6}$ signal (Fig. 11E and F). Similarly to the Elephant grass enzymatic treatment, the decrease in G units occurred to a greater extent than in the S ones, and completely disappeared even in the treatment with lower laccase doses.

A general picture of the composition changes revealed by the NMR analyses of the eucalypt samples after treatment with the different laccase doses (in the presence of HBT) and with laccase alone is shown in Fig. 10B, where the relative amount of carbon in the different wood constituents is indicated. The general tendency at increasing enzyme doses is a significant decrease of lignin carbon (in aromatic, side-chain and methoxyl structures), although in a lower extant than in the Elephant grass samples, and a concomitant increase of polysaccharides (including both normal and acetylated units). In contrast, the effect of laccase alone was very moderate, being basically reduced to the decrease in lignin G units.

The present study shows for the first time that woody and nonwoody biomass can be significantly delignified by enzymes (with 30-50% lignin removal) by applying a sequence consisting of several alternative laccase-mediator and alkaline extraction stages, directly on the whole lignocellulosic material (i.e. without its previous partial deconstruction). The enzymatic pretreatments also resulted in improved cellulase hydrolysis, enabling shorter fermentation times for ethanol production.

b) Elephant grass and eucalypt wood delignification with the low-redox potential MtL and MeS

During this period we also studied the delignification elephant grass and eucalypt wood by a commercial low-redox potential laccase (from *Myceliophthora thermophile; MtL*) and using methyl syringate (MeS) as mediator. Four cycles of enzymatic treatment followed by alkaline extraction were assayed, similarly as previously done with the high-redox potential laccase from *Trametes villosa*. Different laccase and mediator doses were tested. The decrease in the lignin content by the enzymatic treatments was assessed by measuring the Klason lignin content, while the modifications of the lignin structure were evaluated by 2D-NMR.

The lignin contents (as Klason lignin) of elephant grass and eucalypt wood samples after the laccase-mediator treatments (with *MtL* and MeS) were determined and compared with their respective controls, and the data are shown in Table 7.

Table 7**.** Lignin content of elephant grass and eucalypt samples treated with different doses of laccase (from *Myceliophthora thermophila*) and methyl syringate, in a sequence including 4 enzymatic treatments, each of them followed by an alkaline peroxide extraction (using 3% H_2O_2 in 1% NaOH), compared with a control without enzyme and a treatment with laccase alone (in the absence of mediator).

	Elephant grass	Eucalypt
Control	22.0	21.1
Laccase (50 U/g)	21.5	16.8
Laccase (50 U/g)-MeS (3%)	21.5	11.2
Laccase (10 U/g)-MeS (1%)	-	13.3

The lignin content in elephant grass was not modified by the use of this low-redox laccase-mediator system, which contrasts with the high lignin removal obtained when using a high-redox potential laccase. However, and interestingly, when applied to eucalypt wood, the lignin content dramatically decreased after the enzymatic treatments, attaining up to 50% lignin removal when using high doses of *MtL* and MeS. In addition, important lignin removal (ca. 40% removal) was also observed when using lower doses of *MtL* (10 U/g) and MeS (1%). The treatments with laccase alone (without mediator) also produced some decrease in the lignin content in eucalypt wood. The large decrease in lignin observed in eucalypt wood by laccase-mediator treatment with the use of a commercial laccase (*MtL*) and a cheap natural phenol as mediator (MeS) opens up new possibilities to develop and implement an industrially-feasible protocol for the pretreatment of eucalypt wood as feedstocks for the production of bioethanol of second generation.

Further studies on delignification of eucalypt wood with low redox potential laccase (and methyl syringate as mediator) were carried out, including the influence of treatment conditions (reaction time, mediator doses, and presence of organic solvents) and the evaluation of the delignification after each cycle of the enzymatic treatment.

The effect of different parameters of the laccase-mediator treatments in the delignification of eucalypt wood was studied. First of all, we studied the influence of the reaction time on the delignificatoon extent. In Table 8 we present the data of *MtL*-MeS treatment after 6 hours and after 24 hours. While after 24 hours treatment, the lignin removal obtained was 50%, however, after reducing the enzymatic treatment to only 6 hours, the lignin removal was still high, up to 27 % Klason lignin reduction, which indicates that it is possible to optimize and reduce the reaction time without losing delignification efficiency.

Table 8. Lignin content (%) of eucalypt wood treated with different doses of laccase (from *Myceliophthora thermophila*) and methyl syringate, in a sequence including 4 enzymatic treatments, each of them followed by an alkaline peroxide extraction (using 3% H_2O_2 in 1% NaOH), compared with a control without enzyme and a treatment with laccase alone (in the absence of mediator). Effect of reaction time (24 h *vs.* 6 h treatment).

	6 h	24h
Control	19.9	21.1
Laccase (50 U/g)	-	16.8
Laccase (50 U/g)-MeS (3%)	14.6	11.2
Laccase (10 U/g)-MeS (1%)	17.7	13.3

The effect of the increase of mediator doses was studied after 6 hours reaction. However, when increasing the doses of MeS as high as 12%, (and using 50 U/g MtL) only minor lignin removal (16% lignin decrease) was observed. Also, the presence of organic solvents, such as methanol barely modified the lignin removal, even when large reaction times (24 hours) were used for the enzymatic treatments.

The extent of delignification after each cycle of enzymatic treatment was also evaluated. Table 9 shows the lignin content of eucalypt wood feedstock after each cycle of enzymatic treatment using *MtL* and MeS as mediator. This study indicates that the delignification increases with the number of sequences used.

Table 9. Lignin content (%) of eucalypt wood treated with the laccase from *Myceliophthora thermophile* and methyl syringate, in each of the 4 sequences, that include the enzymatic treatment followed by an alkaline peroxide extraction (using 3% H_2O_2 in 1% NaOH), compared with a control without enzyme and a treatment with laccase alone.

	1 cycle	2 cycles	3 cycles	4 cycles
Control	21.6	20.0	20.0	20.9
Laccase (10 U/g)	22.0	-	18.0	18.3
Laccase (10 U/g)-MeS (1%)	20.9	18.2	15.6	13.5

The results obtained indicate, for the first time, that the laccase–mediator treatment using a low-redox laccase and a natural phenolic compound as mediator, that produces up to 50% lignin reduction of eucalypt wood feedstocks, can be an economically feasible procedure from an industrial point of view, since both the laccase (MtL) and the phenolic mediator (MeS) used are commercial and cheap.

In addition to assessing the lignin content decrease, further insight into the lignin structure modification by the enzymatic treatments was achieved by 2D NMR analyses of the

lignocellulosic samples. Figure 12 shows the HSQC NMR spectra of whole eucalypt wood material treated with laccase-mediator and the corresponding controls, obtained at the gel state in DMSO-d_6. The main lignin substructures identified are also shown in this Figure.

The aliphatic oxygenated region of the spectrum of control eucalypt wood showed signals of both lignin and carbohydrates (X) that mainly correspond to xylan since crystalline cellulose is nearly "silent" under solution NMR conditions. In this region, cross-signals of methoxyls and side-chains in β-*O*-4' lignin substructures (A), including C_γ-H_γ, C_β-H_β and C_α-H_α correlations (A_γ, A_β and A_α, respectively) were observed. The main cross-signals in the aromatic region of the HSQC spectrum of control eucalypt wood corresponded to the benzenic rings of the different lignin units, including signals from guaiacyl (G) and syringyl (S) units. The S-lignin units showed a prominent cross-signal for the $C_{2,6}$-$H_{2,6}$ correlation ($S_{2,6}$), while the G-lignin units showed different correlations for C_2-H_2 (G_2), C_5-H_5 (G_5) and C_6-H_6 (G_6). Signals corresponding to $C_{2,6}$-$H_{2,6}$ correlations in C_α-oxidized S-lignin units ($S'_{2,6}$) were hardly observed.

The HSQC spectra of the eucalypt wood after the enzymatic treatments with different laccase doses showed important differences compared to the control ones (Fig. 12). The signals of side-chains in β-*O*-4' lignin substructures (A) present in the aliphatic oxygenated region of the spectrum strongly decreased after laccase-mediator treatment. Likewise, signals of S lignin units present in the aromatic region of the spectrum also strongly decreased after the laccase-mediator treatment with respect to the control, and the cross-signal of oxidized S-lignin units ($S'_{2,6}$) increased concomitantly. The decrease in G units occurred to a greater extent than S ones and disappeared after the enzymatic treatments. The spectra confirmed the extent delignification of eucalypt wood attained after the enzymatic treatments.

The results obtained indicate that the laccase–mediator treatment using a low-redox laccase and a natural phenolic compound as mediator, that produces up to 50% lignin reduction of eucalypt wood feedstocks, can be an economically feasible procedure from an industrial point of view, since both the laccase (*MtL*) and the phenolic mediator (MeS) used are commercial and cheap.

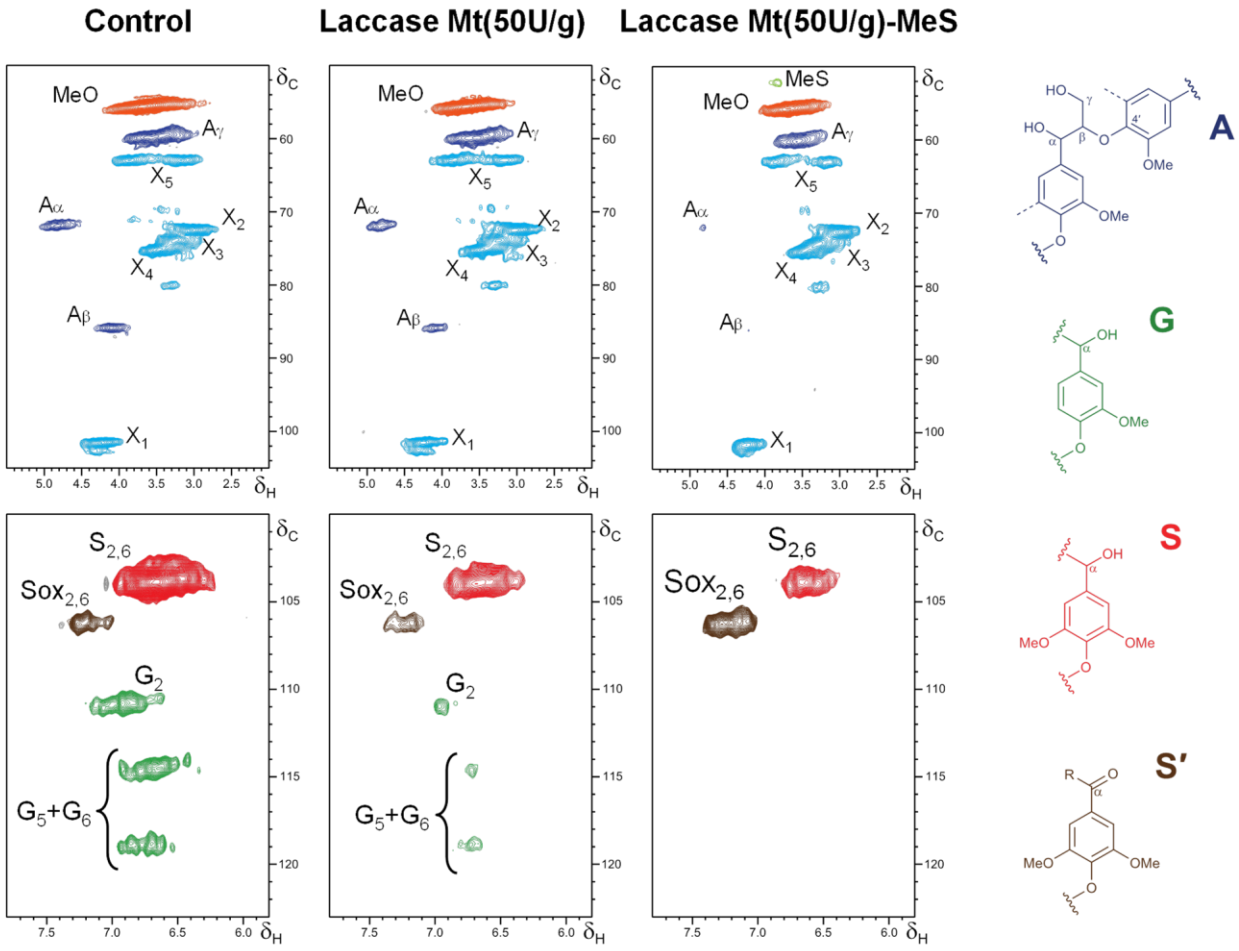


Figure 12.- HSQC NMR spectra of eucalypt wood treated with MtL in the presence of MeS as mediator (four cycles followed by Ep treatment). The spectra corresponded to control eucalypt wood; control eucalypt wood with MtL (50U/g); euclaypt wood trated with MtL (50U/g) and MeS (3%). Main lignin structures identified in the spectra : **A**) β-O-4 lignin substructures; **G**) guaiacyl units; **S**) syringyl units; **S'**) Cα-oxidized syringyl units.

c) Enzymatic pre-treatment of elephant grass with hydrolases

Comparison of the hydrolysis of whole Elephant grass fibres by Ultraflo, Cellulase NS-22086 and Celluclast: Effect of Pressafiner pre-treatment

In previous experiments carried out in the project the enzymatic preparations Ultraflo and Cellulase NS-22086 were found to have the greatest effect in whole stalk Elephant grass (EG) digestion among all the enzymes tested. To complete this study, a comparison of Ultraflo and Cellulase NS22086 with Celluclast, the commercial preparation commonly used in hydrolytic treatments of fibres before fermentation to bioethanol, was performed at **CIB**. The effect of supplementation of Celluclast with a β-glucosidase (BG; Novozym188) was also compared. The treatments were performed on ball-milled EG as well as a

Pressafiner sample (EG-P) which corresponded to whole stalk Elephant grass fibres that were subjected to MSD Pressafiner pre-treatment in water at CTP, dried and balled-milled for 12 h to produce a fine flour. All hydrolysis reactions were performed at 50ºC and pH 6 with 5% dry matter per reaction and 4.5 mg protein/g lignocellulosic biomass enzyme dosage. This corresponds to 1000 U xylanase/g biomass for Ultraflo and NS-22086, and 10 FPU and 200 U xylanase/g biomass for Celluclast. Celluclast was supplemented with BG (5 U against *p*-nitrophenyl-β-D-glucopyranoside/g biomass) where applicable. After 72 h incubation, reactions were terminated by centrifugation and immediate removal of supernatant for determination of reducing sugars (Fig. 13b) and acetic acid release (Fig. 13c). Residual biomass was dried at 65^{o}C before determining the recovered weight (Fig. 13a).

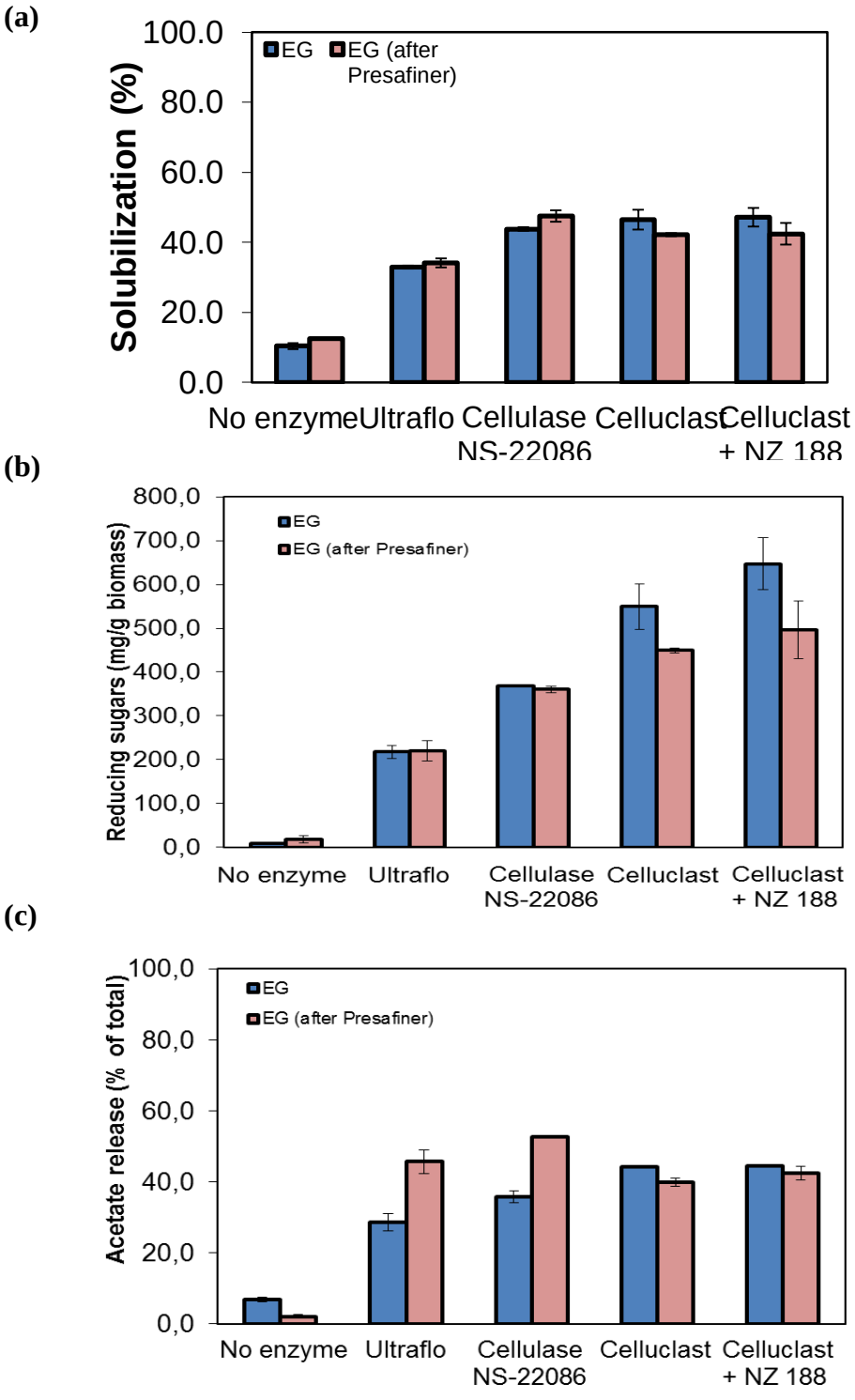


Figure 13.Comparison of the effect of several enzymes on **(a)** Solubilisation of Elephant grass (EG) biomass; **(b)** reducing sugar release (expressed as xylose-equivalents); and **(c)** acetate released after 72h at 50ºC, pH 6.

Slightly more buffer-soluble material is produced from EG by the MSD Pressafiner (12%) than is commonly present (10%). The Pressafiner pre-treatment had no apparent effect on fibre solubilisation by Ultraflo, while with Cellulase NS-22086, 3.5% more EG-P biomass was solubilised compared to the EG sample (Fig. 13a). When Celluclast was used instead of NS-22086 for the hydrolysis, both in the absence and presence of BG, the extent of solubilisation of EG was up to 5% higher than that of EG-P, the converse of that seen with the NS-22086 preparation. NS-22086 has a similar origin to Celluclast but the hydrolytic activity is further boosted by the addition of more cellulolytic and hemicellulolytic activities, making it more suitable for biomass breakdown. While the extent of total solubilisation by the enzymatic treatment remained at 47%, some remaining structural features affected the way the enzymes behave. The extra enzymes present in NS-22086 appear to allow it to degrade more of the EG-P compared to Celluclast, while taking into account the standard error, there is no difference between the enzymes on intact EG. However, the overall increase in biomass solubilisation after pre-treatment is not significantly higher than the enzymatic activity on the intact lignocellulosic biomass to make it a relevant treatment with these enzyme combinations.

Ultraflo was demonstrated to be the poorest performing enzyme preparation on EG of the three preparations examined on an xylanase-equivalent dosage (Fig. 13b). As with biomass solubilisation, there was no increase in reducing group released upon hydrolysis after Pressafiner pre-treatment. There was also no difference in the amount of reducing groups released from the two materials by NS-22086. The increased solubilisation observed in Fig. 13a must therefore be down to the release of non-reducing material or in larger polysaccharides where the number of free reducing ends is comparably low to allow significant changes in the assay response. Therefore, detailed analysis of the residues and solubilised fractions was performed (see section on the effects of enzyme degradation below). With Celluclast, however, the amount of reducing groups generated through hydrolysis of the polysaccharides in the biomass was lower with EG-P compared to the untreated EG**.** It is possible that hydrosolubles present in the Elephant grass are generally easily solubilised and higher reducing group production is a result of further degradation of this soluble material by the enzymes within the Celluclast preparation. Alternatively, pre-treatment with the Pressafiner is generating inhibitory agent(s) of enzyme action. The higher level of reducing ends generated by Celluclast treatment in the presence of BG is due to the further hydrolysis of the reaction products by the endo-acting enzymes.

A significant increase of released acetate levels was observed in the EG-P digestions compared to EG digestion with Ultraflo and NS-22086 (Fig. 2.3.6c), whereas there was little difference in deacetylation with Celluclast. This suggests that the Pressafiner treatment enabled some modifications in the fibres that affect enzyme accessibility and, in the case of NS-22086 and Ultraflo, the higher esterase activities present in the preparation with respect to Celluclast are helping in the material solubilisation and acetate release. Considering the increased solubilisation of the pre-treated biomass observed with NS-22086 (Fig. 2.3.6a), the comparable lack of increased reducing end generation suggests that NS-22086 either is releasing more non-carbohydrate material or that the material released is resistant to further hydrolysis by the enzymes present in this enzyme preparation. Acetylation of the xylan and lignin does not appear to impart an insurmountable barrier to biomass hydrolysis by these enzyme mixtures.

Analyses of the residues recovered from these treatments were performed to determine the amount of Klason lignin, acid-soluble lignin, and residual glucose (Table 10). It is clear that NS-22086 is the most effective of these enzyme preparations in the removal of glucan from the lignocellulosic matrix, and that surprisingly, the addition of the β-glucosidase preparation (NZ-188) resulted in a poorer solubilisation of the available glucose in EG, as well as the poorest degree of solubilisation (49.5%). The residual Klason lignin was not significantly different between the four samples, demonstrating that these enzymes do not act upon this polymer, but the amount of recovered acid-soluble lignin (ASL) was reduced by the action of NS-22086 and Celluclast. It is possible that other material removed through enzymatic activity from EG was affecting what is measured using the spectrophotometric method as ASL, thus giving the lower recovery values expressed in the table.

Table 10. Total recoveries in the residues of EG treated with buffer, NS-22086, Celluclast or Celluclast+NZ188 at 50°C for 24h.

Treatment	Initial amount EG before treatment (mg)	Initial Solubilisation (%)	Residual EG after treatment (mg)	Initial Lignin (mg)	Recovered lignin (mg)	Initial acid-soluble lignin (mg)	Recovered acid-soluble lignin (mg)	Initial glucose (mg)	Recovered glucose (mg)
no enzyme	750	19,3	605,0	135	145,2	11,5	7,3	291	244,3
Cellulase (NS22086)	750	54,0	344,7	135	135,6	11,5	5,5	291	65,7
Celluclast	750	53,7	347,6	135	128,6	11,5	5,2	291	80,1
Celluclast + NZ188	750	49,6	378,2	135	133,6	11,5	6,8	291	79,8

The sugar composition of the residual material was also determined by gas chromatography (Fig. 14). The main sugar removed through the action of these cellulolytic cocktails was not surprisingly glucose, but what was surprising was that arabinose- and xylose-containing polysaccharides within EG were most resistant to the action of these enzymes. This demonstrates that the hemicellulosic material present within the EG matrix does not form a barrier to the removal of glucan. Another surprising observation was with the removal of mannose-containing material during hydrolysis.

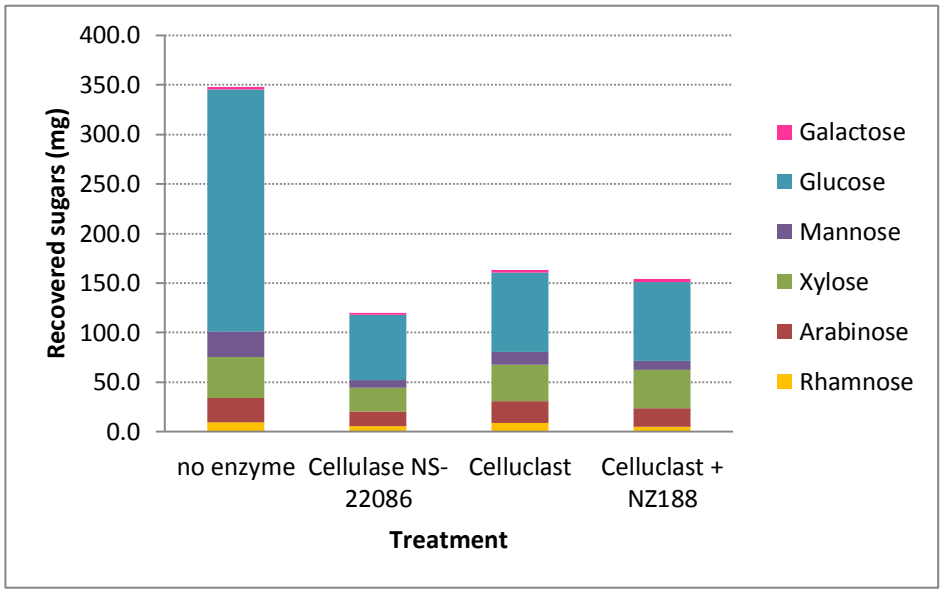


Figure 14. Monosaccharide analysis of the recalcitrant EG recovered after 24 h hydrolysis with NS-22086, Celluclast, Celluclast+NZ188 or only buffer.

Effect of pH on the hydrolysis of Elephant grass fibres by Cellulase NS-22086

In order to check for the optimum pH to perform these hydrolysis reactions, NS-22086 treatment of EG and EG-P was compared at different pH values (Fig. 15) in 72 h reactions at 50ºC. The results show a suitable enzyme performance at pH 5-6 values, with a significant decrease in reducing end production and solubilisation at values higher than pH 6.5. Therefore, the experiment confirms that the reactions performed at pH 6 in the previous and present trials can be considered as optimum for this enzyme preparation. In this experiment, it is clear that EG-P is hydrolysed to a much greater extent by NS-22086 compared to EG, but the trends with respect to pH remained the same irrespective of the substrate.

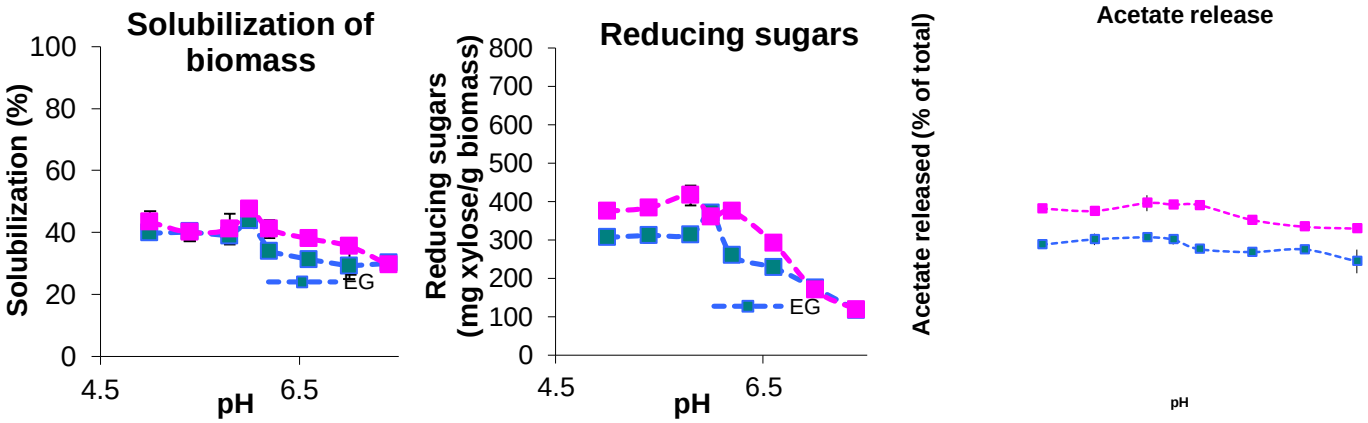


Figure 15. Effect of pH on EG and EG-P hydrolysis by Cellulase NS-22086 after 72 h at 50ºC: Solubilization of biomass, and acetate and reducing sugar release.

Time-course of hydrolysis of Elephant grass by Celluclast and Pressafiner-treated Elephant grass by Cellulase NS-22086

CIB evaluated the extent of biomass hydrolysis over time using the most effective preparations identified so far, i.e. Celluclast for EG and Cellulase NS-22086 for the Pressafiner pre-treated Elephant grass (EG-P) from CTP. Biomass solubilisation (Fig. 16a), reducing sugars release (Fig. 16b) and acetate release (Fig. 16c) were determined at 2, 4, 6, 8, 12 and 24 h (left) time points during the initial day of hydrolysis and subsequently after 2, 3, 4, 7 and 10 days (right). All reactions were performed at 50ºC and pH 6.

The results show a rapid increase in biomass solubilisation (Fig. 16a) and reducing sugar release (Fig. 16b) during the first 3-10 h. After 10 h, little change is observed in all measured parameters for both substrates (EG and EG-P), over the 10-d time course. The increase in reducing sugar levels was higher for the EG-P sample digestion with NS-22086 during the first 12 h, but after 2-3 days similar values were observed for both sample-enzyme treatments. The slight increase in reducing groups measured is probably due to subsequent reduction of solubilised oligosaccharides. Released acetic acid levels (Fig. 16c) reached a more or less constant value after 12-h incubation of both samples, the levels being higher for the EG-P sample treated with NS-22086 in accordance to what was described above.

To examine further the long-term effect of incubation on enzyme activity, residual xylanase activity was determined in the EG+Celluclast and EG-P+NS22086 combinations at 24 h periods (Fig. 17). It is important to note that these enzymes were not dosed on xylanase activity. NS-22086 has over 5-fold more xylanase than Celluclast (communication with Novozymes). What is important to point out is that after 24 h of incubation of NS-22086 at 50^{o}C with EG-P, under 60% of the initial activity remains in solution. The incubations were within Novozymes product specifications for temperature optimization, so either the enzyme is associating with the substrate or it is inactivating either through denaturation or binding soluble inhibitors. Further work is needed to determine this effect. With EG+Celluclast however, measurable xylanase activity increased after 24-h incubation. As this is whole Elephant grass, it is possible that the initial enzymatic hydrolysis has released residual endogenous xylanase, and that the more intact nature of the substrate prevents non-specific interactions between the Elephant grass fibres and the Celluclast enzymes.

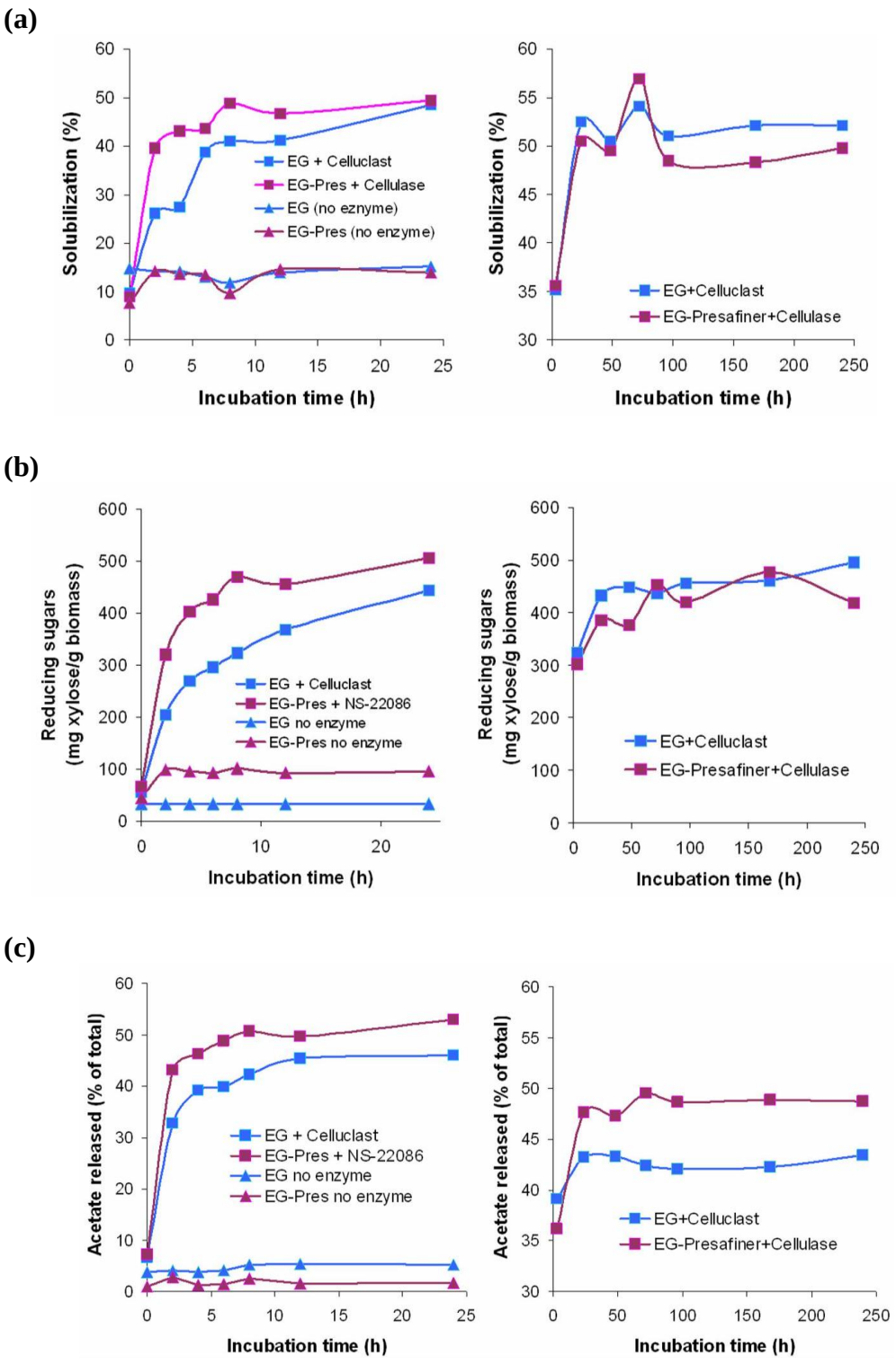


Figure 16. Time-course (0-24 h, **left**, and 0-10 d, **right**) of hydrolysis of Elephant grass by Celluclast and Pressafiner-Elephant grass by Cellulase NS-22086: **(a)** Biomass solubilisation; **(b)** Reducing sugars (as xylose-equivalents); and **(c)** Acetate release.

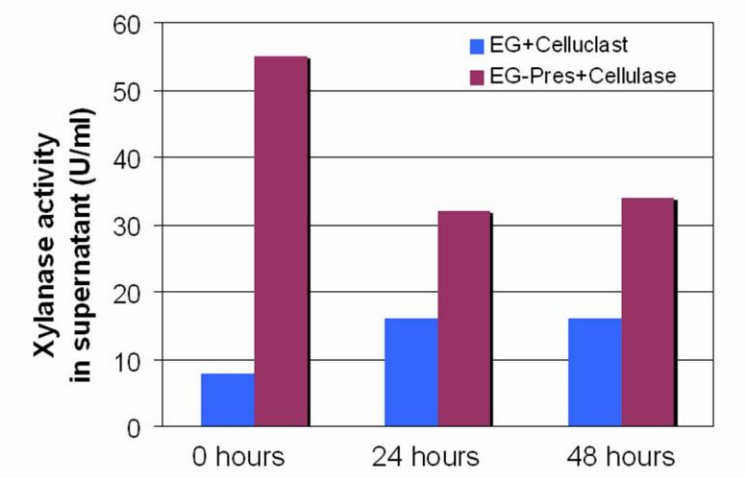


Figure 17. Residual xylanase activity in reaction supernatants after 24-h and 48-h incubation at 50ºC in the presence of Elephant grass biomass.

Effect of supplementation with feruloyl esterases

The effect of supplementing NS-22086 and Celluclast with an A-type feruloyl esterase from *Aspergillus niger* (AnFaeA) or a C-type feruloyl esterase from *Talaromyces stipitatus* (TsFaeC) was examined at **CIB**. Whole stalk EG or EG-P was enzymatically hydrolysed with the above mixtures at 50^{o}C for 24h as before and the effect on biomass solubilisation, reducing sugar release and acetic acid release determined (Fig. 18). The addition of either of the feruloyl esterases had no significant effect on any of the measured parameters with the non-pre-treated EG. The higher amount of reducing sugars produced by NS-22086 is concurrent with its improved activities compared to Celluclast, as described by the manufacturer, **Novozymes**. However, with EG-P, TsFaeC does display a positive effect over and above that of NS-22086 or Celluclast. The increased solubilisation, reducing sugar release and acetic acid release is more pronounced when TsFaeC is added with NS-22086. It is not yet known if TsFaeC is acting on the acetate groups in the biomass or on the hydroxycinnamate linkages present on the hemicellulose and lignin, or on both. The addition of AnFaeA to the enzymes had no measurable effect on the hydrolysis.

Effect of enzymatic hydrolysis on EG whole stalk and the separated pith and cortex fractions

Samples of ball-milled manually-separated EG pith and cortex were received from **IRNAS**. Similar treatments as those performed before with the different enzymatic preparations available from **Novozymes** were carried out in order to compare the effect of the enzymes in the two fractions and the whole material. Initially, a time-course of the hydrolysis of the three materials using Ultraflo was performed measuring biomass solubilization, reducing sugars and acetic acid release after 0, 24, 48, 72 and 96 hours (Fig. 19). Overall, the treatment was more efficient in the pith fraction than in the cortex or the whole material as revealed by the higher values observed in the three measured parameters.

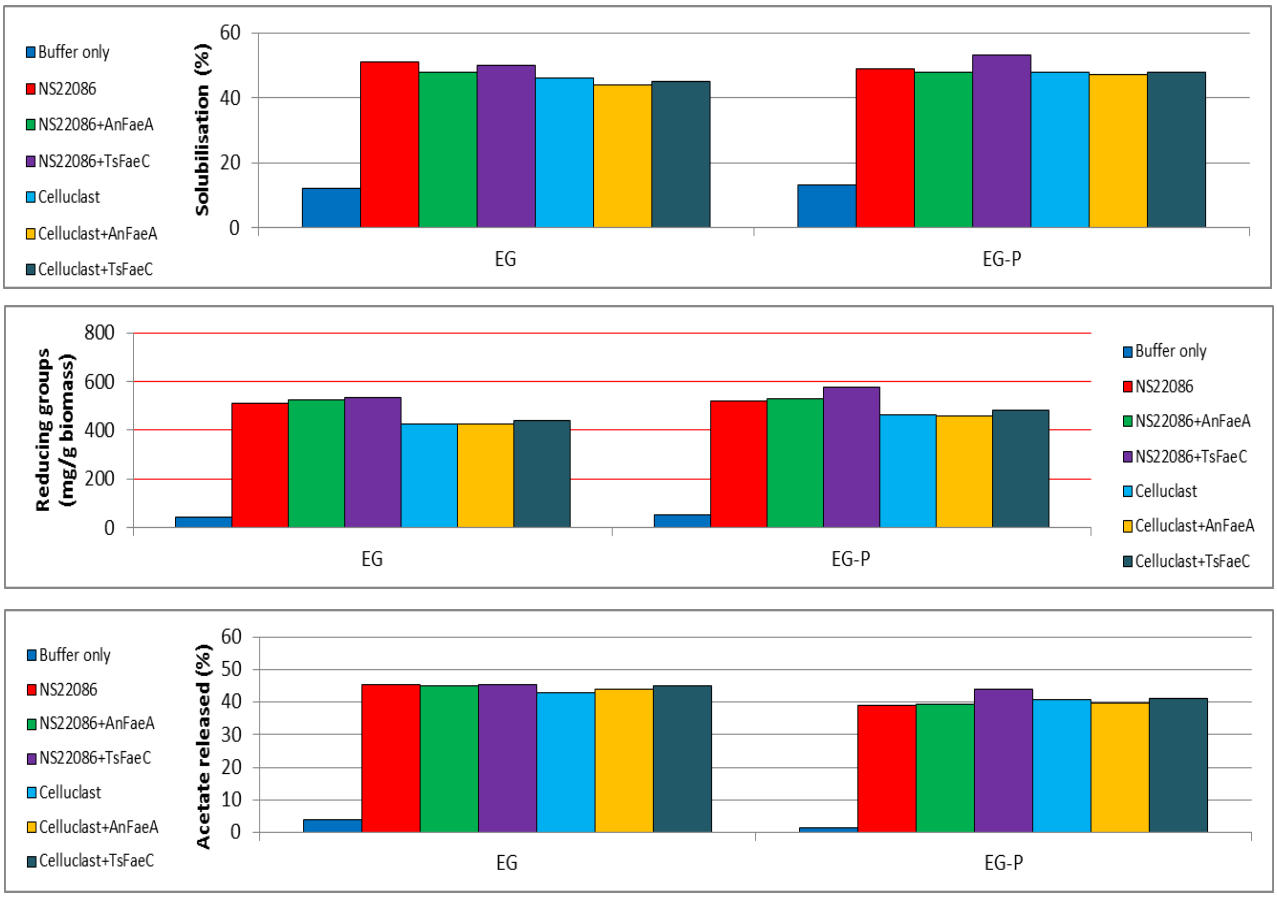


Figure 18. The influence on the (a) solubilisation, (b) reducing sugar release, and (c) acetic acid release from EG and EG-P by supplementation of commercial carbohydrase cocktails with feruloyl esterases.

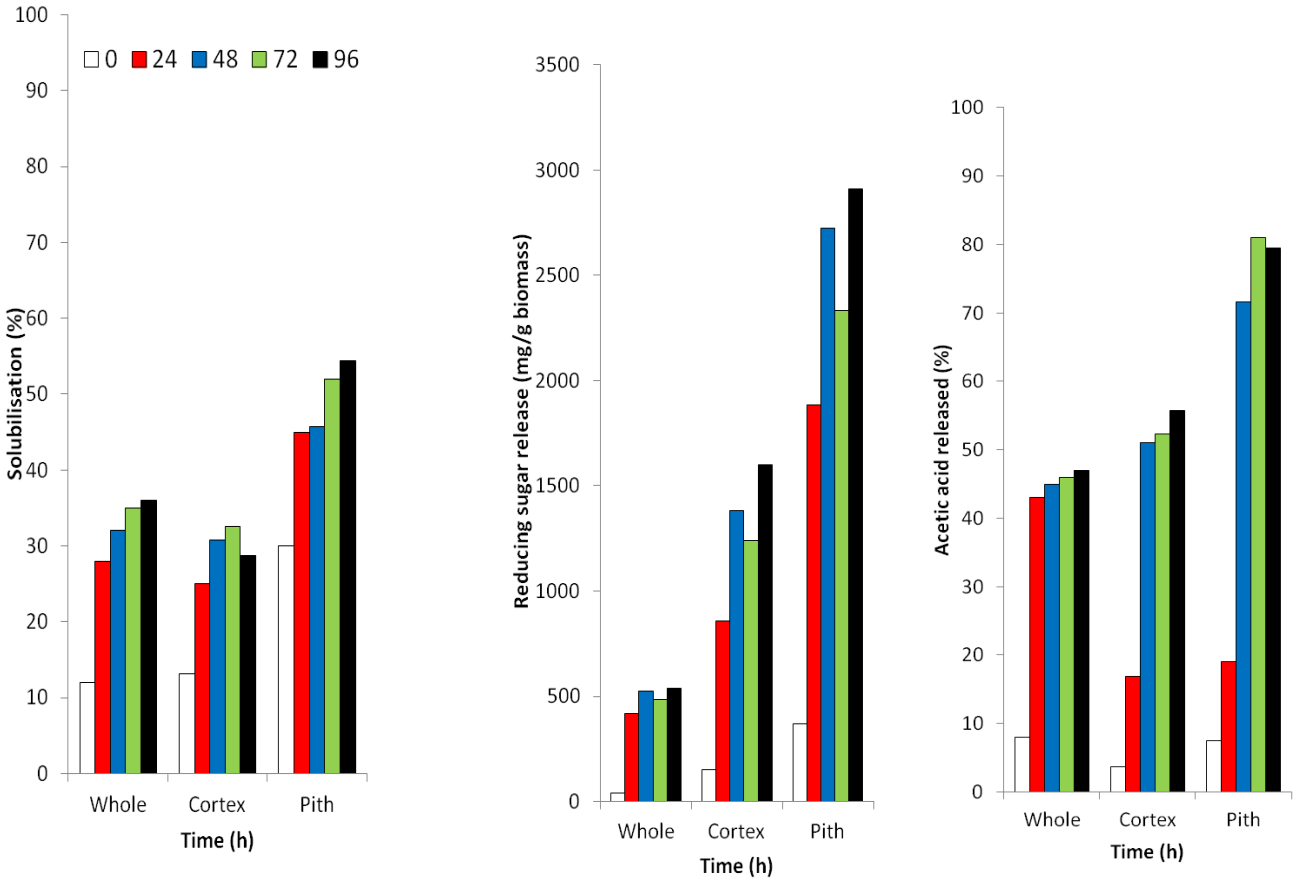


Figure 19. Effect of enzymatic treatment of whole stalk EG, cortex and pith with Ultraflo on (a) biomass solubilization, (b) reducing sugars and (c) acetic acid release as a function of time.

A reaction time of 72 hours was chosen for the comparison with NS-22086 and Celluclast action on both EG fractions as well as in the whole material (Fig. 20).

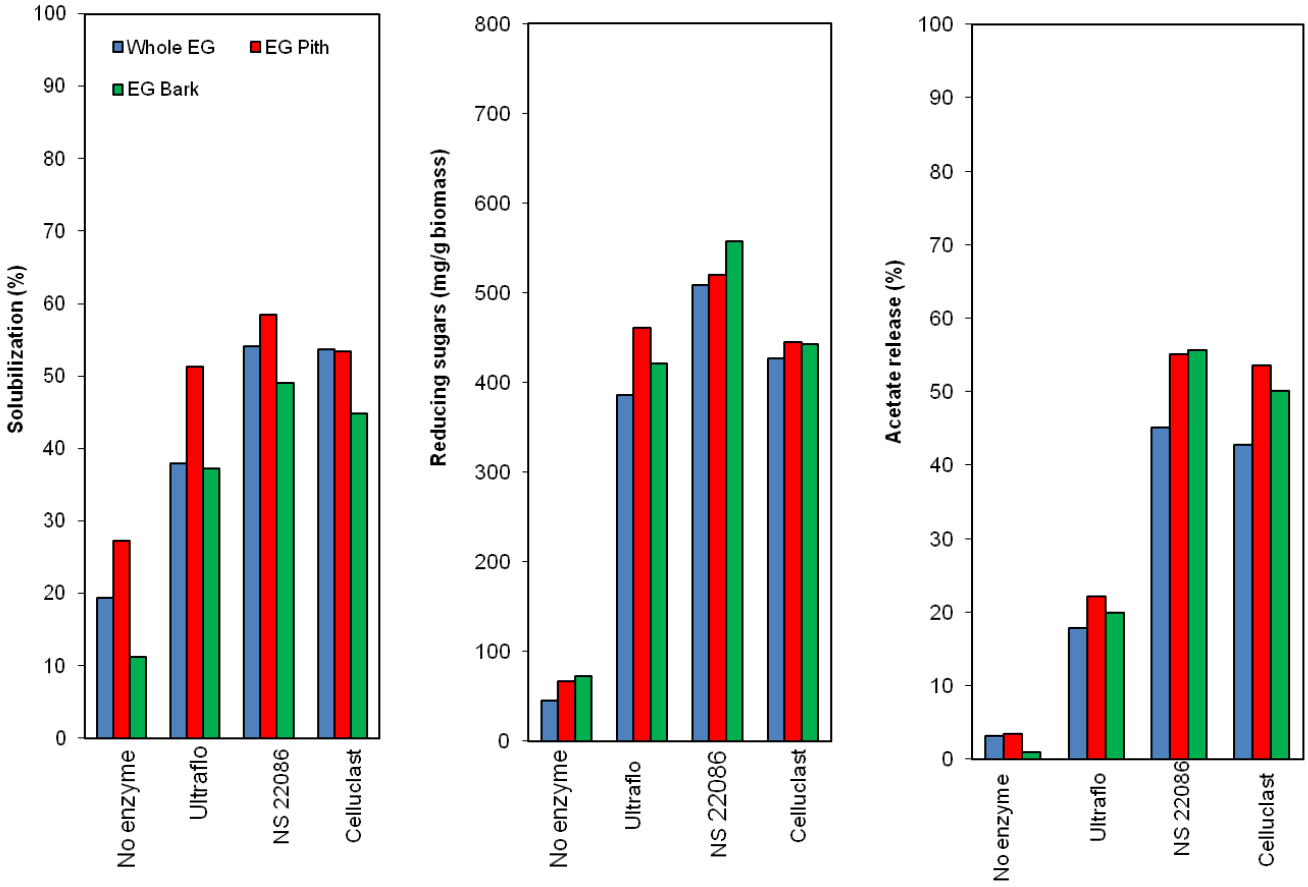


Figure 20. Comparison of the enzymatic treatment of EG whole, pith and cortex with Ultraflo, NS22086 and Celluclast. Effect on biomass solubilization, reducing sugars and acetic acid release.

As in previous experiments, the highest values of solubilization, reducing sugars an acetic acid release were obtained with NS22086 preparation. Overall, extracted EG pith is easier to degrade than the whole stem or cortex/bark as shown by the increased biomass solubilization observed specially with Ultraflo and NS22086 compared to the whole fibre although the effect was not so evident when using Celluclast (yielding similar solubilization values for whole material and pith) (Fig. 20, left). In all cases the cortex was the material less solubilised. When comparing reducing sugars released by NS22086 or Celluclast similar values can be observed, the release being even higher for the cortex fraction with NS22086 (Fig. 20, middle) suggesting that the solubilised sugars may be further degraded by the type of enzymes in NS22086, but that this has no effect on reducing the amount of recalcitrant material. The levels of released acetate were higher for the pith or cortex treated with either enzyme preparation in comparison to the whole fibre (Fig. 20, right). It is possible that the compact structure of the whole stalk material restricts access of the glycoside hydrolases in the enzyme preparations to both the pith and cortex fractions and hence the rate of enzyme degradation is poorer than with the separated material. It has been shown on wheat straw that degradation occurs from inside to outside and the vascular

bundles located at the outer edge of the cortex together with the cuticular epidermis are not significantly affected by the enzymes. The presence of the pith in EG will reduce this accessibility to the cortex further resulting in poor degradation. The presence of less accessible areas on the pith in the whole stalk material will also reduce degradation compared to the exposed pith samples.

Enzymatic hydrolysis of EG by Ultraflo in the presence of DMSO

Organic co-solvents can expand the use of enzymes in lignocellulose deconstruction through making hemicelluloses and lignin more soluble or at least less compact due to disruption of hydrophobic and electrostatic bonds between the polymers and thus more accessible to enzymatic degradation (Quesada-Medina et al., 2010). During the first 18-month period we looked at the effect of DMSO on EG whole fibres hydrolysis by Ultraflo. These studies were completed in this reporting period through examining at the effect of the presence of DMSO on the pith and cortex fractions of EG. The whole stalk EG or pith and cortex material (50 mg) was mixed with 20% DMSO in 100 mM MOPS buffer, pH 6. Ultraflo was then added and the samples incubated at 37^{o}C for 72 h under agitation. Hydrolysis was terminated by centrifugation as before, the resultant pellets being dried at 65^{o}C and the supernatants being tested for reducing group and acetic acid release (Fig. 21).

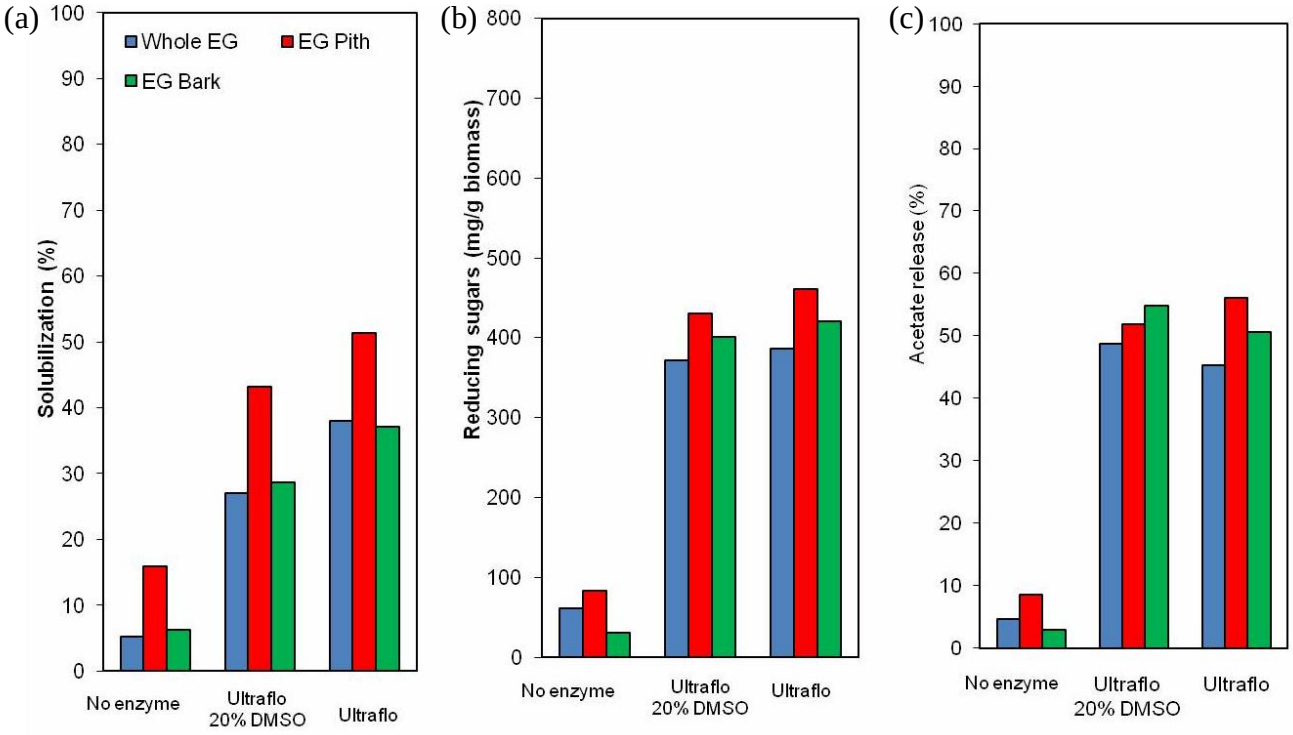


Figure 21. The influence of DMSO on (a) solubilisation, (b) production of reducing groups and (c) acetic acid release from whole elephant grass, pith and cortex by Ultraflo.

The presence of DMSO led to a decrease in solubilisation of the three substrates (Fig. 21a) as we have shown in our previous experiments with the whole fibre. Thus it appears that the use of DMSO does not improve biomass solubility, and generally leads to a reduction in

degradation. However, DMSO addition did not significantly affect the reducing sugar or acetic acid release by Ultraflo (Fig. 21b and c), the latter values being slightly enhanced in whole EG and cortex fraction indicating that xylanase and esterase activities in Ultraflo could be stimulated by the presence of DMSO, which compliments the results reported previously on the stimulation of feruloyl esterase activity on model compounds by the presence of 20% DMSO, as well as the protective effect of DMSO on xylanase stability throughout the reaction time course, also reported in the first periodic report.

To understand the effect DMSO was having on the substrate, comparative analysis regarding the levels of Klason lignin, acid-soluble lignin, and residual glucose was performed on the initial substrate and on the residues recovered from Ultraflo treatment on whole EG, pith or cortex in the absence and presence of 20% DMSO (Table 11). In addition, we also examined the specific effect of DMSO on EG sugars (Table 12).

Table 11. Total recoveries in the residues of whole EG, pith and cortex treated with buffer or Ultraflo in the absence or presence of 20% DMSO at 37^{o}C for 72 h. (--) signifies value not determined.

Treatment	Initial amount EG before treatment (mg)	Initial Solub (%)	Residual EG (mg)	Final lignin (mg)	Final acid-soluble lignin (mg)	Final glucose (mg)
Buffer+DMSO (whole)	750	5	711	--	28	--
Buffer (whole)	750	19	605	--	--	--
Ultraflo+DMSO (whole)	750	27	547	345	21	47
Ultraflo (whole)	750	38	465	324	18	73
Buffer+DMSO (pith)	750	16	630	357	22	14
Buffer (pith)	750	27	546	342	15	20
Ultraflo+DMSO (pith)	750	43	427	256	17	73
Ultraflo (pith)	750	51	365	237	12	81
Buffer+DMSO (cortex)	750	6	703	452	24	3
Buffer (cortex)	750	11	666	450	18	7
Ultraflo+DMSO (cortex)	750	29	535	351	20	12
Ultraflo (cortex)	750	37	471	327	17	59

DMSO appears to be aiding the solubilization of the carbohydrate in preference to the lignin in the whole stalk material as shown with the same Klason lignin content with and without DMSO in all samples and the reduction of glucose in the presence of the co-solvent. However the removal of glucose with DMSO does to correspond to the lower degree of solubility in the presence of DMSO, suggesting that the co-solvent is restricting

more the removal of extractives during the hydrolysis treatment. It is also interesting to point out that Ultraflo is removing material which normally is incorporated in the Klason lignin. It is probably unlikely that lignin is being broken down by this preparation and suggests that the cocktail acts on proteinaceous or lipid-type compounds which are entrapped within the matrix and hence not so easily extractable in an aqueous environment without the aid of enzymes.

Table 12. Specific effect on EG sugars after treatment with Ultraflo in the absence or presence of 20% DMSO at 37^{o}C for 72 h.

Treatment	**Glucose (mg)**		**Xylose (mg)**	
	-DMSO	**+DMSO**	**-DMSO**	**+DMSO**
Whole EG+buffer	5	-	69	113
Whole EG+Ultraflo	73	47 (64%)	161	80 (50%)
Pith+ buffer	20	14 (70%)	230	156 (68%)
Pith+ Ultraflo	81	73 (90%)	105	95 (90%)
Cortex+ buffer	7	3 (43%)	128	70 (55%)
Cortex+ Ultraflo	59	12 (20%)	119	28 (24%)

Table 12 shows that DMSO is removing xylose and glucose from the walls of EG even in the absence of Ultraflo. The values in parenthesis indicate the percentile of the sugar present in the recalcitrant material without the addition of DMSO. The low difference in the pith signifies the efficiency of Ultraflo in the initial solubilisation of the sugars from the matrix and that the xylan and glucan remaining is thus impervious to removal even by DMSO. The co-solvent appears very efficient on the cortex-derived material, and more so in the presence of the enzymes, suggesting that the DMSO is indeed either solubilising the polysaccharides or at least swelling the matrix allowing better enzyme accessibility to their substrates. So in conclusion, while the presence of DMSO aids in the solubilisation of xylan and glucan as well as increasing the activity of enzymes, other material are less soluble in the presence of DMSO and remain in the recalcitrant matrix. It could be possible to perform an initial aqueous extraction to remove such material before performing the hydrolytic reaction in the presence of the co-solvent, thus potentially solubilising even more of the biomass.

d) Enzyme degradation of wheat straw (WS) and Abaca (Aba)

During the first 18-month reporting period, we also looked at the effect of Ultraflo hydrolysis on wheat straw (WS) and Abaca (Aba) fibres. The results showed that Ultraflo was more effective on abaca, solubilising almost 45% of the biomass after 5 days, and that most reducing groups were generated from the hydrolysis of abaca. To examine whether the esterases present in Ultraflo were insufficient for the ester linkages present, 50 µl of the enzyme preparation was supplemented with an A-type feruloyl esterase from *Aspergillus niger* (AnFaeA) or a C-type feruloyl esterase from *Talaromyces stipitatus* (TsFaeC) and the effect on biomass solubilisation, reducing sugar release and acetic acid release was determined as well as acid soluble lignin (ASL), Klason lignin and total glucose (Fig. 2.3.15).

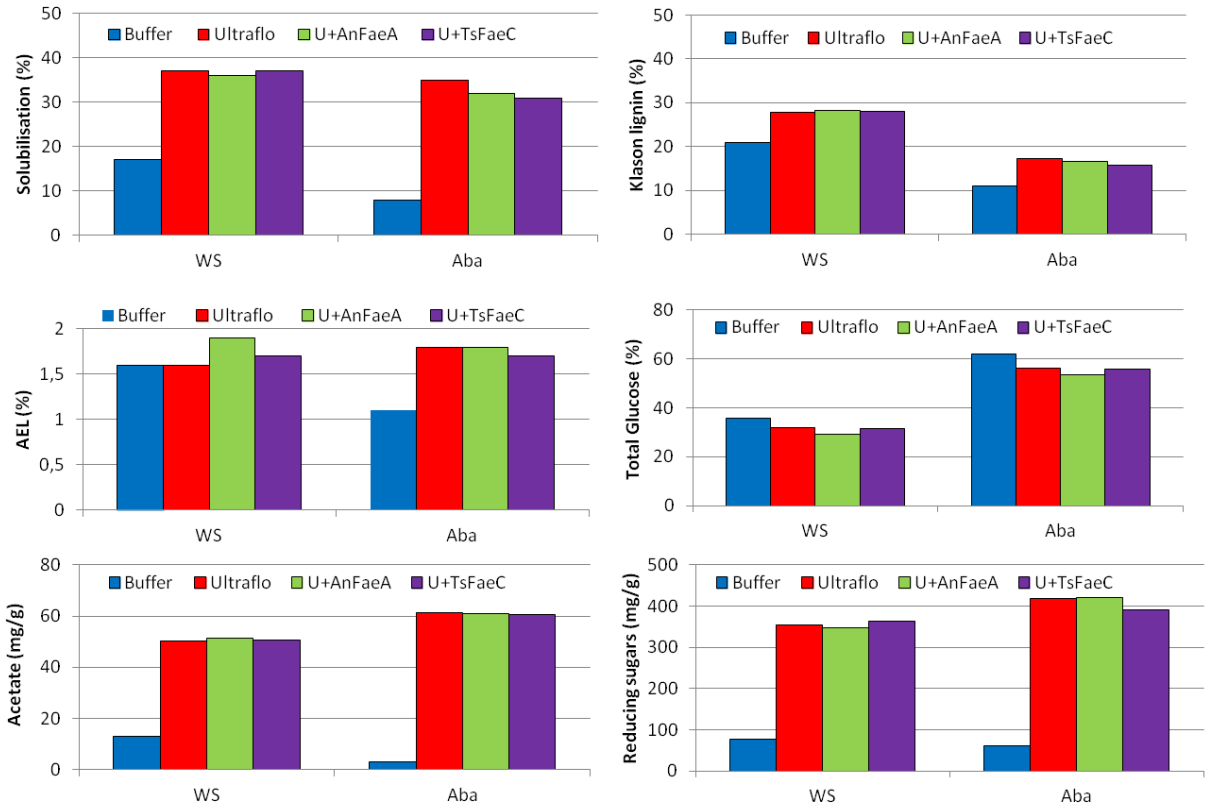


Figure 22. Treatment of wheat straw and Abaca fibres with Ultraflo and feruloyl esterases. Effect on solubilization, Klason lignin, acid soluble lignin (ASL), total glucose, acetic acid release and reducing sugar release.

The first noticeable effect was that the enzymes behaved differently on the 2 plant-derived substrates. While more WS was solubilised than abaca, supplementation with FAE led to a decrease in biomass solubilisation in abaca tissues. This decrease was not due to an effect on global reducing sugar and acetic acid release. As expected, Klason lignin levels increase in the residues after Ultraflo treatment, and with WS, the addition of the feruloyl esterases increased slightly the amount of non-lignin removed while with abaca the opposite was observed.

Furthermore, gas chromatography analysis was carried out to investigate the residual sugars in wheat straw (Fig. 23a) and Abaca (Fig. 23b) after enzymatic hydrolysis with Ultraflo alone or supplemented with AnFaeA or TsFaeC. The y-axis scales of the graph are different to take into account the higher level of sugars in the abaca sample. With WS, very little xylose is being removed by Ultraflo compared to glucose and in particular arabinose and mannose. Mannose appears to being selectively removed when AnFaeA is present. It is also apparent that Ultraflo treatment used in the experiment is only removing 33% of the total sugar. The addition of the feruloyl esterase does not have a significant effect on total sugar release. With abaca, 71% of the total sugar appears to be removed with Ultraflo treatment, and the addition of AnFaeA removed the remaining mannose and arabinose. TsFaeC was not supplementing saccharification of abaca by Ultraflo.

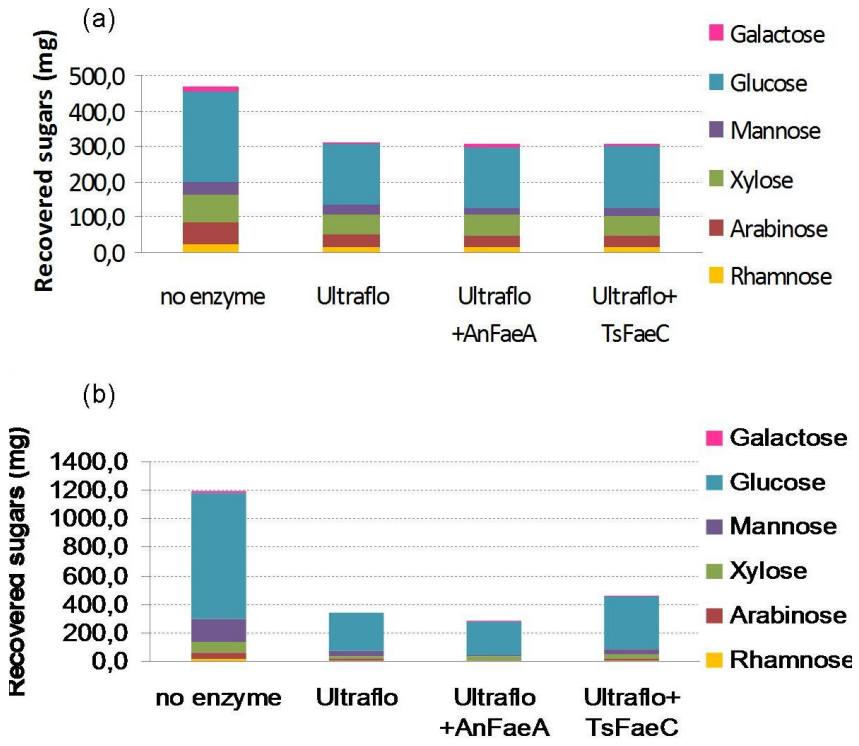


Figure 23. Residual sugars after enzyme treatment of (a) wheat straw and (b) Abaca with buffer, Ultraflo or Ultraflo supplemented with AnFaeA or TsFaeC feruloyl esterases.

Similar gas chromatography analyses were performed to investigate the effect of DMSO on sugar solubilization from wheat straw and Abaca in the Ultraflo treatments (Fig. 24). There was a problem in analysing the sugars after DMSO-buffer treatment in the absence of enzyme, and the sugar recovery is very low. DMSO treatment of WS is selectively removing mannose and arabinose, leaving a residue enriched in glucan and xylose. The same effect was seen with Ultraflo although the recovered sugar levels are lower. It is also interesting to note that Ultraflo is not a suitable preparation to remove WS arabinoxylan, even though there is high xylanase activity present. This technique helps to indicate shortcomings in commercial cocktails on specific substrates. With the abaca sample, the presence of DMSO decreased sugar solubilisation, especially xylose removal, although again mannose is preferentially removed by the addition of DMSO.

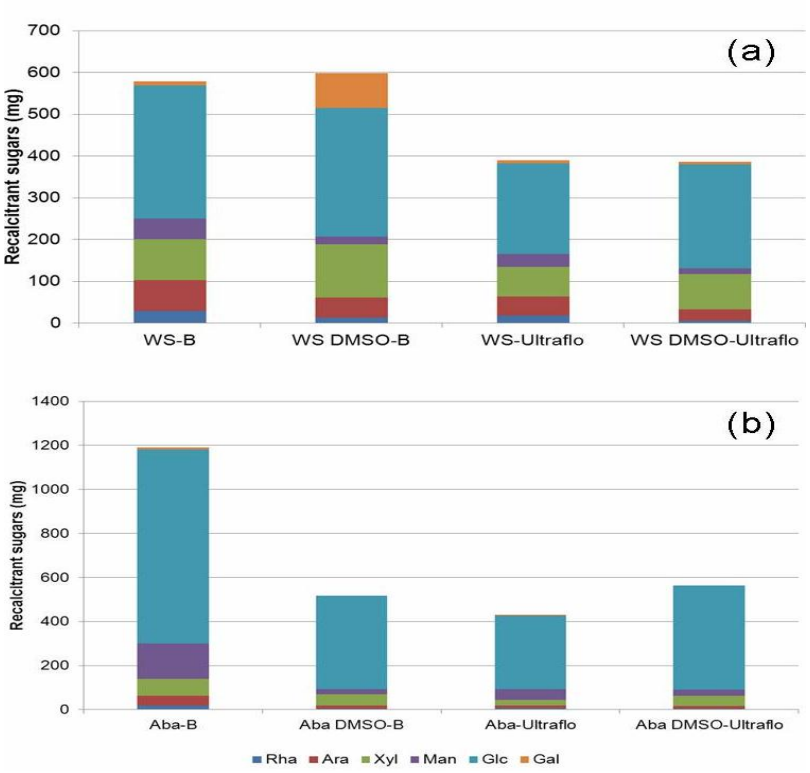


Figure 24. Residual sugars after enzyme treatment of (a) wheat straw and (b) Abaca with buffer (B), 20% DMSO in buffer (DMSO-B), Ultraflo or Ultraflo in the presence of 20% DMSO (DMSO-Ultraflo).

e) Effect of co-solvents on* Myceliophthora thermophila *laccase

The possibility of using certain amounts (10-20%) of organic solvents in some enzymatic treatments to increase substrate (for example, lignin) solubilisation and hence improve the chances of a further biomass deconstruction by the enzymes is of high interest, but depends on the ultimate effect that these solvents produce on the enzyme catalytic properties. In this frame, **CIB** studied and already reported the influence of organic co-solvents on the activity of feruloyl esterases and the effect of dimethylsulfoxide (DMSO) on biomass hydrolysis by Ultraflo, as well as the solvents effect on the high redox potential laccase from the basidiomycete *Pycnoporus cinnabarinus* (PcL). We have now completed the activity measurements for the low redox potential laccase from the thermostable ascomycete *Myceliophthora thermophila* (MtL) in the presence of organic co-solvents.

MtL activity was assayed by following the oxidation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) and 2,6-dimethoxyphenol (DMP) at pH 5.0 and 25ºC (Herpoël et al, *FEMS Microbiol. Lett.* 183:301, 2000) in the absence of mediators. In all cases the solvent was slowly added to the buffer, mixed to insure homogeneity and then enzyme was added. After brief equilibrium at 25ºC, substrate was added to initiate the reaction. Initial activity was calculated and referred to the activity in the absence of solvent to determine the residual activity. As shown in Figure 25, 90-100% of the activity was retained in the presence of 10-20% 1,4-dioxane or acetone (depending

on substrate and solvent) but higher concentrations led to the gradual inactivation of the enzyme. While the hydrolysis of both substrates was equally inhibited by 1,4-dioxane, oxidation of DMP was inhibited by a lower concentration of acetone compared to the oxidation of ABTS, with corresponding $IC_{50\%}$ values of approx. 18% and 35% acetone, respectively. Conversely, an activation effect seemed to occur upon incubation in DMSO or methanol in concentrations up to 20-30% (depending on substrate and solvent).

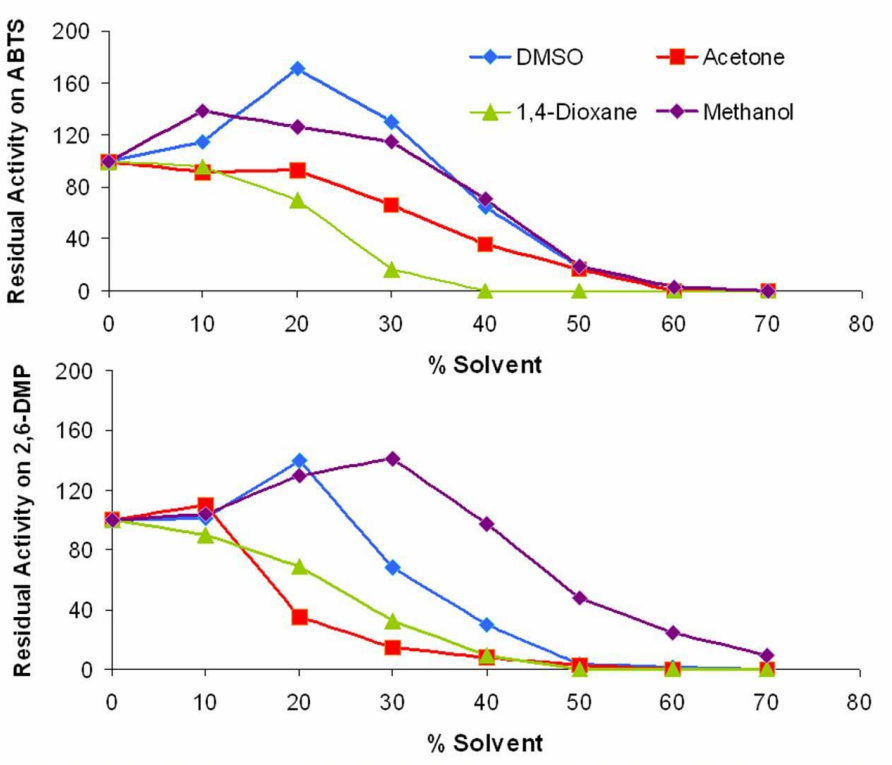


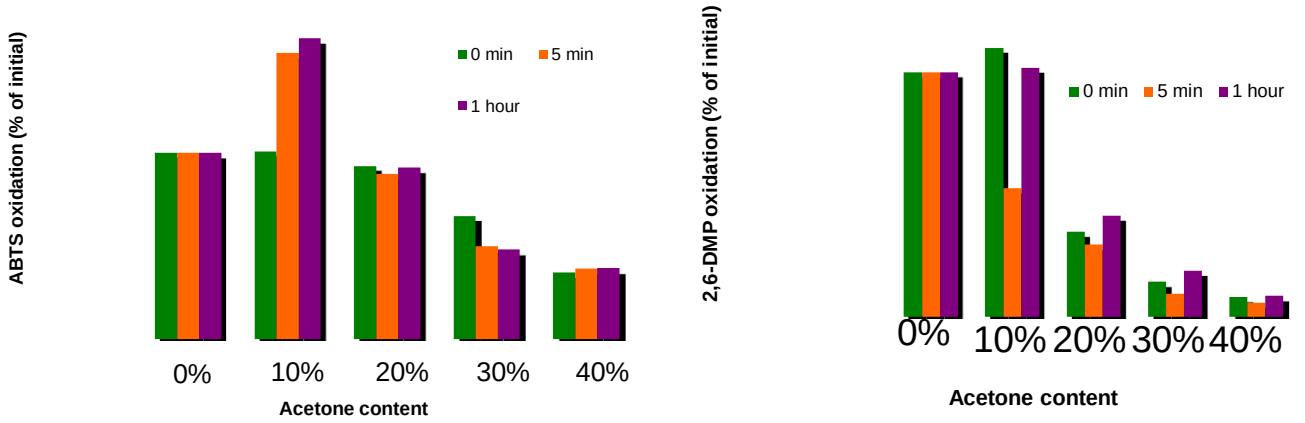
Figure 25. Effect of organic solvents in the activity of *Myceliophthora thermophila* laccase measured by the oxidation of ABTS (top) and DMP (bottom) at pH 5.

In order to further investigate this activation/stabilization effect, MtL was incubated for 5 min or 1 hour in increasing concentrations of acetone, methanol or DMSO, and initial activity on both ABTS and DMP substrates was subsequently determined (Fig. 26).

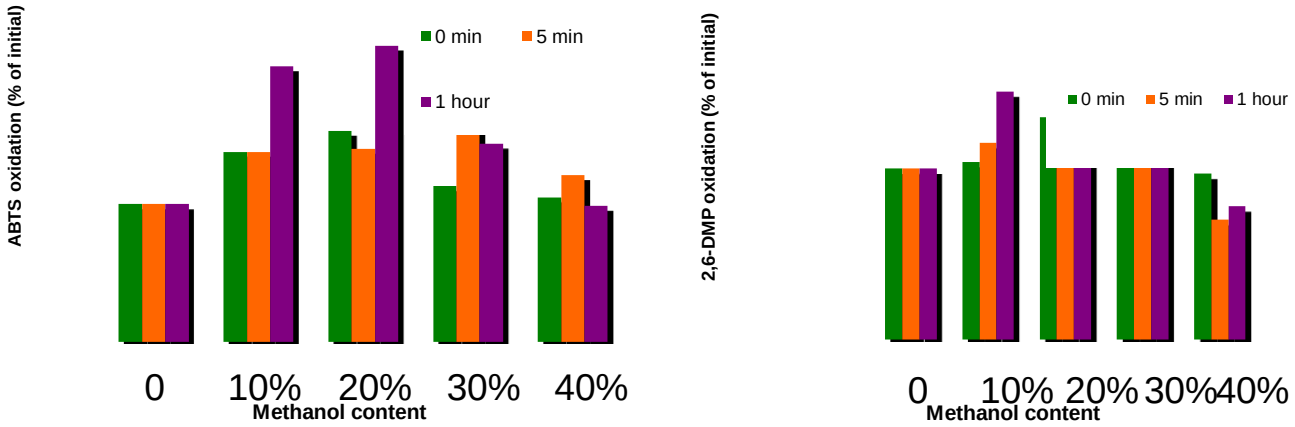
There was a clear positive effect of methanol and DMSO on MtL oxidation of both substrates, the initial activity being 40% and 20% higher on ABTS and DMP, respectively, after 1 hour pre-incubation in 30% methanol (Fig. 26b) and 20-30% higher on both substrates with 20% DMSO (Fig. 26c). In the case of methanol, the initial activity on ABTS measured after 1 hour incubation at 20% solvent content is double the value obtained in the absence of solvent, and 60% higher on DMP in the same conditions. In addition, 100% of the activity on ABTS and 80% on DMP was retained after 1 hour incubation at 40% solvent concentration. The effect of acetone was less significant (Fig.

26a), but still 100% of the activity was retained on ABTS and DMP after 1 hour incubation in 20% and 10% solvent concentration, respectively. Incubation with 10% acetone also resulted in a 60% increase of activity on ABTS.

(a)



(b)



(c)

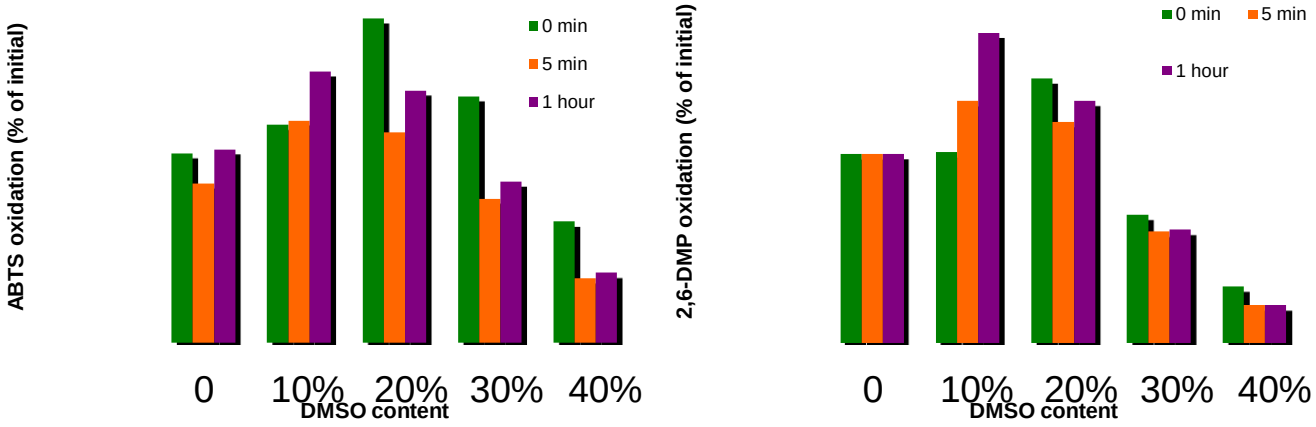


Figure 26. Effect of organic co-solvents on *Myceliophthora thermophila* laccase stability measured as the initial activity on ABTS **(left)** and DMP **(right)** after 0, 5 and 60 min incubation in **(a)** acetone, **(b)** methanol and **(c)** DMSO at 25ºC (as percentages of the initial activity in the absence of co-solvent).

The *Myceliophthora thermophila* laccase used in these trials is a commercial enzyme formulation, most probably including additives aimed to stabilize and support enzyme performance. To check if this stability and activation effect was due to the enzyme itself or driven by the other components of the preparation, a pure sample of MtL was received from **Novozymes** to repeat the incubation with the organic co-solvents. As a comparison, we include here the activity of high redox potential laccase from *Pycnoporus cinnabarinus* (PcL) in the presence of the same mixtures (Fig. 27). Surprisingly, the activation effect observed for pure MtL was more than double the activation of commercial MtL preparation, the best results being obtained for the oxidation of both substrates in the presence of 30% methanol (3.5 and 4.3-fold increased activity on ABTS and DMP, respectively). As observed in previous experiments, for PcL, no activation effect was observed in any case, with methanol being the less affecting solvent, but still producing enzyme inactivation with increased solvent concentrations. A similar activation effect as with MtL, although to a much lesser extent, has been recently described for the laccase produced by another ascomycete, *Chaetomium thermophilum* (Maijala et al (2011) J Mol Catalys Enz B) although in the same paper other ascomycete laccases and one basidiomycete laccase were also investigated, all of them resulting in inactivation by the organic co-solvents. MtL as well as the laccase from *Chaetomium thermophilum* are thermostable enzymes, and the same structural properties that confer thermal stability could influence the general robustness of the enzyme making it more stable to, for example, organic co-solvents. However more studies are required to elucidate what enzyme properties are being affecting by the solvent (i.e. stability, substrate binding, electron transfer, environmental effects) and to determine if this effect is enzyme specific or specific to a certain laccase family, and then to determine if this effect can be transferred to biomass treatment with the laccases.

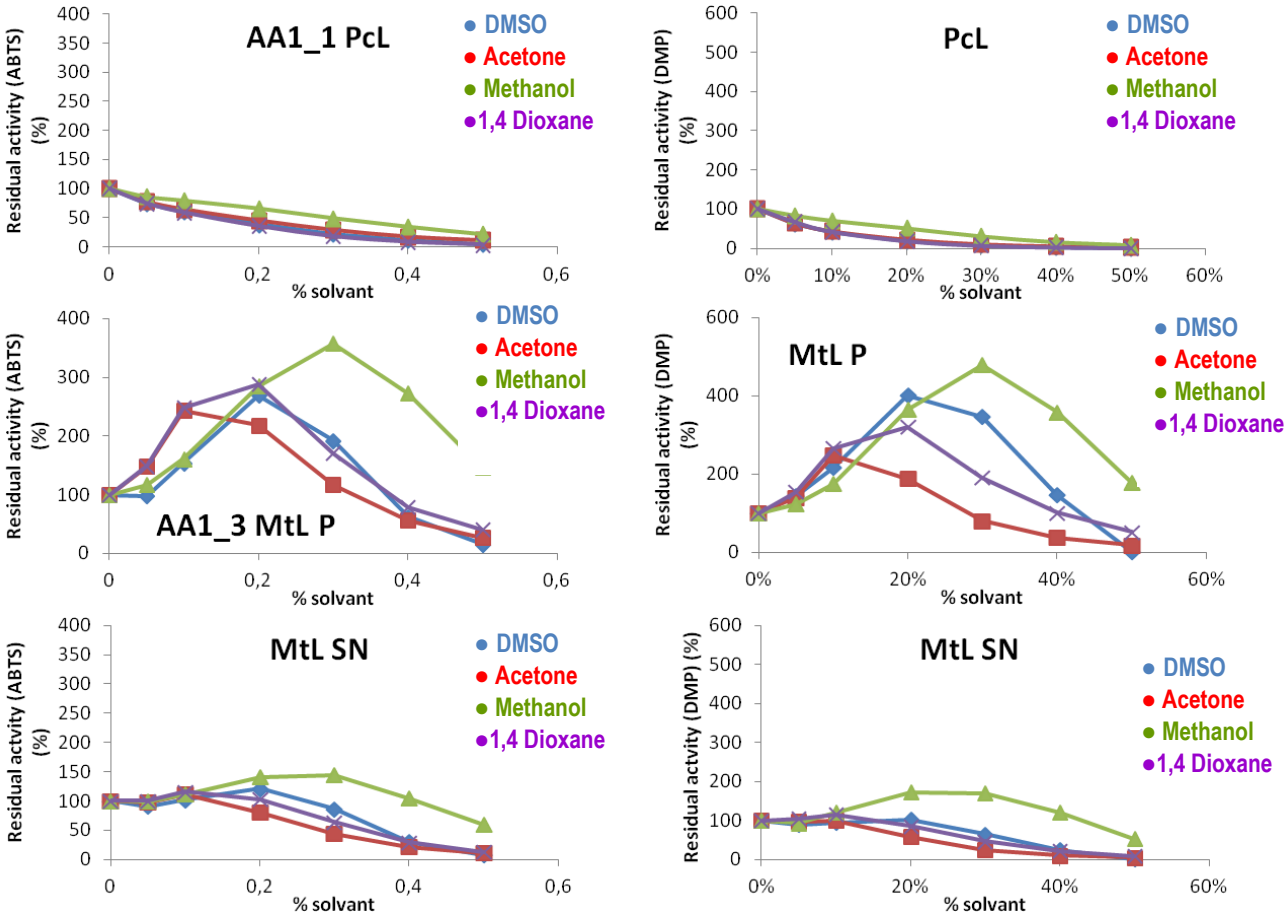


Figure 27. Effect of organic co-solvents on the activity of *Pycnoporus cinnabarinus* laccase (PcL), pure *Myceliophthora thermophila* laccase (MtL P) and *Myceliophthora thermophila* laccase commercial preparation (MtL SN) measured by the oxidation of ABTS (**left**) and DMP (**right**) at pH 4.

Optimization of NS51115 pre-treatment based on a bench-top simulation of mechanical deconstruction

Previous experiments identified a novel xylanase-based product, NS51115, to repeatedly have a significant impact on the integrity of eucalyptus wood when used for pre-treatment prior to mechanical deconstruction. The past 6 months has been devoted to further optimization of the enzymatic deconstruction using this xylanase of the LignoDeco raw materials. The previously used bench-top screening procedure, comprising an enzymatic incubation with the eucalyptus wood followed by a mechanical deconstruction and, ultimately, an estimation of surface area, has been further developed to include an enzyme "impregnation" step in order to simulate an impressafiner. This operation was included in the bench-top simulation of a mechanical deconstruction in order to introduce the enzymes to the interior of the lignocellulosic material and was used for the optimization trials with varying pH and temperature.

Suzano wood chips were comminuted by passing through a Wiley knife mill without a screen installed. These "mini-chips" were further separated into various size fractions by sieving before use in the procedure. Two oven dry grams of wood chip were allowed to pre-soak in 15 ml of 0,2M Britton & Robinson buffer (varying pH) for 30 min. 1 ml of enzyme preparation was added and mixed and the sample was transferred to custom impregnation cups, see Figure 1, immediately prior to compression. The temperature of the press was set according to the temperature used during the incubation and the wood chips were compressed for 5 min at a pressure of 45 kg/cm^2.

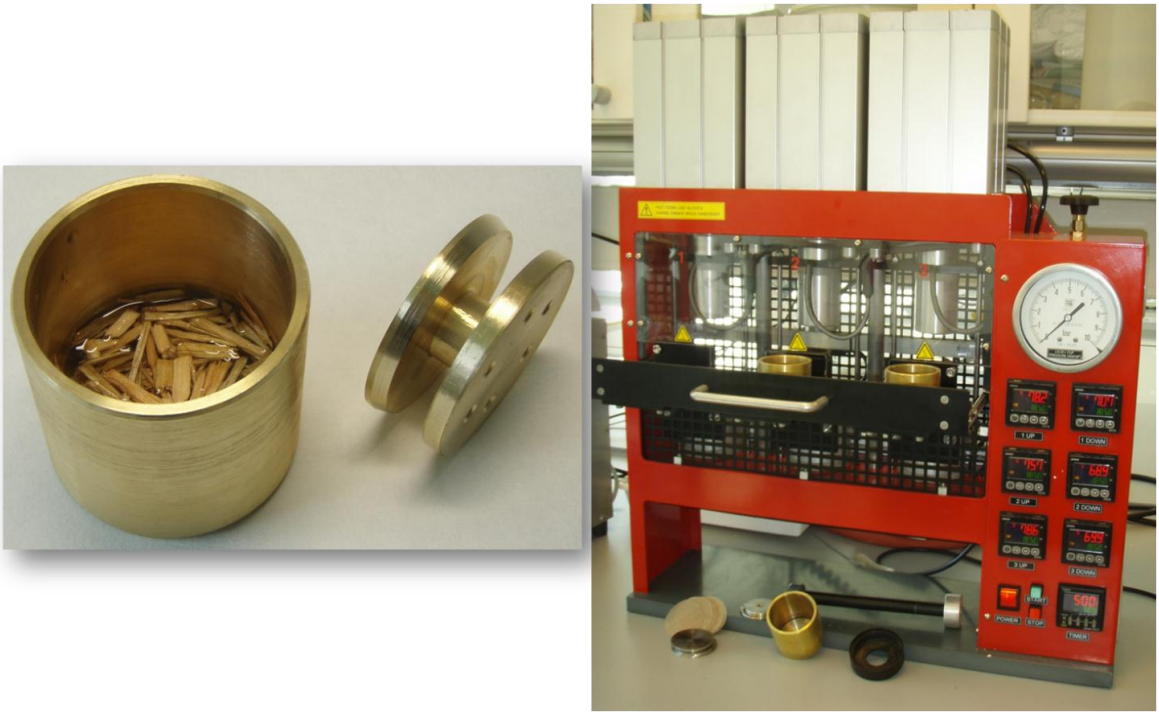


Figure 28. On the left the cup and insertion platen used for the impregnation of eucalyptus wood chips. The perforated platen, allows rapid penetration of the impregnation liquor, containing enzyme, upon pressure relief. On right is shown the mini-impregnator which is used for the actual compression of the wood chips within the cups.

After pressure relief, the samples were transferred to 50 ml Falcon tubes and submerged within preheated water in large 1L Lab-O-Mat beakers. These were incubated for 4 hours with rotary mixing at 20 rpm. After incubation the samples were placed in a 90°C water bath in order to inactivate the enzymes. The contents of the tubes (i.e. chips and liquor) were transferred directly from the 90°C bath to a Waring blender in which the blades were reversed (by reversing the blades to reveal the blunt edges, less "cutting" of the wood chips during mechanical action was observed). The sample was then "refined" within the Waring blender, operating at the low speed setting, for 5 minutes. After recovery from the blender, the samples were centrifuged for 10 minutes at 4000 rpm. After decanting the supernatant, the samples were diluted with 100 ml of fresh MilliQ water and once again concentrated by centrifugation. The supernatant was discarded and the samples were washed once more and 45ml 0,1M sodium acetate buffer pH 5,5 was added and allowed to equilibrate overnight. The samples were centrifuged again and the supernatant was discarded and the samples were suspended to a target volume in MilliQ water. A pre-determined excessive dose of 0,003 N polyDADMAC was introduced and allowed to absorb to accessible surfaces within

the sample during 2 hours of continuous stirring at room temperature. Afterwards, an aliquot of the liquor from each sample was filtered via 0,2 µm syringe filters and 10 ml of each filtered liquor titrated to zero charge within the Mütek PCD-04 using 0,001 N PesNa to determine the overall cationic demand (1) of the original mechanically disrupted and washed sample.

Ideally, a difference in cationic demand between two samples of the same initial substrate is a (partial) function of a difference in surface area between the two samples. Increased cationic demand is presumed an indirect indicator of increased surface area after enzymatic pre-treatment and mechanical disruption. This method was further validated with the more time-consuming Simons Stain method, which shows good correlation to the cationic demand measurements (data not shown refer to LignoDeco 18 months report for details).

The optimization trials were conducted under these conditions and SAS JMP statistical software (version 8.0.1) was used to design the varying experiential conditions allowing for a surface plot to be generated in order to identify the optimal conditions for the enzymatic pre-treatment. A total of 120 data points was generated by the above assay for the NS51115 pre-treatment with pH ranging from 3-8 and temperatures from 30-90°C. These data were used to generate a model by the Standard Least Squares method describing the development of cationic demand by enzymatic pre-treatment as a function of pH and temperature.

The prediction surface plot can be viewed in Figure 29 and identifies the optimal pH for the enzymatic pre-treatment with NS51115 to be between pH 4,5 and 5,5 at temperatures ranging from 40-60°C using a predicted 20% increase in cationic demand as the cut-off value. At pH 5 and 55°C the actual increase in cationic demand is 45% and shows good repeatability with the screening trials conducted at similar conditions which further substantiate the destabilizing effect of the xylanase on the cell wall structure. It is therefore recommended that the enzymatic pre-treatment of eucalyptus wood for the mechanical deconstruction pilot scale trials is to be conducted at a pH of 5 and a temperature of 55°C.

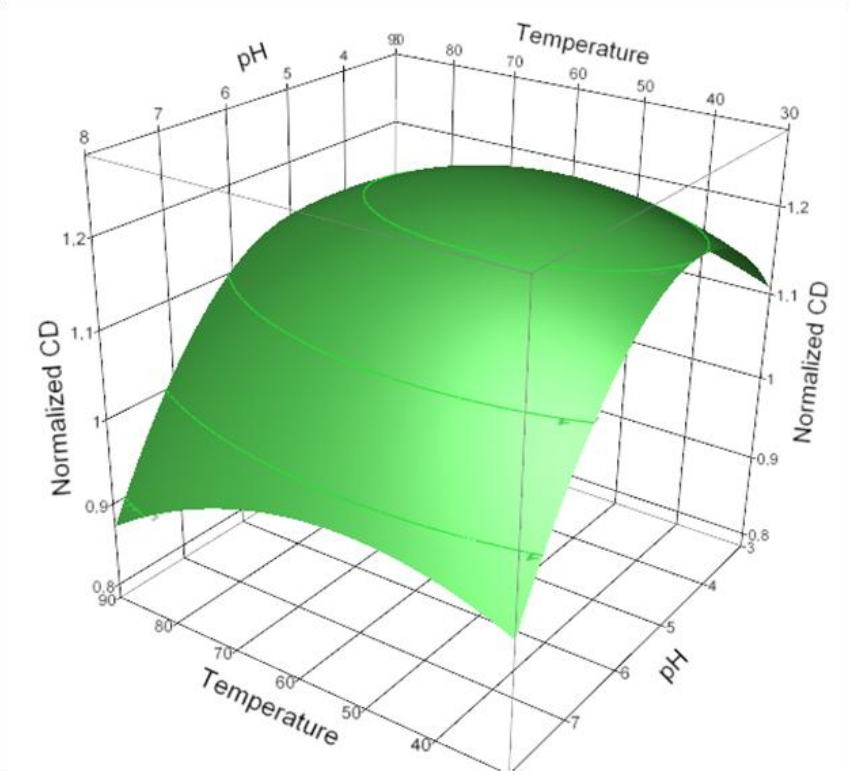


Figure 29. Prediction surface plot describing the development of cationic demand as a function of pH and temperature based on 120 data points using the Standard Least Squares method.

Conclusions

In optimised LGF cooking conditions (3.5% H_3PO_2, 15% water, 130˚C), sufficient delignification with higher polysaccharide yield could be obtained already during 20h cooking. To reach industrially applicable process, further reduction in LGF cooking time could be possible by impregnation, more efficient mixing/liquor circulation, higher temperature or using fresh chips. After 20h cooking and alkaline extraction, the DGxU2 clone with highest pulp yield seemed most potential raw material for bioethanol production.

The establishment and validation of the previous described technique enabled us to identify a novel xylanase-based product, NS51115, among more than 30 enzymes and enzyme blends, both commercial and experimental, for the pre-treatment of eucalyptus wood prior to mechanical deconstruction. This candidate repeatedly showed to have a destabilizing effect on the cell wall structure and was chosen for the optimization trials which generated a prediction model based on 120 data points revealing the optimum conditions.

3.1.3 Progress on WP3 - Chemical and physical characterization of pretreated materials

Task 3.1: Fibre morphology and strength

The different eucalyptus raw materials, selected in WP1, were treated with Kraft (as a reference) or Soda/ Anthraquinone cooking process in order to obtain pulps at kappa 20 and

15. Morphological characteristics of the corresponding pulps were measured in order to determine which eucalyptus pulp had the best fibres morphological characteristics and which process was the most interesting.

The comparison of morphological analysis results obtained before and after cooking was not possible because the chemical treatment applied on the wood to determine the initial fibres characteristics affected the fibres (there was already a chemical impact). However, results obtained on the Soda/ Anthraquinone pulps confirmed tendencies observed on the raw materials, except for the vessels.
DGxU2 eucalypt hybrid seemed to be one of the most interesting raw material for pulp manufactured with Soda-AQ process.
E. globulus raw material was also interesting for the pulp manufacture. After Soda-AQ cooking, the fibres were the longest, the most flexible with a high bonding potential and this pulp contained fewer vessels.

On the other hand, after Kraft cooking, tendencies observed on pulps were not similar as tendencies observed on the raw materials.
IP and *E. globulus* eucalyptus woods were the most interesting raw materials for Kraft pulp manufacture. IP pulp presented the longest and flexible fibres with a high bonding potential, and *E. globulus* pulp has the lowest vessels content and the longest fibres.

The best process depended on the raw materials. Soda-AQ cooking seemed to be the most interesting process for the manufacture of *E. globulus* and DGxU2 pulps. This cooking induced a reduction of vessels content and an improvement of the fibre flexibility and seemed to reduce the broken fibres content compared to the Kraft process.
On the other hand, Kraft cooking was the most interesting process for the manufacture of IP eucalyptus pulp. The IP Kraft pulp contained longest fibre with bonding potential and lower vessels content than the IP pulp manufactured with Soda-AQ process.

The morphological analysis on elephant grass pulp (EG1) showed that Soda-AQ process was slightly more interesting than Kraft cooking. This process allowed reducing vessels content in the pulp and fibres were more flexible. However, hydrogen bonding potential was higher after Kraft process.

Task 3.2 Polysaccharide (cellulose and hemicellulose) analyses

Cellulose crystallinity by solid state NMR spectroscopy
The cellulose crystallinity (CrI) was determined from the areas of crystalline and amorphous C4 signals of ^{13}C CPMAS NMR spectra by deconvolution:

$$CrI = A_{86\text{-}92ppm} / (A_{79\text{-}86ppm} + A_{86\text{-}92ppm}) \cdot 100\%.$$

For pure cellulose the method gives comparable results with X-ray (Teeäär *et al.* 1987). However, in wood and pulp samples, also hemicelluloses and lignin side-chains contribute to the amorphous C4 signal, and the CrI values obtained rather describe the crystallinity of the whole pulp/wood. To determine the actual cellulose crystallinity, the interfering signals of other amorphous components must be removed either chemically or spectroscopically

before determination of CrI. For spectroscopic removal of hemicelluloses and lignin from cellulose spectra, a proton spin-relaxation based spectral edition (PSRE) method was implemented at VTT, as described previously. In the PSRE method the differences in the proton spin-relaxation times ($T_{1\rho H}$) of crystalline cellulose and amorphous lignin and hemicelluloses are utilized to separate the components into subspectra of their own (Newman and Hemmingson 1990; Newman 1999, Liitiä et al 2003). From the cellulose subspectrum, the crystallinity of cellulose (CrI_{PSRE}) was determined without the interference of the less ordered pulp/wood components.

For NMR measurements the dry wood chips and elephant grass were wiley milled (2 mm screen), and re-wetted with deionised water (~50 wt %). The LGF pulp samples were equally pretreated. All the measurements were performed either with a Chemagnetics CMX 270 MHz or 400 MHz NMR spectrometer. The spinning speed was 5000 Hz with 270 MHz equipment and 8000 Hz at higher field of 400 MHz to improve the resolution. In all cases, the acquisition time was 20 ms, contact time 1 ms and delay between pulses 2 s. The delayed contact measurements were conducted with spin-lock times of t_{sl} =0 and 3-7 ms, and the subspectra of components were obtained by linear combination using intensities of crystalline cellulose C4 and lignin methoxyl signals at 89 and 56 ppm, respectively (Newman and Hemmingson 1990; Newman 1999). This way better linear combinations and cellulose subspectra could be obtained than using signal intensities at 89 and 80 ppm, as described earlier. The biomass crystallinity (CrI_{CPMAS}) was determined using the ordinary CPMAS spectra measured with t_{sl}=0 ms and the cellulose crystallinity (CrI_{PSRE}) was determined from the subspectra of cellulose.

Crystallinity of raw materials and corresponding LGF pulps

Crystallinities determined for the available raw materials before and after the LGF cooking and alkaline extraction are given in Table 13. Cellulose crystallinity in eucalyptus clones varied between 40-57 %, being highest in *G1xUGL* and *Suzano* clones. The cellulose crystallinity of elephant grass was clearly lower compared to eucalyptus, and remained lower also after the LGF cooking and alkaline extraction. During LGF cooking and alkaline extraction, the **pulp crystallinity** determined from the ordinary CPMAS spectra (CrI_{CPMAS}) increased when more amorphous lignin and xylan was removed. In most cases, also the **cellulose crystallinity** determined from the cellulose subspectra after spectral edition (CrI_{PSRE}) increased, as reported also for kraft pulps during cooking (Liitiä 2002). This is due to the removal of more amorphous cellulose, and also some cellulose ordering/aggregation, taking place when amorphous lignin and hemicelluloses are removed between the fibrils.

Table 13. The biomass crystallinity (CrI_{CPMAS}) determined using the ordinary CPMAS spectra and the cellulose crystallinity (CrI_{PSRE}) determined from the subspectra of cellulose.

	Crystallinity of raw materials		Crystallinity of LGF pulps after NaOH extraction	
Feedstock	**Wood CrI_{cpmas} %**	**Cellulose CrI_{PSRE} %**	**Pulp CrI_{cpmas} %**	**Cellulose CrI_{PSRE} %**
G1xUGL	37	57	45	56
Suzano	40	56	43	59
IP	37	49	43	54
U1XU2	33	40	-	-
DGXU2	33	42	48	50
E.Globulus	-	-	48	61
EG1	24	33	36	39

No clear correlation between the hydrolysability of the LGF pulps and crystallinity of the raw materials or LGF pulps could be detected. Although cellulose crystallinity would affect the cellulose hydrolysability, there are clearly also other factors, *e.g.* lignin and hemicellulose content, contributing more.

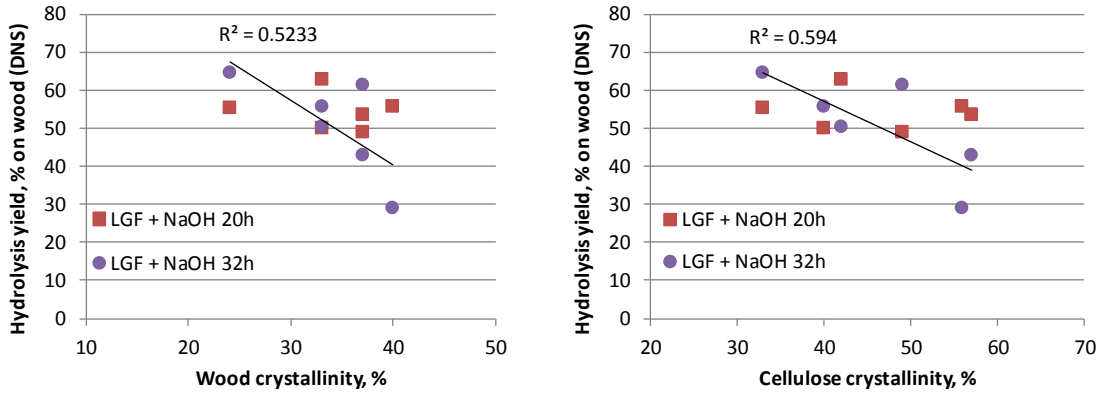


Figure 30. Correlation of wood (left) and wood cellulose (right) crystallinity with LGF pulp hydrolysability after 20 and 32 h LGF cooking followed by alkaline hydrolysis.

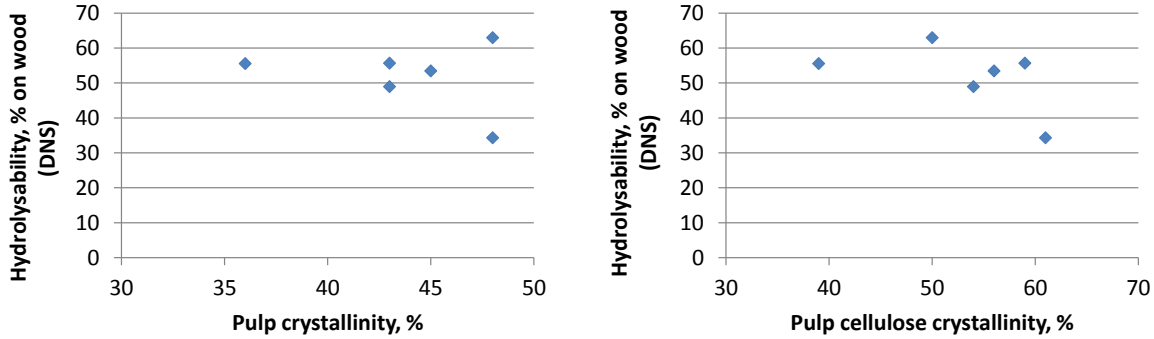


Figure 31. Correlation of LGF pulp (left) and pulp cellulose (right) crystallinity with the corresponding pulp hydrolysability after 20 h LGF cooking followed by alkaline hydrolysis.

Effect of alkaline cooking on cellulose crystallinity

Alkaline oxidation ($NaOH+O_2$) and soda-anthraquinone (Soda-AQ) treatments were studied as potential alkaline deconstruction methods for bioethanol production, and the effect of these alkaline treatments on cellulose crystallinity was also evaluated by solid state NMR spectroscopy. The alkaline treatments were performed in Suzano for elephant grass (*EG1*) and *G1xUGL* eucalyptus hybrid (kappa levels of 50, 30 and 15).

After alkaline deconstruction the cellulose crystallinity of elephant grass was clearly lower compared to eucalyptus pulps, as detected also with LGF pulps. With both raw materials, the **pulp crystallinity** determined from the ordinary CPMAS spectra (CrI_{CPMAS}) increased throughout cooking when more amorphous lignin and xylan were removed. There was increasing trend also in **cellulose crystallinity,** as detected with LGF pulps and reported also previously for kraft pulps (Liitiä 2002). No clear correlation between the crystallinity of alkaline pulps and their hydrolysability determined at WP4 could be detected, as was reported also for the LGF pulps.

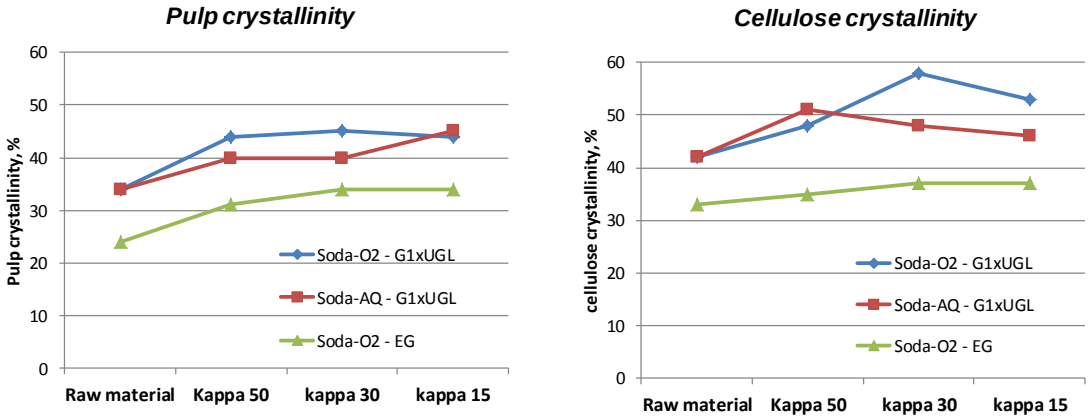


Figure 32. Pulp and cellulose crystallinities determined for alkaline pulps of elephant grass and G1xUGL clone.

Distribution of precipitated EG xylan on alkaline Eucalyptus pulp fibres

Immunolabelling microscopy was used for surface mapping of xylan on alkaline *Eucalyptus* pulp fibres after precipitation of elephant grass (EG) xylan. Two pulps with reprecipitated xylan loadings, and a corresponding reference was studied. The reprecipitated xylan was isolated using alkali charges of 400kg/t NaOH and 700kg/t NaOH. Labelling of the samples was carried out as previously reported and described (Lappalainen et al, 2004). A primary antibody was used, which recognises an epitope consisting of a short xylooligosaccharidic stretch carrying a 4-*O*-methyl-glucuronic acid residue (MeGlcA-Xyl_{2-3}). Previously, in the project the antibody was shown to recognise the xylan both on eucalyptus and elephant grass fibres.

Images on the labelled samples are shown in Figures 33-35. In general, xylan specific antibody was successfully used for labelling of xylan on *Eucalyptus* fibres. It is noteworthy that the method does not discriminate between the native and re-precipitated EG xylan and therefore in the image contribution of both components are merged.

On the untreated reference sample moderate labelling of fibres was observed (Fig. 10). Labelling appeared unevenly as faint and brighter patches on fibre surfaces. Especially, on damaged fibres, fibrillated fines and around pits more extensive labelling was observed. This is undoubtedly due to increased accessibility of xylan at kinks and dislocations and high surface area of fines for recognition by the rather large immunoglobulin molecules.

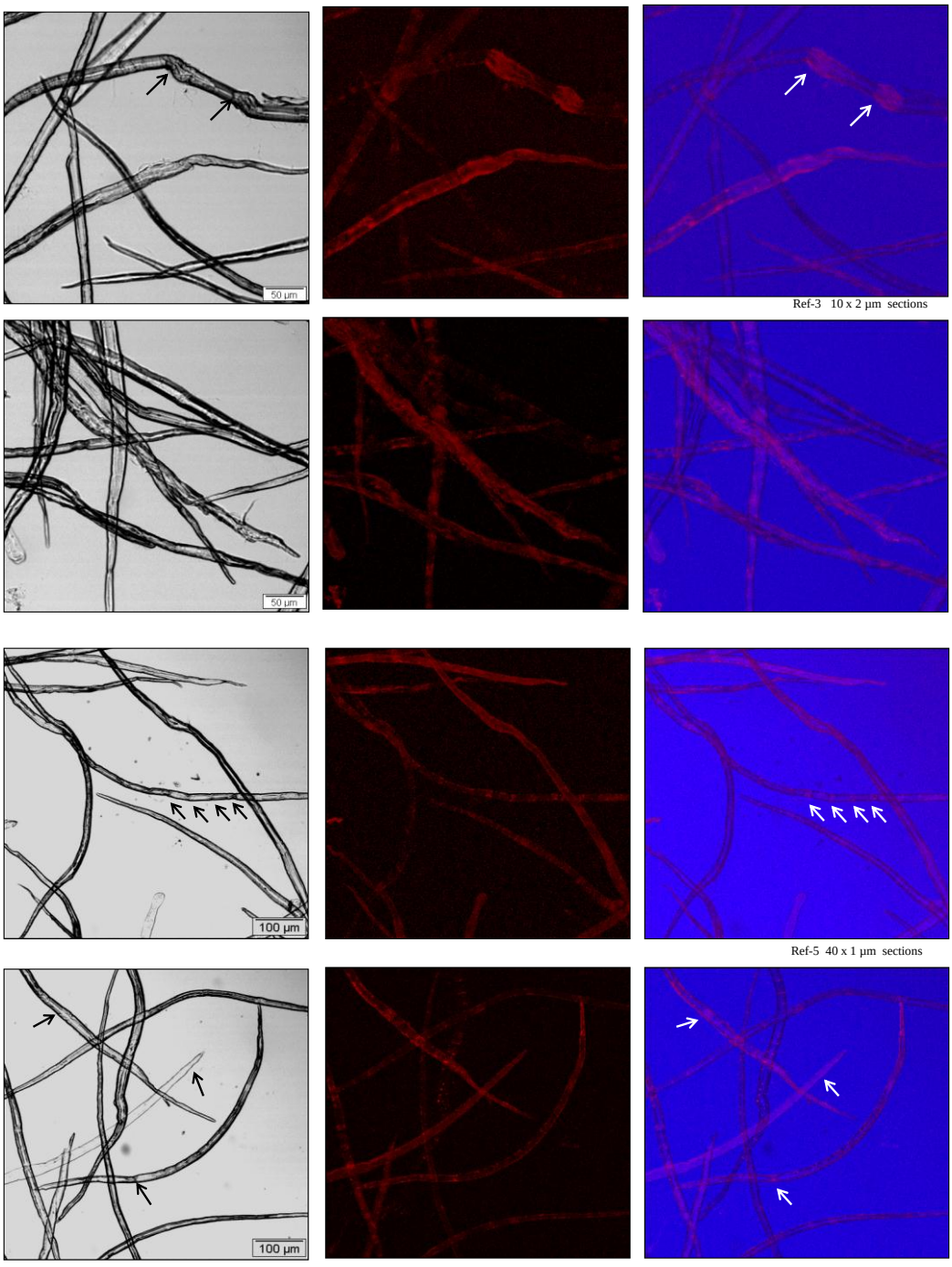


Figure 33. Immunolabelling of xylan on the untreated reference Eucalyptus fibres

Increased labelling of xylan was detected on the samples with precipitated EG xylan as compared with the reference (Figs. 34-35). Overall labelling of bulk fibres was increased and especially fine fibrils and damaged fibres were subjected to dense labelling. Some fibres were very evenly and heavily labelled. However, high background labeling of the 700kg/t NaOH sample indicated desorption of xylan (or antibodies) during preparation of the samples. No unspecific binding of the secondary antibody was detected with the labelling procedure used, *i.e.* the labelling was specific to xylan.

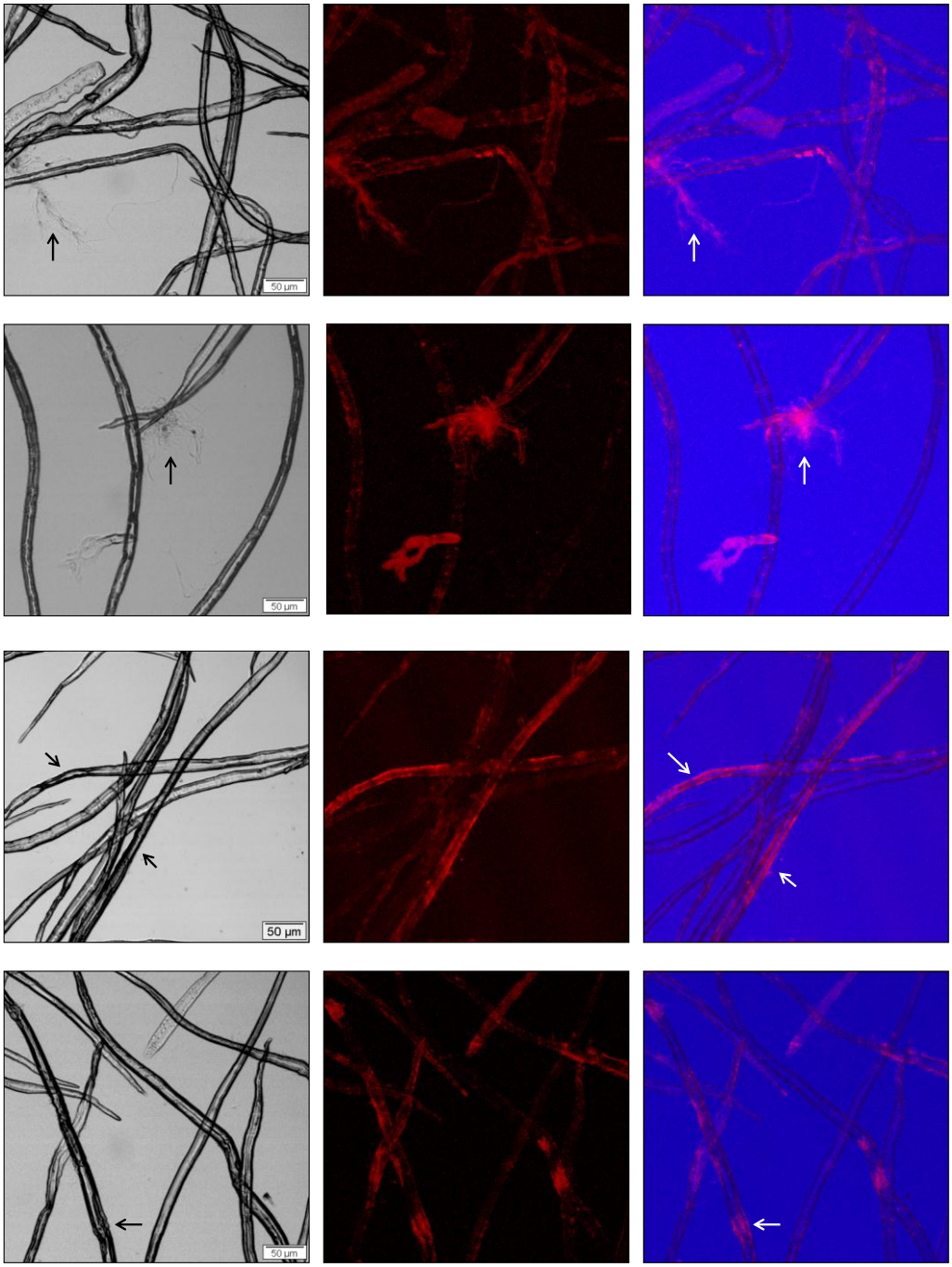


Figure 34. Immunolabelling of xylan on Eucalyptus fibres treated with 400 kg/t NaOH.

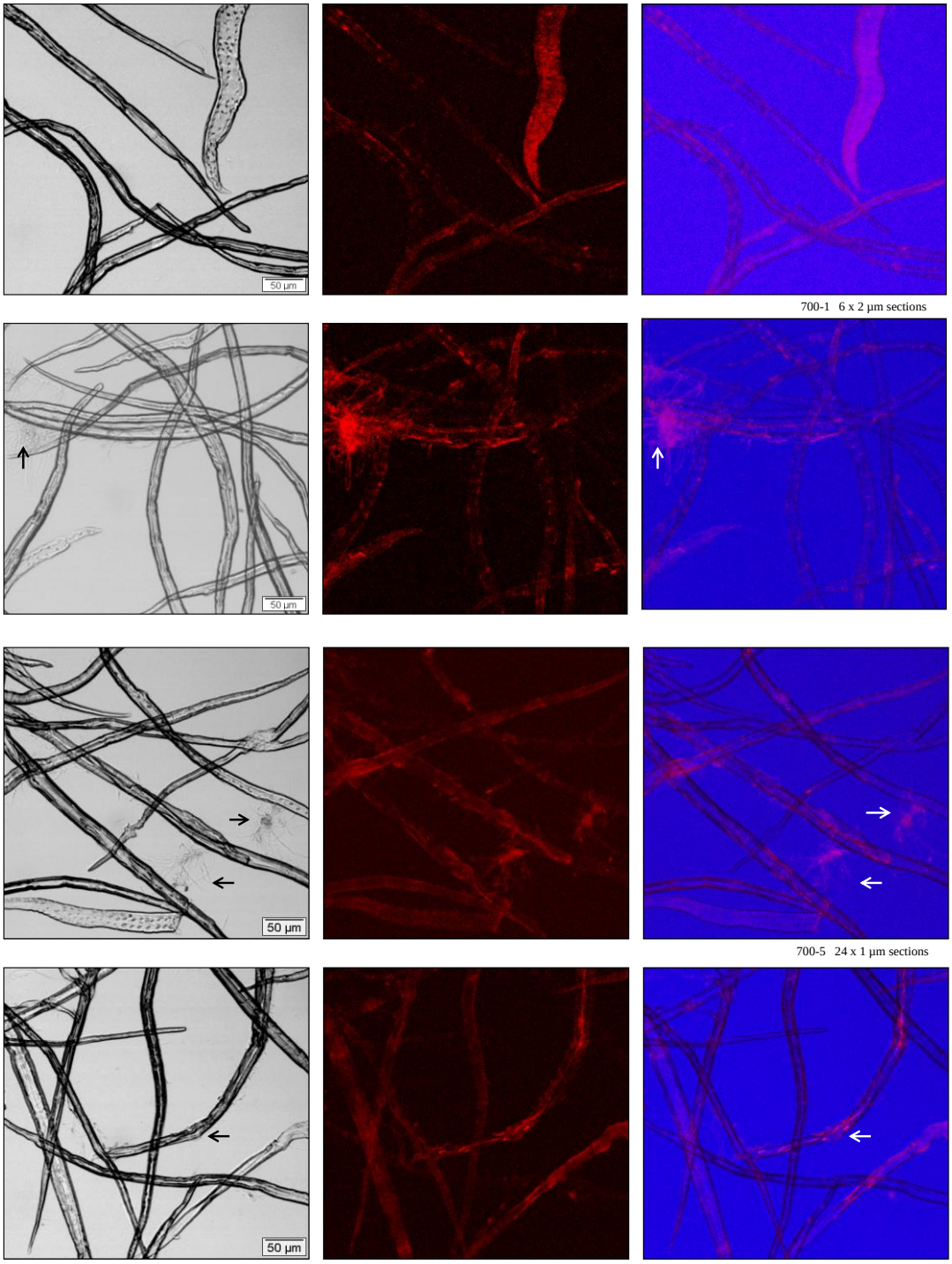


Figure 35. Immunolabelling of xylan on Eucalyptus fibres treated with 700 kg/t NaOH.

Task 3.3 Analysis of lignin and minor components

a) Structural characterization of sound woody and non-woody feedstocks

We have completed the characterization of the samples of elephant grass and eucalypt woods by calculating the molecular weights (M_w) of their lignins. In the case of elephant grass, the cortex and the pith were separated manually and analysed independently. For this purpose, the milled wood lignins were isolated according to classical procedures and the M_w estimated by Gel permeation Chromatography (GPC).

Gel Permeation Chromatography. GPC analyses of the isolated MWLs were performed on a Shimadzu LC-20A LC system (Shimadzu, Kyoto, Japan) equipped with a photodiode array (PDA) detector (SPD-M20A; Shimadzu) using the following conditions: TSK gel α-M + α-2500 (Tosoh, Tokyo, Japan) column; 0.1 M LiBr in dimethylformamide (DMF) as eluent; 0.5 mL min-1 flow rate; 40 °C oven temperature; PDA detection at 280 nm. The data acquisition and computation used LCsolution version 1.25 software (Shimadzu). The molecular weight calibration was via polystyrene standards.

The values of the weight-average (M_w) and number-average (M_n) molecular weights of the MWL isolated from the cortex and pith fractions of elephant grass stems, estimated from the GPC curves (relative values related to polystyrene standards), and the polydispersity (M_w/M_n), are indicated in Table 14. The two lignins exhibited similar molecular weight distributions, in the range 6920-6720 g mol-1, being slightly higher in the case of the lignin from the cortex. In addition, both lignins exhibited relatively narrow molecular weight distributions, with $M_w/M_n < 3$. Those values are comparable to literature values for various isolated lignins.

Table 14. Weight-average (*Mw*) and number-average (*Mn*) molecular weights (g mol-1), and polydispersity (*Mw/Mn*) of the MWLs isolated from the cortex and pith of elephant grass (*P. purpureum*).

	MWL cortex	MWL pith
M_w	6920	6720
M_n	2390	2490
M_w/M_n	2.9	2.7

The values of the weight-average (M_w) and number-average (M_n) molecular weights, estimated from the GPC curves (relative values related to polystyrene), and the polydispersity (M_w/M_n) of the MWL from the selected eucalypt hybrids, are indicated in Table 15. The MWLs exhibited similar molecular weight distributions, in the range 11300-

15040 g mol-1, being slightly higher in the case of the MWL from IP and lower for the MWL from DG×U2. In addition, all the MWL exhibited relatively narrow molecular weight distributions, with $M_w/M_n < 4$.

Table 15. Weight-average (*Mw*) and number-average (*Mn*) molecular weights (g mol-1), and polydispersity (*Mw*/*Mn*) of the MWL from the woods of the different eucalypt hybrids selected in this study

	IP	U1×U2	G1×UGL	DG×U2
M_w	15000	12900	13300	11300
M_n	4300	3900	3500	3000
M_w/M_n	3.5	3.3	3.8	3.8

b) Characterization of residual lignins from kraft, soda-AQ pulps and soda-O_2 pulps (intended for paper and/or bioethanol and biogas production)

Different sets of pulps produced from elephant grass and eucalypt hybrid G1xUGL by different cooking processes were received from **Suzano** (partner 5):

- Pulps intended for paper production: pulp samples from eucalypt G1xUGL and elephant grass prepared by the kraft and soda-AQ processes at kappa 20 and 15. The respective black liquors were also received and analyzed, and the main results are reported in the next section (**Task 3.4**).

- Pulps intended for bioethanol and biogas production: pulp samples from eucalypt G1xUGL and elephant grass prepared by the soda-AQ and soda-O_2 processes at kappa 50, 35 and 15 (these pulps include the rejects).

The residual lignins from the different pulps were isolated by acidolysis and subsequent analyzed by 2D-NMR in HSQC experiments, and by Py-GC/MS.

Isolation of the residual lignins. The isolation of the residual lignins was performed by acid hydrolysis. The extractives-free pulp sample (100 g dry weight) was refluxed for 2 h with 150 ml of 0.1 M HCl in dioxane–water 82:18 (v/v) under nitrogen. The pulp was the filtered and washed with dioxane–water 82:18. The filtrate was evaporated at 40 °C and then the lignin was precipitated in water.

2D-NMR spectroscopy. NMR spectra of isolated lignins were recorded at 25 ºC using a Bruker AVANCE 600 MHz instrument equipped with a cryogenically-cooled z-gradient triple resonance probe. Around 40 mg of lignin were dissolved in 0.75 mL of deuterated dimethylsulfoxide (DMSO-d_6) and 2D-NMR spectra were recorded in HSQC (heteronuclear single quantum coherence) experiments using Bruker's 'hsqcetgp' pulse program with spectral widths of 5000 Hz and 13200 Hz for the ^{1}H- and ^{13}C-dimensions.

The number of collected complex points was 2048 for the ^{1}H-dimension with a recycle delay of 1 s. The number of transients was 64, and 256 time increments were recorded in 13C-dimension. The $^{1}J_{CH}$ used was 140 Hz. Processing used typical matched Gaussian apodization in ^{1}H and a squared cosine-bell in^{13}C. Prior to Fourier transformation, the data matrices were zero-filled up to 1024 points in the 13C-dimension. The central solvent peak was used as an internal reference (δ_C 39.5; δ_H 2.49). A semiquantitative analysis of the volume integrals of the HSQC cross-correlation signals was performed. As the volume integral depends on the particular $^{1}J_{CH}$ value, as well on the T_2 relaxation time, absolute quantitation is impossible but relative integrals (between spectra) allow valid comparisons. Thus, the integration of the cross-signals was performed separately for the different regions of the HSQC spectrum, which contain signals that correspond to chemically analogous carbon-proton pairs. In the aliphatic oxygenated region, the relative abundances of side-chains involved in inter-unit linkages or present in terminal units were estimated from the C_α–H_α correlations to avoid possible interference from homonuclear ^{1}H–^{1}H couplings. In the aromatic region, C_2-H_2 correlations from H, G and S lignin units and from *p*-coumarate and ferulate were used to estimate their relative abundances.

Py-GC/MS. Pyrolysis of isolated residual lignins (approximately 100 μg) was performed with a 2020 micro-furnace pyrolyzer (Frontier Laboratories Ltd.) connected to an Agilent 6890 GC/MS equipment using a DB-1701 fused-silica capillary column (30 m x 0.25 mm i.d., 0.25 μm film thickness) and an Agilent 5973 mass selective detector (EI at 70 eV). The pyrolysis was performed at 500 ºC. The oven temperature was programmed from 50 ºC (1 min) to 100 ºC at 30 ºC min-1 and then to 300 ºC (10 min) at 10 ºC min-1. He was the carrier gas (1 mL min-1). The compounds were identified by comparing their mass spectra with those of the Wiley and NIST libraries and those reported in the literature. Peak molar areas were calculated for the lignin-degradation products, the summed areas were normalized, and the data for two repetitive analyses were averaged and expressed as percentages.

b1) Structural characteristics of the residual lignins isolated from pulps intended for paper production (kraft and soda-AQ processes).

The residual lignins isolated from eucalypt hybrid G1xUGL and elephant grass pulps produced by the kraft and soda-AQ pulps at kappa 20 and 15, as well as the lignins precipitated from their respective black liquors, were analysed by 2D-NMR (in HSQC experiments). The data regarding the lignins from black liquors belongs to ***Task 3.4*** and will be explained in detail latter in that section.

The HSQC spectra (δ_C/δ_H 50-125/2.5-8.0) of a representative residual lignin (isolated from the eucalypt wood G1xUGL kraft pulp at kappa 20), and the precipitated lignin from its respective black liquor, are shown in Figure 36. The spectrum of the MWL isolated from G1xUGL is also shown for comparison. The main substructures found are also depicted in Figure 36. Likewise, the HSQC spectra (δ_C/δ_H 50-150/2.5-8.0) of a representative residual lignin isolated from the elephant grass kraft pulp at kappa 20, and the precipitated lignin from its respective black liquor, are shown in Figure 37. The spectrum of the MWL isolated

from elephant grass is also shown for comparison. The main substructures found are also depicted in Figure 37.

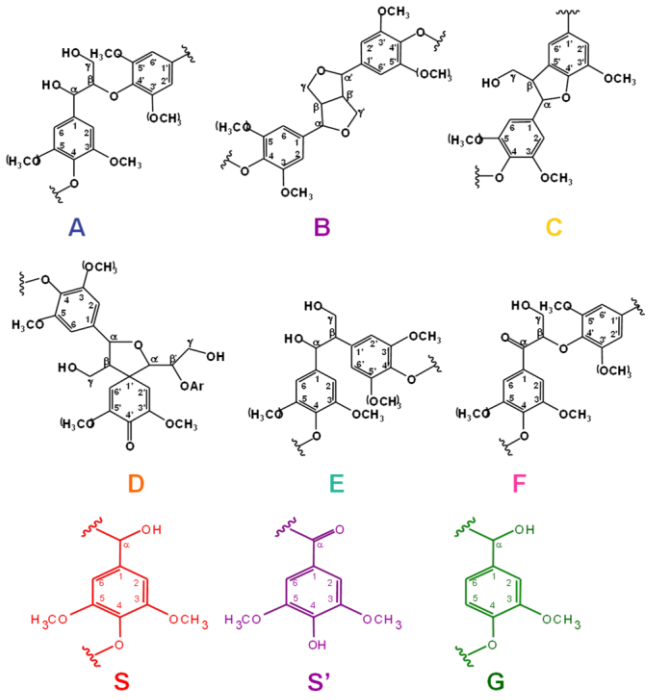


Figure 36. 2D-NMR spectra of a selected residual lignin (isolated from eucalypt G1xUGL kraft pulp at kappa 20) and the lignin precipitated from the respective black liquor. The spectrum of the MWL from eucalypt G1xUGL is also shown for comparison. The main inter-units linkages and lignin structures are also depicted here

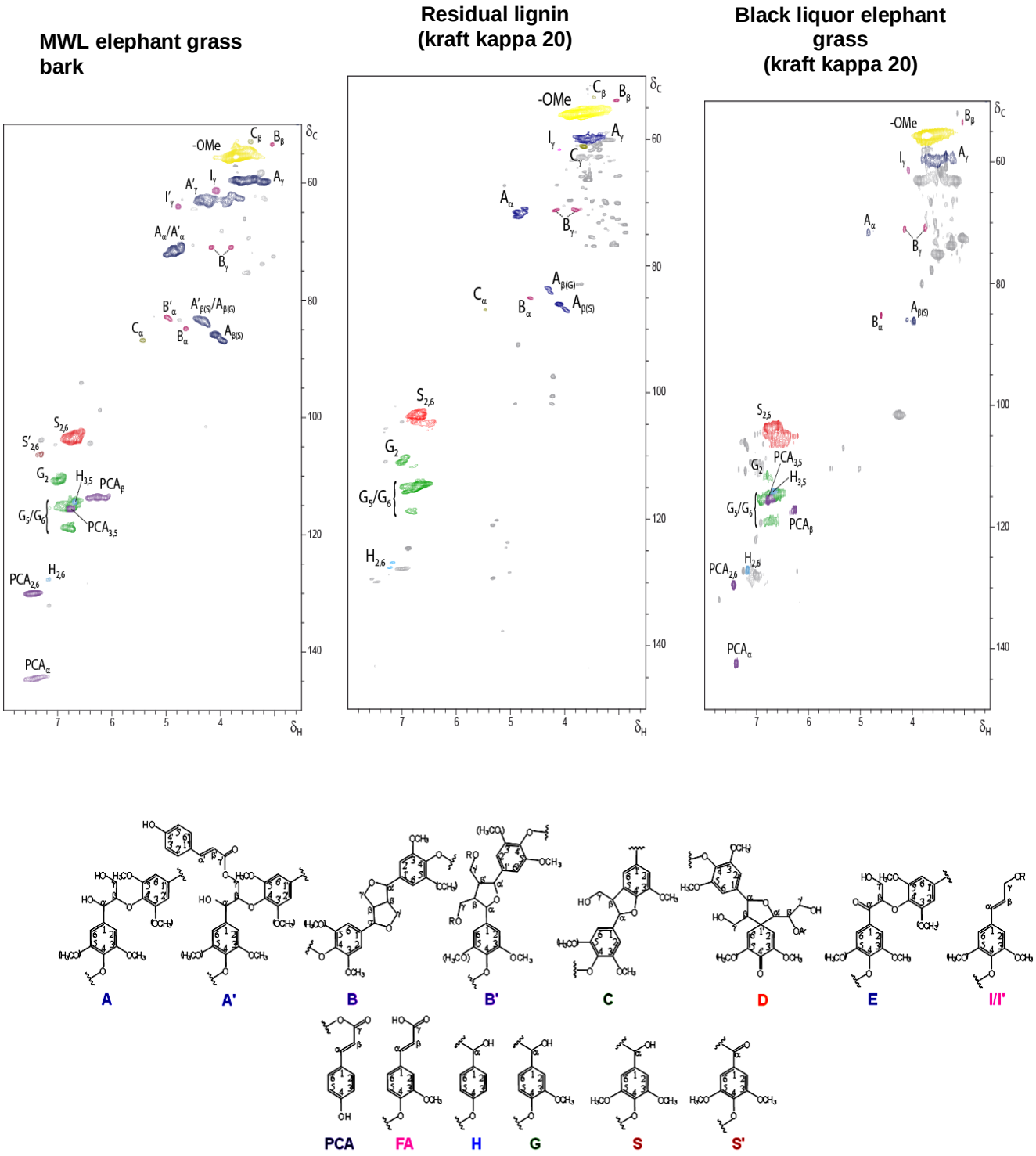


Figure 37. 2D- NMR spectra of a selected residual lignin (isolated from elephant grass kraft pulp kappa 20) and the lignin precipitated from the respective black liquor. The spectrum of the MWL from elephant grass is also shown for comparison. The main inter-units linkages and lignin structures are also depicted here.

A quantitation of the abundance of the main lignin inter-unit linkages present in the different residual lignins from eucalypt G1xUGL pulps, as well as the abundance of the G and S lignin units was performed by integration of the volume contours of their cross-signals and was referred to as per 100 aromatic units (Table 16). The main linkages observed in the residual lignins from G1xUGL were β–*O*–4 aryl ether, β–β resinol and β–5 phenylcoumaran structures, in both the kraft and soda-AQ pulps. No oxidized lignin moieties were observed by 2D-NMR in these residual lignins. The distributions of the different inter-unit linkages in the pulp residual lignins is similar to that observed in the native lignin in wood, with a predominance of β–*O*–4 alkyl-aryl ether linkages, followed by lower amounts of resinols and phenylcoumaran, although with a drastic reduction in their content. This reduction in the content of linkages was more evident in the pulps with lower kappa number (kappa 15) than in pulps with higher kappa number (kappa 20) due to the more drastic pulping conditions at lower kappa numbers. Moreover, at similar kappa number, the content of β–*O*–4 aryl ether linkages in the residual lignin were lower for the soda-AQ process that for the kraft process, indicating a higher efficiency of the soda-AQ process for delignifying the G1xUGL eucalypt wood.

Table 16. Main lignin structural characteristics (linkages per 100 aromatic units, and S/G ratio) of the residual lignins isolated from the pulps produced from eucalypt G1xUGL after kraft and soda-AQ processes at kappa 20 and 15. The composition of the MWL isolated from eucalypt hybrid G1xUGL is shown for comparison.

Linkages (per 100 aromatic units)	MWL	Kraft process		Soda-AQ process	
		Kappa 20	Kappa 15	Kappa 20	Kappa 15
β–*O*–4 alkyl-aryl ether	90.0	16.0	14.0	18.0	12.0
β–β resinols	10.5	7.4	7.4	6.5	6.7
β–5 phenylcoumarans	5.3	0.3	0.1	0.4	0.4
S/G ratio	2.8	3.0	3.0	2.8	2.8

In the case of elephant grass pulps, the structural characteristics (obtained from the HSQC spectra) of the residual lignins are reported in Table 17. In the case of the MWL from elephant grass, 2D-NMR showed a predominance of alkyl aryl ether (β–*O*–4) linkages, with low amounts of the so-called 'condensed' substructures, such as resinols (β–β) and phenylcoumarans (β–5). Moreover, the NMR spectra indicated that these lignins are extensively acylated at the γ-carbon of the side-chain, and predominantly with *p*-coumarate groups. The main lignin substructures present in the residual lignins were β–*O*–4 aryl ether, with lower amounts of β–β resinol and β–5 phenylcoumaran structures, in both the kraft and soda-AQ pulps, with a similar distribution as observed in the native lignin of elephant grass. However, a reduction of the main substructures was observed after the cooking, this reduction being more evident in the pulps with lower kappa number (kappa 15) due to the higher extent of delignification. At similar kappa numbers, the content of β–*O*–4 aryl ether linkages in the residual lignin were lower for the soda-AQ process than for the kraft

process, as already observed in the case of eucalypt wood shown above, indicating a higher efficiency of the soda-AQ process for delignifying the elephant grass. As already observed in the eucalypt pulps, no oxidized lignin moieties were observed by 2D-NMR in the residual lignins from elephant grass. On the other hand, both kraft and soda-AQ cooking produced a complete hydrolysis of the *p*-coumarate groups that are acylating the γ-carbon of the lignin side-chain, and only minor amounts of *p*-coumarates are found in the pulps.

Table 17. Main lignin structural characteristics (linkages per 100 aromatic units, percentage of γ-acylation, S/G and H/G ratios, and percentage of *p*-coumaric acid) of the residual lignins isolated from the pulps produced from elephant grass after kraft and soda-AQ processes at kappa 20 and 15. The composition of the MWL isolated from elephant grass is shown for comparison.

Linkages (per 100 aromatic units)	MWL	Kraft process		Soda-AQ process	
		Kappa 20	Kappa 15	Kappa 20	Kappa 15
β–*O*–4 alkyl-aryl ether	73.0	41.0	11.0	15.0	11.0
β–β resinols	2.4	2.5	0.7	1.8	1.5
β–5 phenylcoumarans	2.9	3.6	0.2	0.3	0.5
% of γ-acylation	39.0	14.0	0.0	0.0	0.0
S/G ratio	1.7	1.1	0.9	1.1	0.8
H/G ratio	0.1	0.1	0.2	0.2	0.2
p-coumaric acid	25.5	13.3	0.3	0.5	0.4

On the other hand, the isolated residual lignins, as well as the lignins precipitated from the black liquors were also analyzed by Py-GC/MS (the data regarding the black liquors are described in *Task 3.4*).

The Py-GC/MS of a selected residual lignin from eucalypt G1xUGL (from kraft pulp at kappa 20) and the lignin precipitated from the respective black liquor are shown in Figure 38. The Py-GC/MS of the MWL from eucalypt G1xUGL is also shown for comparison. The main lignin structural characteristics obtained from the Py-GC/MS data (percentage of H, G and S units, and S/G ratio, and percentage of short-chain pyrolysis products) of the residual lignins isolated from the pulps produced from eucalypt G1xUGL after kraft and soda-AQ processes at kappa 20 and 15, are shown in Table 18. Interestingly, and as already observed by NMR, there is a similarity between the residual lignins from kraft and soda-AQ processes and the native lignin (as reflected by the MWL), observed upon Py-GC/MS. However, the residual lignin is being depleted in S-lignin units and enriched in G- and H-lignin units, with decreasing kappa number (with increasing the extent of delignification), resulting in a decrease of the S/G ratio, as a consequence of the preferential removal of S-lignin during alkaline delignification. A small increase of the amounts of pyrolysis compounds with shorter chain indicates a partial degradation of the residual lignins, which is more evident at lower kappa numbers.

The residual lignins isolated from elephant grass after kraft and soda-AQ processes, were also studied by Py-GC/MS. The Py-GC/MS of a selected residual lignin from elephant grass (from kraft pulp at kappa 20) and the lignin precipitated from the respective black

liquor are shown in Figure 39. The Py-GC/MS of the MWL from elephant grass is also shown for comparison. The main lignin structural characteristics obtained from the Py-GC/MS data (percentage of H, G and S units, and H/G and S/G ratio, and percentage of short-chain pyrolysis products) of the residual lignins isolated from the pulps produced elephant grass after kraft and soda-AQ processes at kappa 20 and 15, are shown in Table 19. As occurred with the residual lignins from eucalypt wood, the residual lignins from elephant grass are similar to the native lignin, although being depleted in S-units with decreasing kappa number. In addition, the pyrogram showed important amounts of 4-vinylphenol, that indicates the presence of *p*-coumarates in these residual lignins; most probably *p*-coumarates are linked by ether bonds since ester bonds are supposedly hydrolyzed during alkaline cooking. The small increase of the amounts of pyrolysis compounds with shorter chain indicates a partial degradation of the residual lignins, which is more evident at lower kappa numbers, as also occurred in the case of eucalypt pulps.

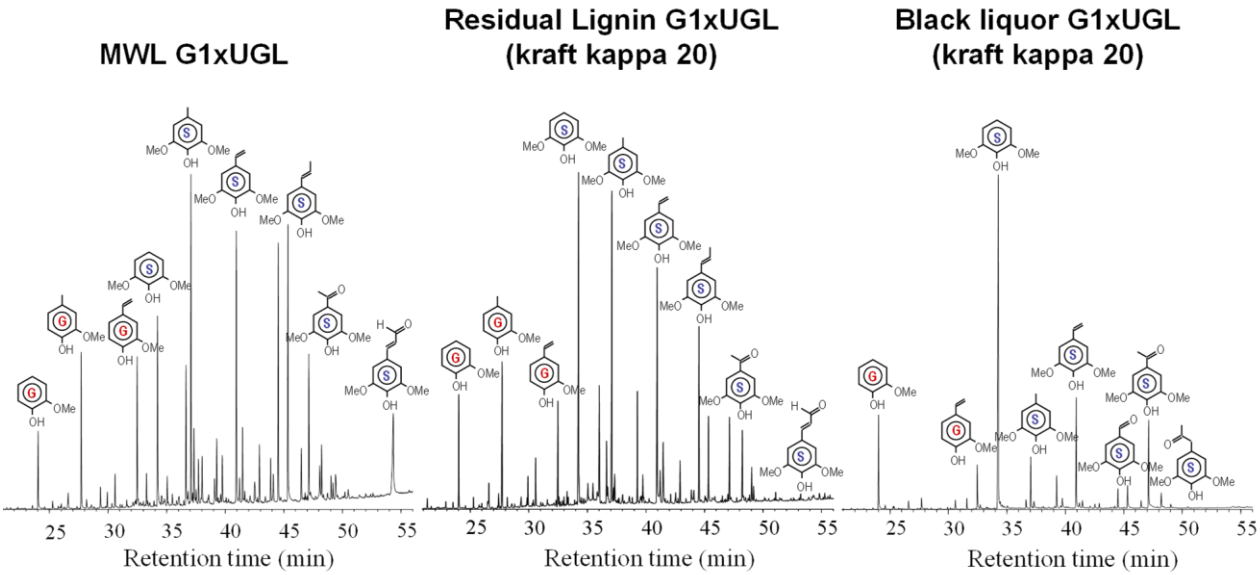


Figure 38.- Py-GC/MS of a selected residual lignin (from eucalypt G1xUGL kraft pulp at kappa 20) and the lignin precipitated from the respective black liquor. The Py-GC/MS of the MWL from eucalypt G1xUGL is also shown for comparison. The main lignin structures are also depicted here.

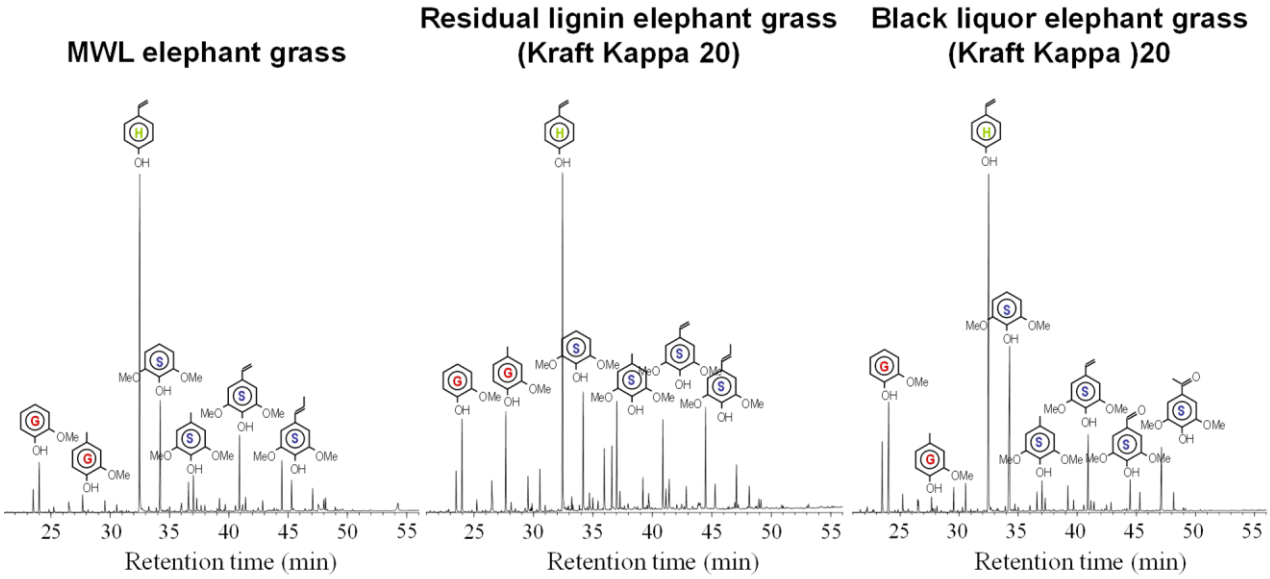


Figure 39**.** Py-GC/MS of a selected residual lignin (from elephant grass kraft pulp kappa 20) and the lignin precipitated from the respective black liquor. The Py-GC/MS of the MWL from elephant grass is also shown for comparison. The main lignin structures are also depicted here.

Table 18. Main lignin structural characteristics (percentage of H, G and S units, and S/G ratio, and percentage of short-chain pyrolysis products) of the residual lignins isolated from the pulps produced from eucalypt G1xUGL after kraft and soda-AQ processes at kappa 20 and 15. The composition of the MWL isolated from eucalypt hybrid G1xUGL is shown for comparison.

Aromatic units	MWL	Kraft process		Soda-AQ process	
		Kappa 20	Kappa 15	Kappa 20	Kappa 15
H	1.7	2.7	3.6	2.6	4.1
G	33.1	30.3	33.5	31.3	35.6
S	65.2	67.0	62.8	66.1	60.4
S/G ratio	2.0	2.1	1.9	2.1	1.7
% short chains (C_{0-2})	64	76	85	81	84

Table 19. Main lignin structural characteristics (percentage of H, G and S units, and H/G and S/G ratios, and percentage of short-chain pyrolysis products) of the residual lignins isolated from the pulps produced from elephant grass after kraft and soda-AQ processes at kappa 20 and 15. The composition of the MWL isolated from elephant grass is shown for comparison.

Aromatic units	MWL	Kraft process		Soda-AQ process	
		Kappa 20	Kappa 15	Kappa 20	Kappa 15
H	49.8	30.4	40.2	34.7	37.0
G	19.4	34.8	31.2	33.1	35.4
S	30.8	34.8	28.5	32.1	27.6
S/G ratio	1.6	1.0	0.9	1.0	0.8
H/G ratio	2.6	0.9	1.3	1.0	1.0
% short chains (C_{0-2})	86	83	88	88	90

b2) Structural characteristics of the residual lignins isolated from pulps intended for bioethanol and biogas production (soda-AQ and soda-O_2 processes)

Eucalypt hybrid G1xUGL and elephant grass pulps were prepared by the soda-AQ and soda-O_2 processes at kappa 15, 35 and 50 by **Suzano** (**partner 5**) and sent to **IRNAS** for subsequent analysis of the residual lignins. For this, the residual lignins were isolated by acidolysis and subsequently analyzed by 2D-NMR (in HSQC experiments), and by Py-GC/MS, as shown above.

The structural characteristics (obtained from the HSQC experiments) of the residual lignins isolated from eucalypt G1xUGL pulps prepared by the soda-AQ and soda-O_2 processes are reported in Table 20. The main linkages observed in the residual lignins isolated from the soda-AQ and soda-O_2 pulps from G1xUGL were β–*O*–4 aryl ether, β–β resinol and β–5 phenylcoumaran structures, as also observed in the native lignin. A reduction in these

linkages was observed as the kappa number decreases, due to the increasing extent of delignification. The pulps with higher kappa number (kappa 50 and 35) still presented high contents of β–*O*–4 aryl ether linkages, which were drastically reduced in the pulps with the lowest kappa number (kappa 15). Comparing both processes, it is apparent that, at the same kappa number, the soda-O_2 process produces pulps with lower content of β–*O*–4 aryl ether linkages for the pulps with higher kappa. Therefore, it seems that the soda-O_2 process is more efficient than soda-AQ process for delignification of eucalypt wood, at least at high kappa numbers.

Table 20**.** Main lignin structural characteristics (linkages per 100 aromatic units, and S/G ratio) of the residual lignins isolated from the pulps produced from eucalypt G1xUGL after soda-AQ and soda-O_2 processes at kappa 15, 35 and 50. The composition of the MWL isolated from G1xUGL is shown for comparison.

Linkages (per 100 aromatic units)	MWL	Soda-AQ process			Soda-O_2 process		
		K50	K35	K15	K50	K35	K15
β–*O*–4 alkyl-aryl ether	90.0	56.0	47.0	12.0	41.0	29.0	12.0
β–β resinols	10.5	11.0	11.0	6.7	13.0	10.3	8.2
β–5 phenylcoumarans	5.3	1.0	0.9	0.4	0.9	1.0	0.9
S/G ratio	2.8	5.9	5.9	4.8	4.6	4.5	4.6

On the other hand, the structural characteristics (obtained from HSQC spectra) of the residual lignins isolated from elephant grass pulps prepared by the soda-AQ and soda-O_2 processes are reported in Table 21. The main lignin substructures present in the residual lignins were β–*O*–4 aryl ether, with lower amounts of β–β resinol and β–5 phenylcoumaran structures, as also observed in the native lignin. A reduction in the content of these linkages was observed as the kappa number decreases, due to the higher extent of delignification. At similar kappa numbers, the content of β–*O*–4 aryl ether linkages in the residual lignins from elephant grass were lower for the soda-O_2 than for the soda-AQ process, as already observed in the case of eucalypt wood shown above, indicating a higher efficiency of the soda-O_2 process for delignifying the elephant grass. Both, soda-AQ and soda-O_2 cooking produced a complete hydrolysis of the *p*-coumarate groups that are acylating the γ-carbon of the lignin side-chain, and only minor amounts of free *p*-coumaric acid are found in the soda-AQ pulps.

Table 21. Main lignin structural characteristics (linkages per 100 aromatic units, percentage of γ-acylation, S/G and H/G ratios and percentage of *p*-coumaric acid) of the residual lignins isolated from the pulps produced from elephant grass after soda-AQ and soda-O_2 processes at kappa 15, 35 and 50. The composition of the MWL isolated from elephant grass is shown for comparison.

Linkages (per 100 aromatic units)	MWL	Soda-AQ process			Soda-O_2 process		
		K50	K35	K15	K50	K35	K15
β–*O*–4 alkyl-aryl ether	73.0	54.0	52.0	11.0	56.0	56.0	24.0
β–β resinols	2.4	4.6	3.3	1.5	4.5	5.0	3.0
β–5 phenylcoumarans	2.9	4.0	3.0	0.3	4.0	4.0	1.5
% of γ-acylation	39.0	6.0	8.0	0.0	4.0	2.0	0.0
S/G ratio	1.7	1.8	1.1	0.8	1.4	1.4	0.8
H/G ratio	0.1	0.0	0.1	0.1	0.1	0.1	0.2
p-coumaric acid	25.5	9.0	10.0	0.0	8.0	5.0	1.0

The residual lignins isolated from eucalypt G1xUGL and elephant grass pulps obtained from the soda-AQ and soda-O_2 processes (at kappa 50, 35 and 15), were also analyzed by Py-GC/MS (Fig. 40 and Fig. 41, respectively). The main structural characteristics of these residual lignins from eucalypt G1xGL and elephant grass pulps, obtained upon Py-GC/MS, are shown in Table 22 and Table 23, respectively.

In the case of the eucalypt wood, the residual lignins are enriched in H- and G-lignin units, and depleted in S-units with decreasing kappa number. Moreover, there is also an increase in the amounts of lignin pyrolysis compounds with shorter chain, indicating a partial lignin degradation with decreasing kappa number. In addition, and in agreement with the NMR data shown above, soda-O_2 process seems to be more efficient than soda-AQ process to delignify eucalypt wood, at least at higher kappa numbers.

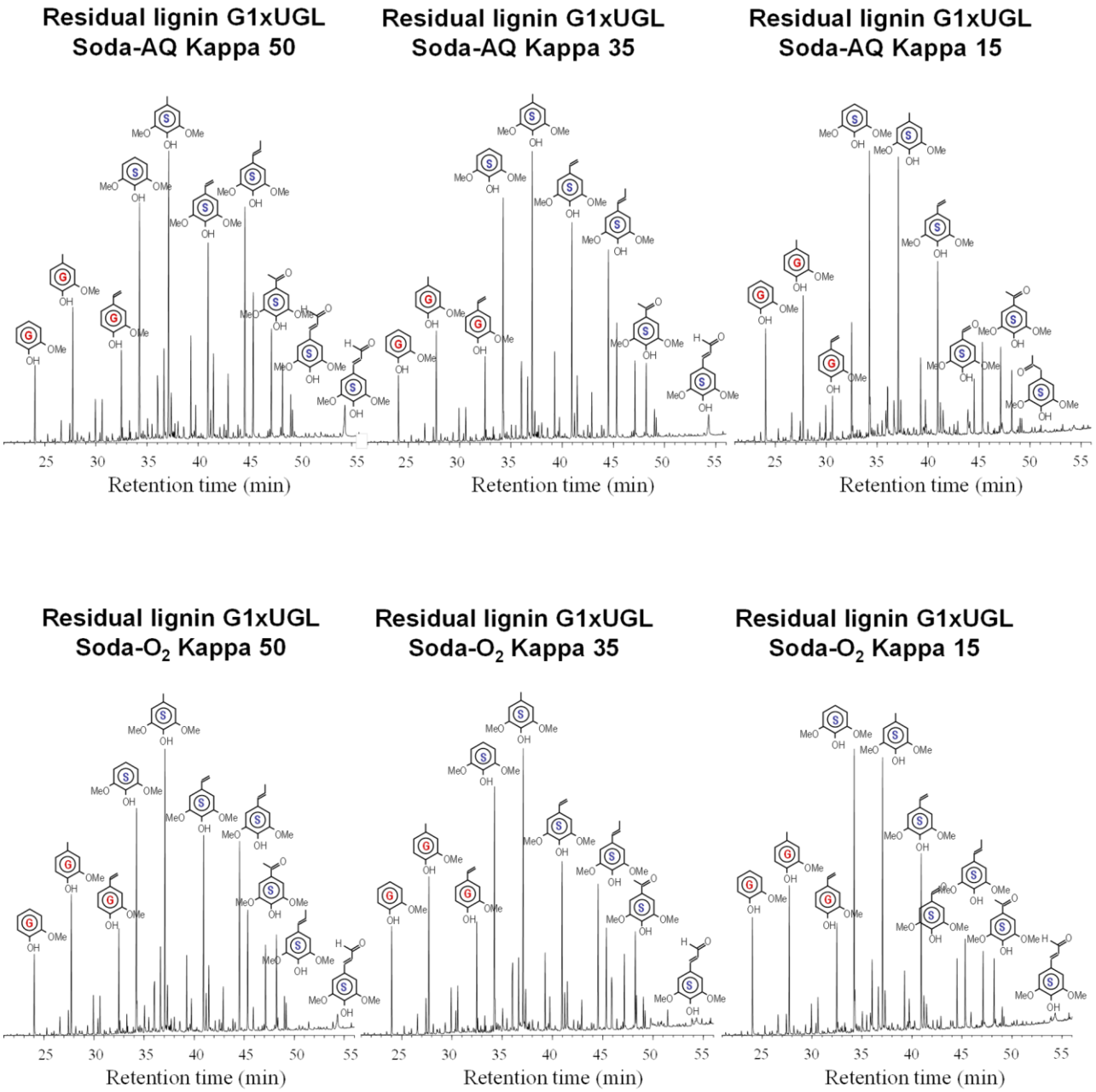


Figure 40.- Py-GC/MS of the residual lignins isolated from eucalypt G1xUGL after soda-AQ and soda-O_2 at kappa 50, 35 and 15.

In the case of the elephant grass, the residual lignins are also enriched in H- and G-lignin units, and depleted in S-units with decreasing kappa number. Moreover, there is also an increase in the amounts of lignin pyrolysis compounds with shorter chain, indicating a partial lignin degradation with decreasing kappa number. In addition, and in agreement with the NMR data shown above, there is a similarity between the soda-O_2 and soda-AQ processes to delignify elephant grass.

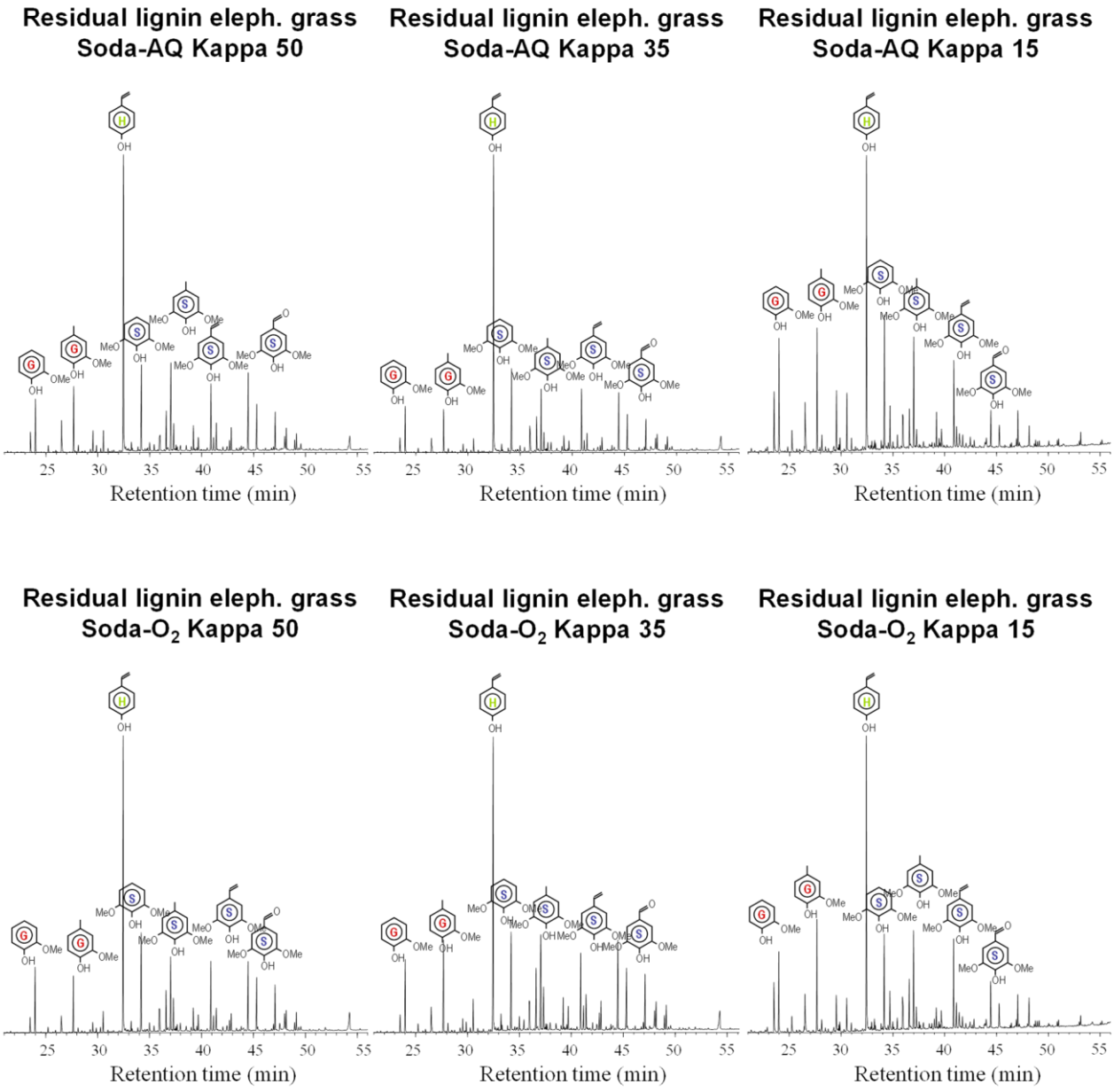


Figure 41. Py-GC/MS of the residual lignins isolated from elephant grass after soda-AQ and soda-O_2 at kappa 50, 35 and 15.

Table 22. Main lignin structural characteristics (percentage of H, G and S units, and S/G ratio, and percentage of short-chain pyrolysis products) of the residual lignins isolated from the pulps produced from eucalypt G1xUGL after soda-AQ and soda-O_2 processes at kappa 15, 35 and 50. The composition of the MWL isolated from G1xUGL is shown for comparison.

Aromatic units	MWL	Soda-AQ process			Soda-O_2 process		
		K50	K35	K15	K50	K35	K15
H	1.7	1.6	1.8	4.0	1.8	2.2	2.8
G	33.1	27.5	27.6	35.6	33.3	35.3	36.2
S	65.2	70.9	70.5	60.4	64.9	62.5	61.0
S/G ratio	2.0	2.6	2.5	1.7	2.0	1.8	1.7
% short chains (C_{0-2})	64	70	73	84	72	76	84

Table 23**.** Main lignin structural characteristics (percentage of H, G and S units, and H/G and S/G ratios, and percentage of short-chain pyrolysis products) of the residual lignins from the pulps produced from elephant grass after soda-AQ and soda-O_2 processes at kappa 15, 35 and 50. The composition of the MWL isolated from elephant grass is shown for comparison.

Linkages (per 100 aromatic units)	MWL	Soda-AQ process			Soda-O_2 process		
		K50	K35	K15	K50	K35	K15
H	49.8	35.0	34.8	37.0	27.3	23.2	33.9
G	19.4	29.4	29.1	35.4	32.9	35.6	37.1
S	30.8	35.7	36.1	27.6	39.7	41.2	29.0
S/G ratio	1.6	1.2	1.2	0.8	1.2	1.2	0.8
H/G ratio	2.6	1.2	1.2	1.0	0.8	0.8	0.9
% short chains (C_{0-2})	86	80	81	90	79	80	86

Effect of LGF cooking conditions on lignin structure

The spent liquor lignins (SLL) produced in LGF organosolv cooking experiments were characterized to better understand the delignification mechanisms, and also the utilization potential of the by-product lignins.

- Effect of phosphinic acid (PA) charge (0, 1.5, 3.5, 5, 15%) was evaluated with lignins originating from LGF cooking of G1xUGL clone (130°C, 15 % water content, 48h)
- Effect of cooking time (10, 16, 20, 32 h) was evaluated with lignins originating from LGF cooking of Suzano clone (3.5% PA, 130°C, 15 % water content)
- Effect of raw material was evaluated with lignins originating from LGF cooking of *EG, E.globulus, Suzano, G1xUGL, U1xU2, IP, and DGxU2* clones (3.5% PA, 130°C, 15 % water content, 20h)

Experimental

To minimize the scattering caused by impurities in analysis, the lignins were purified by dissolving 0.5g SLL into 35 ml 0.1M NaOH for 30 min at room temperature. After that the samples were filtered (Whatman no 4) to remove the insoluble impurities, and washed through the filters with 15 ml 0.1 M NaOH. The lignins were precipitated by acidification with 1M HCl to pH 2.4. The samples were centrifuged (25 min, 9000 rpm, 4°C) and washed with acidified milliQ water (pH 2.4) two times. Finally, the samples were freeze dryed and extractives removed by Soxhlet extraction (8h) using hexane.

The molar mass distributions and average molar masses (Mn, Mw) were determined by Size Exclusion Chromatography (SEC) which in this case is a relative method. The SEC measurements were performed by Waters HPLC in 0.1M NaOH eluent using PSS's MCX 1000 and 100 000 Å columns with UV detection at 280 nm. The average molar masses (Mn, Mw) were calculated relative to the polystyrene sulphonate (Na-PSS) standards using Waters Empower 2 software. For SEC analysis, lignins were dissolved overnight in 0.1M NaOH (1 mg/ml) and filtered with 0.45 μm PTFE membrane.

Lignin functionalities (phenolic and aliphatic hydroxyls and COOH groups) were analysed by ^{31}P NMR spectroscopy. For the ^{31}P NMR measurements, the phosphitylation of dry lignin samples was performed according to Granata and Argyropoulos (1995). The ^{31}P-NMR spectra were recorded on a Bruker Avance III 500 MHz spectrometer using a 90º pulse width and an inverse gated decoupling sequence. $CDCl_3$ was used as a locking solvent and 512 scans were accumulated with a delay time of 5 s between pulses. For processing data, the chemical shifts were calibrated to the sharp signal (132.2 ppm) of the reaction product between water and phosphitylation reagent. After establishing an optimal deconvolution parameters used for all samples, the deconvolutions of the syringylic - and condensed hydroxyl group signals were processed with a line broadening factor of 8 Hz. At the 144.2 – 141.ppm region, 12 and 20 peaks were used to obtain an exact deconvolution results (Bruker topspin software).

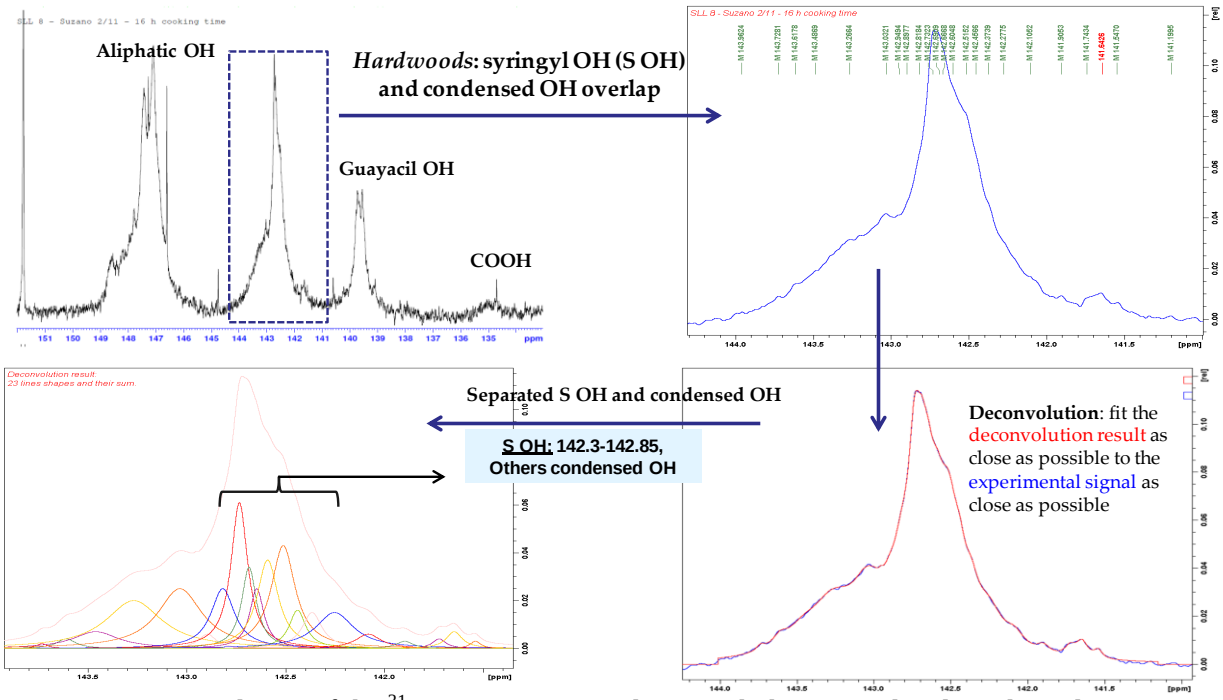


Figure 42. Deconvolution of the ^{31}P NMR spectra to distinguish the syringyl and condenced G units overlapping at 144.2 – 141.ppm region.

Structural features of LGF spent liquor lignins

Table 24. Average molar masses of LGF spent liquor lignins (SEC in 0.1M NaOH, UV-detection, Na-PSS standards).

Raw material	H_3PO_2, %	Cooking time, h	Mn, g/mol	Mw, g/mol	Polydispersity
G1*UGL	0	48	1900	2900	1,50
	1,5	48	2200	3400	1,54
	3,5	48	**2400**	**3700**	1,58
	5	48	2200	3600	1,62
	15	48	2000	3100	1,53
Suzano	3,5	10	2000	3300	1,53
	3,5	16	2100	3600	1,72
	3,5	20	2100	3500	1,67
	3,5	32	**2100**	**3400**	1,62
EG	3,5	20	**1400**	**2200**	1,55
E. Glob.	3,5	20	**2100**	**3500**	1,66
Suzano	3,5	20	2100	3500	1,67
G1*UGL	3,5	20	2200	3700	1,67
U1*U2	3,5	20	2200	3600	1,64
IP	3,5	20	2100	3300	1,63
DG*U2	3,5	20	2200	3700	1,65
Euc. Glob., pilot plant, not purified	3,5	20	**2000**	**3000**	1,52
Euc. Glob.	3,5	20	**2100**	**3200**	1,51

The molar mass results given in Table 24 show that small molecular easily soluble lignin fraction was dissolved in LGF cooking when no PA was used. The molar mass of dissolved lignin increased with increasing PA charge, reaching maximum with 3.5-5% PA charges. The same amount of PA was previously determined optimal for cooking efficiency in respect of pulp hydrolysability. With higher PA charges, the dissolved lignin was more extensively degraded. Cooking time or eucalyptus clone had no significant effect on lignin molar mass. Only for the EG lignin clearly lower Mw was detected. The lower molar mass of pilot *E.globulus* lignin compared to the corresponding lab-scale lignin supports more efficient delignification process at pilot scale, as discussed in WP5.

As shown in Figures 43 and 44, the content of aliphatic hydroxyl groups reduced and proportion of phenolic hydroxyls increased as a function of cooking time and phosphinic acid charge. Without any added PA, more phenolic small molecular lignin was dissolved.
Also the S/G ratio of SSLs increased as cooking proceeded or higher PA charges were used. In both cases, the proportion of S-OH increased continously, whereas the content of G-OH was relatively stable. Interestingly, guaiacyl rich lignin was dissolved when no PA was used in LGF cooking. The proportion of condensed structures also increased with prolonged cooking times and increasing PA charge.

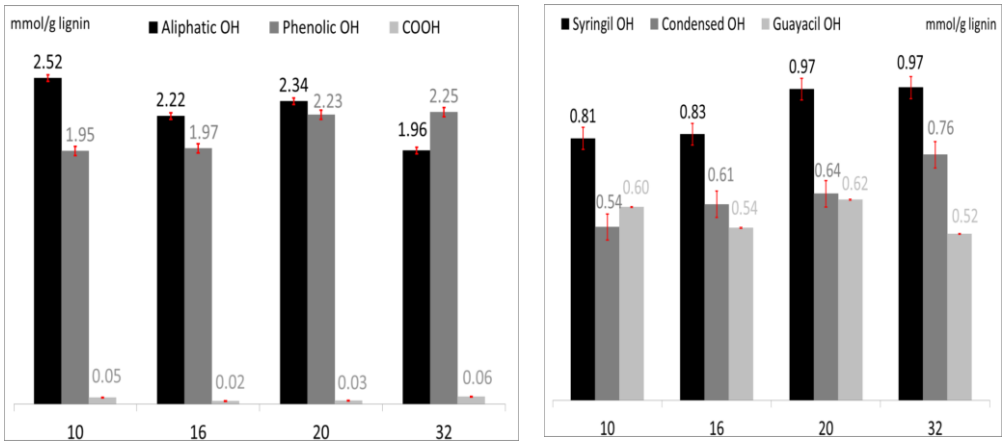


Figure 43. Effect of cooking time (h) on lignin functionalities (Suzano clone, 3.5% PA, 130°C, 15% water content).

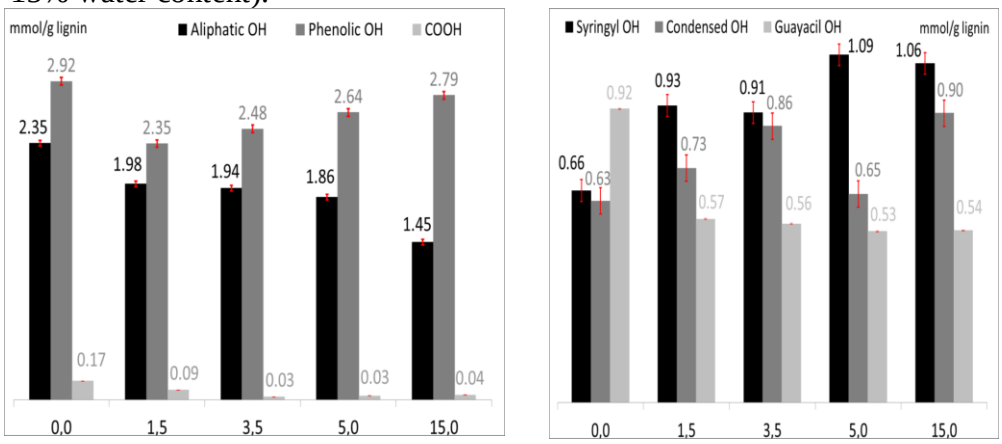


Figure 44. Effect of phosphinic acid charge (%) on lignin functionalities (G1xUGL clone, 130°C, 15% water content, 48h).

In comparison of raw materials, the SLL of *E. globulus* was most phenolic, whereas the *IP* and *EG* SLLs were least phenolic. Also the S/G ratio was highest in *E.globulus* SLL, whereas the proportion of condenced OH was lowest in *EG* and *IP*. This correlates well with the delignification efficiency.

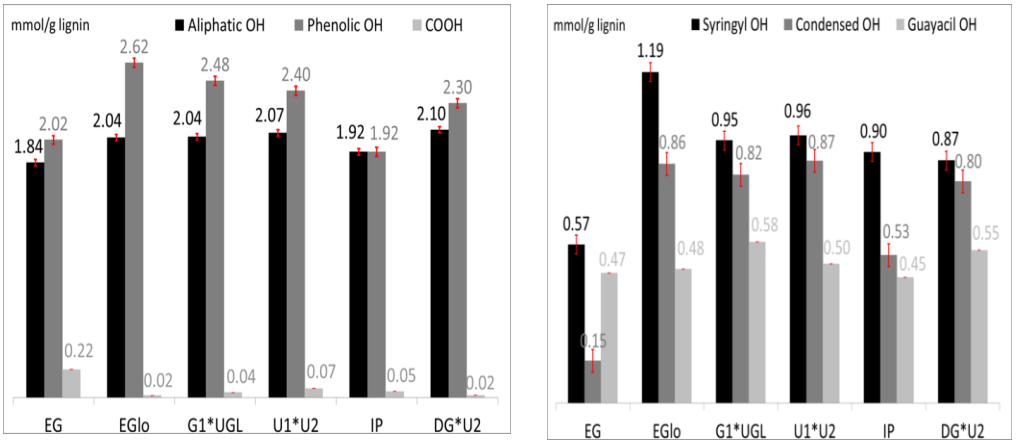


Figure 45. Comparison of lignin functionalities in SSLs of differenct raw materials (3.5% PA,130°C, 15% water content, 20h).

Interestingly, a previously unidentified lignin signals (A and B) were detected in most of the SLLs (Figure 17). The both signals most likely originate from the same structure, as their signal intensities correlated linearly. The identification of the structure is on-going.

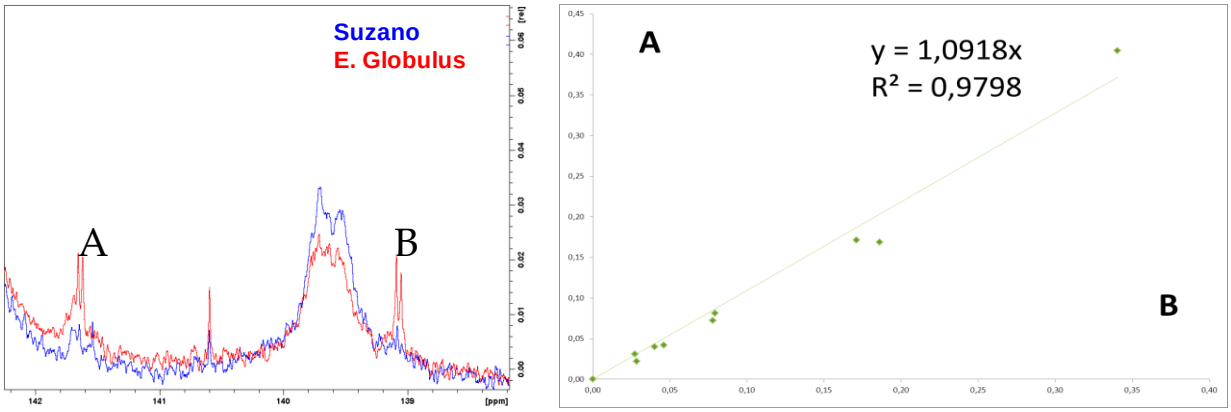


Figure 46. Previously unidentified lignin signals (A,B) and correlation of their signal intensities.

Effect of alkaline cooking on lignin structure

Pulp residual and spent liquor lignins of eucalyptus clone *G1xUGL* and elephant grass were isolated at IRNAS after alkaline cooking (kraft, Soda-AQ, Soda-O2) to kappa levels 50, 35, 20 and 15. The lignins were analysed at VTT during student exchange of Pepijn Prinsen/IRNAS.

The lignin functionalities were analysed by ^{31}P NMR spectroscopy as reported above. During alkaline cooking the content of aliphatic OH groups decreased, whereas the proportion of phenolic units increased mainly due to the cleavage of β-O-4 linkages. As expected, in spent liquor lignins the changes were higher compared to the corresponding pulp residual lignins. At high kappa levels, the decrease of aliphatic hydroxyls (Figure 46) and increase of phenolic units (Figure 47) was most efficient with Soda-O2 and lowest with kraft cooking. At kappa level 15, the content of aliphatic hydroxyl groups of residual lignins was quite same after all the studied alkaline cooking methods, whereas the phenolic content of Soda-O_2 residual lignin remains lower compared to the others. This is expected as the oxidation proceed via phenolic lignin units. This may affect the pulp reactivity in the following bleaching stages if paper pulp is produced, although the pretreatment was mainly tested for bioethanol production purposes. Also the Figure 48 shows, that the removal of aliphatic OH was higher than the formation of new phenolic units at lower kappa levels of Soda-O_2 cooking.

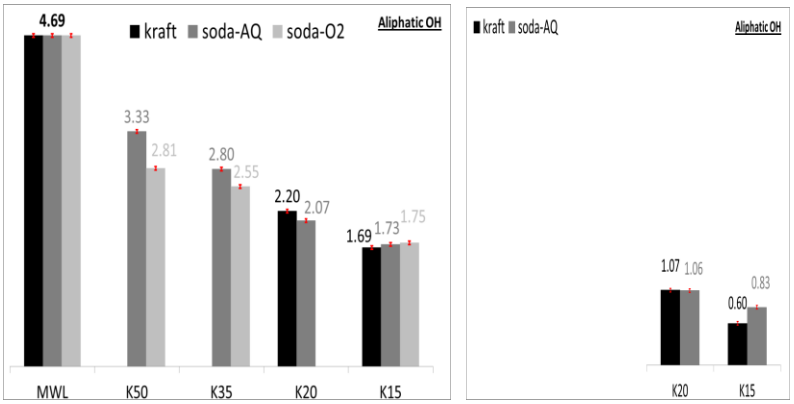


Figure 47. Effect of alkaline cooking on aliphatic hydroxyl content in pulp residual lignin (left) and spent liquor lignin (right).

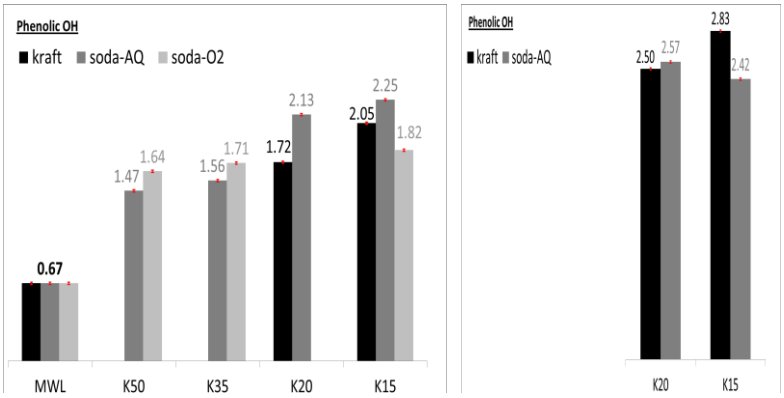


Figure 48. Effect of alkaline cooking on phenolic hydroxyl content in pulp residual lignin (left) and spent liquor lignin (right).

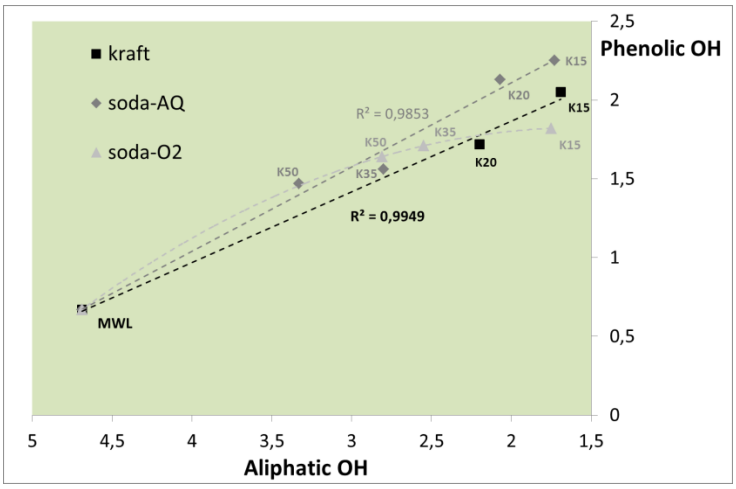


Figure 49. Correlation between phenolic hydroxyl groups formed and aliphatic hydroxyls removed.

The content of phenolic syringyl units increased in residual lignins during kraft and Soda-AQ cooks, but during Soda-O2 cooking remained rather constant at kappa levels 50-15 (Figure 50). The content of reactive phenolic syringyl units was higher in Soda-AQ pulp lignin compared to others. The changes in phenolic guaiacylic groups were lower, remaining relatively constant in pulp residual lignin throughout the alkaline cooks (Figure 50).

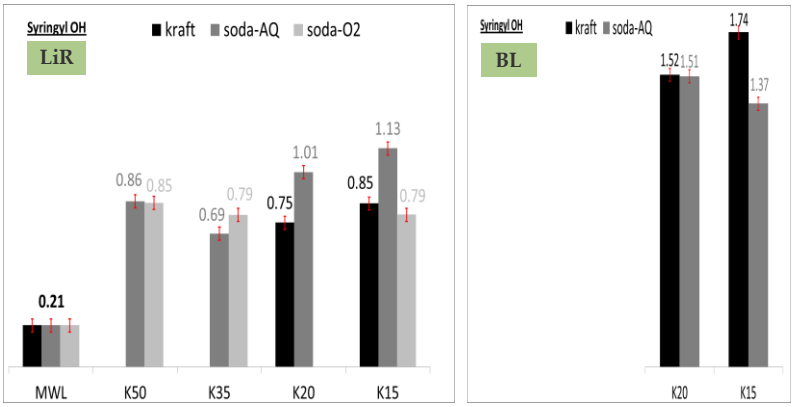


Figure 50. Effect of alkaline cooking on phenolic syringyl units in pulp residual lignin (left) and spent liquor lignin (right).

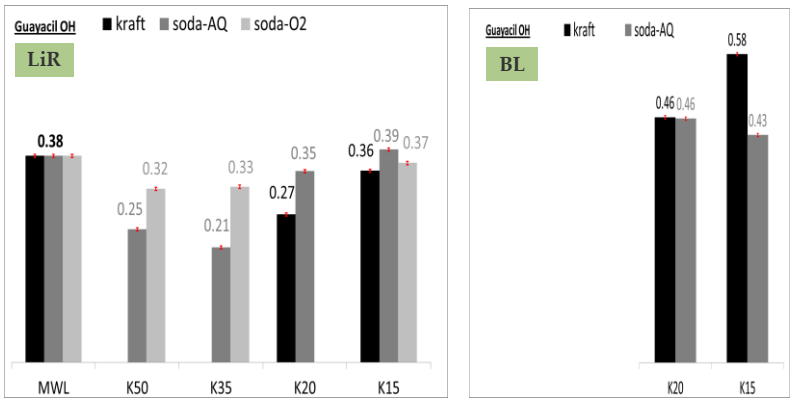


Figure 52. Effect of alkaline cooking on phenolic guaiacyl units in pulp residual lignin (left) and spent liquor lignin (right).

The structure of lignin became more condensed during all the alkaline cooks (Figure 53). In residual lignin, the condensation is most extensive at the end of kraft cooking (kappa 15). After the Soda-O2 cooking the residual lignin was least condensed, which may also be related to the lower phenol content in general. In spent liquor lignins, an opposite order was observed between the cooking methods. Also the content of carboxylic groups increased, being higher in black liquor lignins compared to the residual lignins (Figure 54).

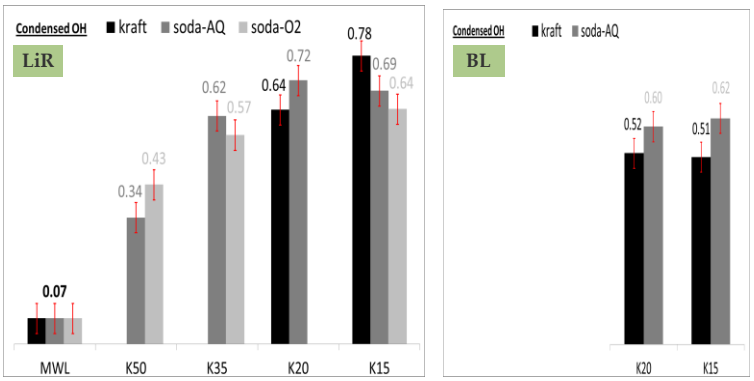


Figure 53. Effect of alkaline cooking on condensed phenolic units in pulp residual lignin (left) and spent liquor lignin (right).

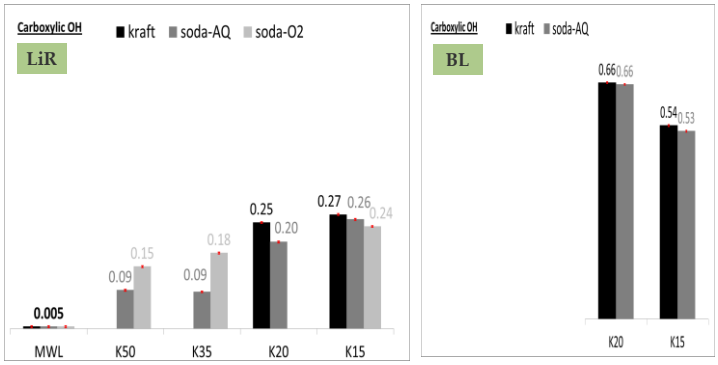


Figure 54. Effect of alkaline cooking on carboxylic acids in pulp residual lignin (left) and spent liquor lignin (right).

Unlike in MWL lignins, also in residual lignins and spent liquor lignins of the alkaline pretreatments, the A (141.65 - 141.61 ppm) and B (139.05 – 139.01 ppm) signals of a novel unidentified lignin structure were detected. Confirmation of the detailed structure is still on-going, but tentative identification suggests condensed catechol structure.

The molar mass of the alkaline lignins was determined by SEC as described above. The molar mass of residual lignin decreased with prolonged cooking, and as expected the molar mass of dissolved lignin was lower compared to the residual lignin remaining in pulp. The decreasing molar mass of Soda-AQ pulp residual lignin correlates well with the cleavage of β-O-4 linkages. Molar mass (Mw) of Soda-AQ pulp residual lignin was highest, whereas the Mw of lignin remaining in kraft pulps at the same kappa level was the lowest.

Table 25. The average molar mass(Mn, Mw) and polydispersity of alkaline lignins.

G1xUGL	**Mn (g/mol)**	**Mw (g/mol)**	**PD**
MWL	1900	4400	2.34
Residual lignins			
Kraft			
Kappa 20	2400	4300	1.78
Kappa 15	2400	3500	1.47
Soda-AQ			
Kappa 50	2500	10600	4.25
Kappa 35	2500	8300	3.30
Kappa 20	2700	4800	1.76
Kappa 15	2700	4200	1.54
Soda-O2			
Kappa 50	2400	9400	3.93
Kappa 35	2400	6100	2.58
Kappa 15	2300	3600	1.58
Black liquors			
Kraft			
Kappa 20	1700	2400	1.42
Kappa 15	1600	2300	1.39
Soda-AQ			
Kappa20	1700	2500	1.53
Kappa 15	1600	2300	1.48

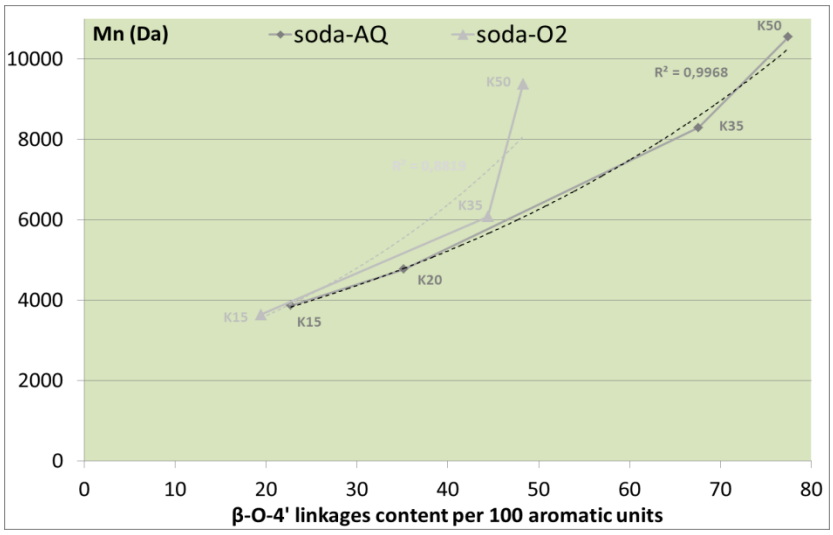


Figure 55. Correlation between residual lignin molar mass (Mw) and the content of β-O-4 linkages.

Task 3.4. Analysis of black liquors and other side streams

The lignins precipitated from the black liquors of eucalypt wood G1xUGL from the kraft and soda-AQ processes, at kappa 20 and kappa 15, were also analyzed by 2D-NMR (see Fig. 56). The quantitation of the main inter-unit linkages and lignin units is shown in Table 26. It is clear from the NMR spectra that these precipitated lignins are enriched in β–β resinol structures, while the other linkages (β–*O*–4 alkyl-aryl ether and β–5 phenylcoumarans), if present, are in much lower amounts. An increase of the S/G ratio is observed, that indicates that S-lignin units, which are predominantly forming β–*O*–4 alkyl-aryl ether structures, are preferentially removed from the eucalypt during pulping and are being enriched in the black liquors. It is interesting to note that the lignins from soda-AQ process are more enriched in S-lignin units that the lignins from kraft process. In addition, while minor amounts of β–*O*–4 alkyl-aryl ether structures are still present in the lignins from kraft process, they were completely absent in the lignins from soda-AQ process. Therefore and as already observed in the analysis of the residual lignins from these pulps, the comparison between the kraft and soda-AQ process indicates that the latter process seems to be more efficient to depolymerize the lignin from eucalypt wood than the kraft process.

Table 26**.** Main lignin structural characteristics (linkages per 100 aromatic units, and S/G ratio) of the lignins precipitated from the black liquors produced from eucalypt G1xUGL after kraft and soda-AQ processes at kappa 20 and 15. The composition of the MWL isolated from eucalypt hybrid G1xUGL is shown for comparison.

Linkages (per 100 aromatic units)	MWL	Kraft process		Soda-AQ process	
		Kappa 20	Kappa 15	Kappa 20	Kappa 15
β–*O*–4 alkyl-aryl ether	90.0	1.0	0.0	0.0	0.0
β–β resinols	10.5	9.7	9.8	9.4	9.3
β–5 phenylcoumarans	5.3	0.0	0.0	0.0	0.0
S/G ratio	2.8	8.9	8.2	8.9	8.5

On the other hand, the lignins precipitated from the black liquors from the pulping of elephant grass by the kraft and soda-AQ processes at kappa 20 and kappa 15 were also studied by 2D-NMR (in HSQC experiments). A quantitation of the abundance of the main lignin inter-unit linkages present in the lignins precipitated from the different black liquors, as well as the abundance of the H, G and S lignin units and *p*-coumaric acid, and the percentage of acylation of the γ-carbon, was performed by integration of the volume contours of their cross-signals and was referred to as per 100 aromatic units (Table 27). The composition of the lignins precipitated from the black liquors was completely different from that of the MWL. First of all, the *p*-coumarate esters in the γ-carbon have been completely hydrolyzed, and the *p*-coumaric acid has been liberated. However, only in the black liquor from kraft kappa 20, important amounts of *p*-coumaric acid could be detected. β–*O*–4 linkages are only present in low amounts, and its abundance are reduced with decreasing kappa number due to the more drastic pulping conditions. Comparing kraft and soda-AQ processes, the abundance of β–*O*–4 linkages is lower, in the latest, which seems to indicate that soda-AQ process performs more efficiently in elephant grass, as also occurred with eucalypt woods. Condensed structures, such as β–β resinols and β–5 phenylcoumarans, are present in relatively more abundance in the black liquor compared to the MWL, and especially the resinols, as also occurs in eucalypt wood, despite its very low abundance in the native lignin. Finally, the S/G ratio is higher in the lignins precipitated from the black liquors, which indicates that S-lignin units, that form predominantly β–*O*–4 linkages are preferentially removed during cooking. The higher S/G value observed in the liquors of the soda-AQ process compared to the kraft process is another indication of the better performance of soda-AQ for delignifying elephant grass, as already observed in eucalypt wood.

Table 27. Main lignin structural characteristics (linkages per 100 aromatic units, percentage of g-acylation, S/G and H/G ratios and percentage of *p*-coumaric) of the lignins precipitated from the black liquors produced from elephant grass after kraft and soda-AQ processes at kappa 20 and 15. The composition of the MWL isolated from elephant grass is shown for comparison.

Linkages (per 100 aromatic units)	MWL	Kraft process		Soda-AQ process	
		Kappa 20	Kappa 15	Kappa 20	Kappa 15
β–*O*–4 alkyl-aryl ether	73.0	4.0	0.0	1.0	1.0
β–β resinols	2.4	2.0	1.8	3.0	3.0
β–5 phenylcoumarans	2.9	0.0	0.0	0.0	0.0
% of γ-acylation	39.0	0.0	0.0	0.0	0.0
S/G ratio	1.7	2.0	1.9	2.0	1.9
H/G ratio	0.1	0.3	0.3	0.3	0.3
p-coumaric acid	25.5	12.0	0.0	0.0	0.0

The lignins precipitated from the black liquors were also analysed by Py-GC/MS. The main structural characteristics obtained upon Py-GC/MS are shown in Table 28 (for the case of eucalypt G1xUGL) and in Table 29 (for elephant grass). The data indicate that the lignin in black liquors is completely different from the native lignin and from the residual lignin in the pulp. The lignin in black liquors is highly enriched in S-lignin units (due to the preferential solubilisation of S-lignin units) and depleted in G-units. In addition, the higher amounts of pyrolysis compounds with shorter chain and/or oxidized, indicates that this lignin is heavily degraded and oxidized. In the case of elephant grass, important amounts of H-units, arising from *p*-coumaric acid, can also be observed, indicating that *p*-coumaric acid is present in free from or etherified to the lignin.

Table 28. Main lignin structural characteristics (percentage of H, G and S units, and S/G ratio, and percentage of short-chain pyrolysis products) of lignins precipitated from the black liquors produced from eucalypt G1xUGL after kraft and soda-AQ processes at kappa 20 and 15. The composition of the MWL isolated from eucalypt hybrid G1xUGL is shown for comparison.

Aromatic units	MWL	Kraft process		Soda-AQ process	
		Kappa 20	Kappa 15	Kappa 20	Kappa 15
H	1.7	1.1	1.9	2.0	2.3
G	33.1	22.9	24.1	26.1	25.7
S	65.2	76.0	74.0	72.0	72.0
S/G ratio	2.0	3.3	3.1	2.8	2.8
% short chains (C_{0-2})	64	94	95	93	93

Table 29. Main lignin structural characteristics (percentage of H, G and S units, and H/G and S/G ratios, and percentage of short-chain pyrolysis products) of the lignins precipitated from the black liquors produced from elephant grass after kraft and soda-AQ processes at kappa 20 and 15. The composition of the MWL isolated from elephant grass is shown for comparison.

Aromatic units	MWL	Kraft process		Soda-AQ process	
		Kappa 20	Kappa 15	Kappa 20	Kappa 15
H	49.8	39.4	41.8	38.9	39.2
G	19.4	27.0	27.5	27.4	28.6
S	30.8	33.8	30.7	33.7	32.2
S/G ratio	1.6	1.4	1.2	1.3	1.2
H/G ratio	2.6	1.5	1.5	1.4	1.4
% short chains (C_{0-2})	86	94	96	95	96

Task 3.4 of WP3: Analysis of black liquors and other side stream

The composition of black liquors and other side streams derived from alkali deconstruction processes (Kraft and Soda-AQ) was characterized in *Task 3.4*. The following characteristics were determined: solid contents, including organic and inorganic, residual effective alkali, heating values and elemental analysis including Na, S, K, Cl, SiO_2, C, H, O and N. The analyses were carried out using elemental analyzer, atomic absorption spectrometer and ion chromatograph. Also studied was the potential of using the black liquor as a source of xylans, since it is known that the Kraft black liquor contains reasonable amounts of high molecular weight xylan.

Black liquor characterization: The material dissolved in the black liquor consist mainly of lignin and degraded carbohydrates (hemicelluloses and cellulose) while the minor part are extractives, proteins and inorganic constituents. Table 30 shows black liquor heating value, total solids, inorganic solids, organic solids and residual alkali. Results of inorganic /organic solids indicate that the eucalypt as well as the elephant grass black liquors obtained by kraft and Soda-AQ processes possess an average of 46% (44.1-52.8%) inorganic matter and 54% (50.2-56.1%) organic matter. Cooking terminated at kappa number twenty resulted black liquors of higher heating value and lower solids content in relation to cooking terminated at kappa 15, indicating less carbohydrate loss in the former case. The Soda-AQ process would be an excellent solution compared to kraft process, since the black liquor from this process presents a higher heating value than the kraft black liquor and is sulphur free. The absence of sulfur compounds in the Soda-AQ black liquor enormously facilitates its further fractionation into valuable components.

Chemically, black liquor is a mixture of several basic elements. The results of Na, S, K, Cl, SiO_2, C, H, O and N are presented in the Table 31 and are expressed in terms of the percentage of the element mass to the total mass of dry solids existing in the liquor. Potassium and chloride are particularly dangerous for their ability to decrease the ash melting point during combustion, thus causing sticky ash problems in recovery boiler systems. In addition, chlorides are highly corrosive and troublesome for most equipment

regardless of metallurgy. The amounts of Cl, SiO_2, C, N, H and O contents were very close among the black liquors derived from woody materials. Kraft cooking terminated at kappa 20 resulted black liquor of lower sulfur content than that of cooking terminated at kappa 15.

Black liquors from elephant grass presented very high contents of potassium and SiO_2, which is very undesirable in most industrial processes for their ability to cause deposits in equipment during evaporation of liquid streams and combustion of solid streams. In addition, these liquors showed high nitrogen content, probably due to protein presence in the juvenile grasses with intense metabolic activities. The high nitrogen content is not favorable in combustion processes since nitrogen enhances NOx emissions. Another interesting observation is that the black liquors from elephant grass present low carbon content and, consequently, low gross heating values.

Table 30: Heating value, total solids, inorganic solids, organic solids and residual alkali of black liquors derived from Kraft and Soda-AQ cooking of eucalypt and elephant grass, with cooking ending in kappa numbers 15 and 20.

.

		Kappa 15					**Kappa 20**				
		Heating value, cal/g	Total Solids,%	Inorganic Solids,%	Organic Solids, %	Residual AA, g/L NaOH	Heating value, cal/g	Total Solids,%	Inorganic Solids,%	Organic Solids, %	Residual AA, g/L NaOH
Kraft	U1 x U2	3963.1	13.0	46.5	53.5	10.2	3949.6	10.9	45.4	54.6	3.1
	G1 x UGL	3591.7	13.4	44.1	55.9	9.6	3970.6	12.8	49.4	50.6	2.7
	DG x U2	3669.0	12.7	46.3	53.7	9.0	4028.8	11.2	41.4	53.7	3.8
	IP	3657.0	12.5	46.8	53.2	9.4	3920.3	11.0	49.4	50.6	2.6
	IB	3818.5	11.3	48.5	51.5	5.7	3917.6	10.2	43.3	56.7	2.3
	E. Grass	3395.0	12.9	46.0	54.0	5.1	3554.0	10.4	47.5	52.5	4.2
Soda-AQ	U1 x U2	3442.5	13.4	45.4	54.6	22.4	4000.2	13.0	45.2	54.8	12.2
	G1 x UGL	3705.1	13.5	49.7	50.3	20.2	4049.1	11.2	47.6	52.4	10
	DG x U2	3691.3	15.7	46.9	53.1	22.7	4071.3	12.4	43.7	56.3	8.6
	IP	3602.3	13.0	49.8	50.2	18.9	4072.6	11.3	46.1	53.9	7.2
	IB	3748.7	11.7	52.8	47.3	11.8	3851.3	10.3	43.1	56.9	5.3
	E. Grass	3510.5	11.0	46.6	53.4	2.7	3696.0	10.4	43.9	56.1	1.2

Table 31: Elemental analyses of black liquors derived from Kraft and Soda-AQ cooking of eucalypt and elephant grass, with cooking ending in kappa numbers 15 and 20.

		Kappa 15									**Kappa 20**								
Element, %		**Na**	**SiO_2**	**Cl**	**K**	**C**	**H**	**N**	**S**	**O**	**Na**	**SiO_2**	**Cl**	**K**	**C**	**H**	**N**	**S**	**O**
Kraft	U1 x U2	18.1	1.6	0.08	0.08	41.1	3.7	0.1	3.5	33.7	20.7	1.3	0.07	0.08	43.0	3.9	0.1	2.8	34.1
	G1 x UGL	17.8	1.1	0.10	0.07	39.2	3.7	ND	4.5	34.7	17.4	1.4	0.10	0.08	42.0	3.8	0.1	3.6	34.7
	DG x U2	22.3	1.1	0.06	0.09	41.2	3.8	0.1	3.9	34.3	14.6	1.2	0.06	0.07	43.0	3.9	ND	3.3	34.4
	IP	20.5	1.4	0.05	0.06	40.6	3.8	0.1	3.4	34.8	17.9	1.4	0.12	0.07	44.7	4.0	0.1	2.9	34.0
	IB	14.7	1.0	0.06	0.06	41.3	3.9	ND	4.0	35.5	13.8	1.3	0.06	0.07	41.7	3.9	ND	3.1	35.8
	E. Grass	14.9	4.0	1.32	0.33	35.8	3.6	0.5	3.4	34.2	15.4	3.3	1.98	0.50	36.0	3.8	0.6	3.0	34.2
Soda-AQ	U1 x U2	18.3	1.4	0.06	0.06	40.2	3.9	ND	ND	35.2	14.9	1.9	0.07	0.09	44.4	4.0	ND	ND	34.3
	G1 x UGL	17.8	1.0	0.09	0.10	40.5	3.8	ND	ND	35.0	15.3	0.7	0.08	0.07	44.1	4.0	ND	ND	34.9
	DG x U2	17.8	1.4	0.07	0.06	41.9	3.8	ND	ND	35.4	15.6	1.7	0.07	0.06	44.4	3.9	ND	ND	34.5
	IP	17.9	1.2	0.08	0.09	40.4	3.9	0.1	ND	34.7	14.7	1.5	0.76	0.08	44.4	4.0	0.1	ND	34.7
	IB	16.2	1.5	0.07	0.05	43.7	3.8	ND	ND	34.9	16.3	1.6	0.13	0.05	45.6	3.9	ND	ND	35.1
	E. Grass	14.9	3.1	2.60	0.44	38.6	3.9	0.4	ND	35.3	15.4	3.4	2.21	0.56	39.3	4.0	0.4	ND	34.8

Black liquor application studies

Black liquor contains a variety of low molecular weight materials, mainly organic acids, but also contain sizeable amounts of lignin and a small but significant amount of polymeric xylans. Many efforts have been made in the isolation and utilization of the lignin, but not so much has been done on the utilization of the xylans. The aim of this study was developing a novel and practical way of reclaiming the polymeric xylans existing in black liquor streams. The technique involved the addition of black liquor in the oxygen delignification stage under proper conditions for the xylans contained therein to precipitate. The proposed technique is easy to implement on an industrial scale, since most of the infrastructure required for its implementation already exists in most Kraft pulp mills. The technique has the potential of increasing the content of xylans in the pulp by 2%, thus improving yield and pulp quality. The xylans would otherwise be burnt along with the black liquor in the recovery system, but producing very little heat since they are highly oxygenated.

Generation and characterization of pulp and black liquors: Kraft black liquors from *Eucalyptus urograndis* (IP) and *Eucalyptus globulus* were used. The two woods were cooked at 4L/1 kg liquor to wood ratio, 165^{o}C, 90 min to maximum temperature, 60 min at maximum temperature and 35% sulfidity. The kraft pulping results are presented in Table 32, which shows a slightly better performance for the European *Eucalyptus globulus* attributable to its high S/G ratio of 4/1 (Table 2) in comparison with the *IP* clone (2.7/1). The fate of the xylans of the two different woods was determined. The xylans in the pulps were measured according to Wallis et 1996 and the xylans in the black liquors derived from the two woods were extracted according to the procedure described by Teleman et al. (1995). The amount of xylans distributed in the pulps and black liquors are presented in Table 6.

Table 32. Cooking results of the eucalypt clones evaluated.

Wood species	AA, % NaOH	Screen Yield, %	Rejects, %	Viscosity, dm^3/kg	HexA, mmol/kg	Xylans, %	Balck liquor solids, %
European Eucalyptus globulus	17.2	54.5	0.7	1350	63.3	17.4	15.1
Eucalyptus urograndis (IP)	18.9	53.3	0.0	1290	57.4	15.8	15.8

*Kraft conditions: 4L/1 kg liquor to wood, 165^{o}C, 90 minutes to maximum temperature, 60 minutes at maximum temperature and 35% sulfidity.

About 54-56%% of the xylans from the original wood ended up in the pulp and the remaining dissolved in the black liquor either in the polymeric form (9-10%) or degraded (34-38%). No significant differences were observed between the xylan fate in the kraft process for *E. urograndis and E. globulus*. Although only 9-10% of the xylans remained in the polymeric form and were recovered, it is a significant a number if they can be adsorbed back onto the fibers by some ingenious technique. Table 33 shows the molecular weight of the wood, pulp and black liquor xylans. It is noticeable that that the polymeric xylans in the black liquor are smaller than the ones in the original chips and in the kraft pulp, but still valuable if they could be adsorbed back onto the pulp. Since these xylans still contain significant amounts of uronic acids (Table 34), i.e., approximately 1 molar weight unit of uronic acids (4-O-methyl-D-glucuronic acid and hexenuronic acids) per ten xylose units, they present good potential for improving pulp properties in addition to their role in improving process yield.

Table 33. Distribution of Eucalyptus wood xylans in pulp and black liquor after kraft pulping to Kappa 20.

Wood species	Xylans, % of original wood		
	Pulp	Black Liquor	
		Polymeric	Degraded
Eucalyptus globulus	55.7	9.9	34.4
Eucalyptus urograndis (IP)	53.8	8.6	37.6

Table 34. Molecular weight (g.mol-1) of xylans in the wood, kraft pulps and corresponding black liquor after cooking at Kappa 20.

Wood species	**Wood**	**Kraft Pulp**	**Black Liquor**
New *Eucalyptus globulus*	27,160	20,567	15,257
Eucalyptus urograndis (IP)	27,512	20,314	15,322

Table 35. Molar ratios of MeGlcA and HexA/10 xyloses in kappa 20 pulp and corresponding black liquor as measured by 1H NMR.

Wood Species	**Kraft Pulp at Kappa 20**			**Black Liquor at Kappa 20**		
	MeGlcA	HexA	**Total**	MeGlcA	HexA	**Total**
New *Eucalyptus globulus*	0.60	0.47	**1.07**	0.40	0.55	**0.95**
Eucalyptus urograndis (IP)	0.54	0.34	**0.88**	0.28	0.70	**0.98**

MeGlcA: 4-O-methyl-D-glucuronic acid.
HexA: hexenuronic acids.

Reclaiming Xylans from Black liquor in the Oxygen Delignification Stage: The recovery of polymeric xylans from Kraft black liquor has always been a challenge, since it is mixed with a large variety of organic and inorganic materials, including sulfur based ones. Instead of separating the xylans from the black liquor, an approach of redepositing them directly from the black liquor onto the pulp in the oxygen delignification stage was evaluated in this study. Black liquor from *Eucalyptus urograndis* (IP) produced according to Table 32 was added to kraft pulp also produced according to Table 32. The xylan contents of the pulps produced under reference and black liquor assisted processes were 15.2% and 16.4%, respectively. Therefore, about 1.4% of xylans were reclaimed from the black liquor across the oxygen delignification stage, and that reflected in a process yield increase of the same proportion. However, the oxygen delignification performance was negatively affected by the addition of black liquor, mainly the brightness gain and the kappa drop across the stage. This loss of performance was anticipated since black liquor contains lignin, which consumes alkali an oxygen, thus decreasing overall O-stage performance.

Table 36 Results of the black liquor* assisted oxygen delignification** of *Eucalyptus urograndis* kraft pulp***.

Parameter	Reference	Black Liquor Assisted
Kappa number	12.7	13.1
Viscosity, dm^3/kg	1052	1073
Brightness, % ISO	56.1	52.3
Kappa Drop, %	35.5	33.5
Viscosity Drop, dm^3/kg	238	217
Brightness gain, % ISO	19.3	15.5
Xylans, %	**15.2**	**16.4**
Yield, %	**98.1**	**99.5**

*15.8% solids, 45.7% organics, 54.3% inorganics, 8.6% xylans on wood weight.
**10% consistency, 600 kPa, 105°C, 60 min, 20 kg NaOH/adt, 30 kg O_2/adt, 200 kg COD/adt added to the O-stage in the process with black liquor.
***kappa number 9.7, viscosity 1290 dm^3/kg, brightness 36,8% ISO, xylans 15.8%, 5.5 kg COD/odt pulp.

Impact of black liquor application on oxygen delignification stage on bleaching performance: The pulps produced according to Table 36 were bleached with the D-P-D sequence in order to determine the impact of black liquor addition in the O-stage on the chemical consumption during bleaching (Table 37). As anticipated, the pulp produced by the black liquor assisted O-stage required a higher quantity of chlorine dioxide (1.8 kg ClO_2/odt pulp) to reach full brightness in relation to the reference. This is explained by the higher kappa number (0.4 units) and lower brightness (3.8% ISO) of the black liquor treated pulp. The cost related to the additional chlorine dioxide demand must be weighed against the yield gain caused by the xylan deposition. The xylan deposition also resulted in decreased bleached pulp viscosity, which can be explained by the low molecular weight of the xylans in relation to cellulose. The deposited xylans were stable across the bleaching process. The xylans losses across bleaching for the reference and xylans enriched pulps were similar. After bleaching, the xylans content were 14.9% and 16.2% for the pulps produced with conventional and black liquor assisted oxygen delignification stages, respectively.

Table 37. Bleaching results with the sequence D-P-D*, for the pulps produced with conventional and black liquor assisted oxygen delignification stages

Parameter	Reference	Black Liquor Assisted
ClO_2, kg/odt	10.8	12.6
H_2O_2, kg/odt	5	5
NaOH, kg/odt	10.0	11.0
H_2SO_4, kg/odt	3	5
Brightness, % ISO	90.2	90.3
Viscosity, dm^3/kg	798	769
Xylans, %	14.9	16.2
Yield, %	97.9	97.8

*D: 10% consistency, 90°C, 120 min, pH 3, 8 kg ClO_2/odt pulp; P: 10% consistency, 80°C, 90 min, pH 11, 10-11 kg NaOH/odt pulp, 5 kg H_2O_2/odt pulp; 10% consistency, 80°C, 120 min, pH 5.5, 2.8 and 4.6 kg ClO_2/odt pulp for reference and black liquor assisted, respectively,

Black Liquor studies by Suzano

Analysis done for this task: kraft and Soda-AQ process black liquors. This characterization was done for each material at each process: Elementary analysis, lower heating value, solids content, organic and

inorganic content, Cl, K, Na, Mg, P, SiO_2, residual alkali and pH. The main kappa numbers were chosen for each process, according to the chosen process and material in each case. For the alternative process developed in the project, also the relevant side streams were analyzed. The same analysis was performed (Elementary analysis, lower heating value, solids content, organic and inorganic content, Cl, K, Na, Mg, P, SiO_2, residual alkali and pH), and some additional analysis was included to complete the entire characterization of each stream (viscosity, S/G ratio, for instance). In each case it was defined after receive the sample for each partner.

The organic composition includes alkali lignin, hydroxy acids extractives, acetic acid, formic acid, methanol, etc. The elemental analyses was carried out using elemental analyzer, atomic absorption spectrometer and ion chromatograph. The organic compounds were measured by GC and py-GC-MS. A number of standard procedures for paper pulp and residual liquor analysis were used including the ones listed below:

Table 38: List of methodologies utilized in the side streams analysis

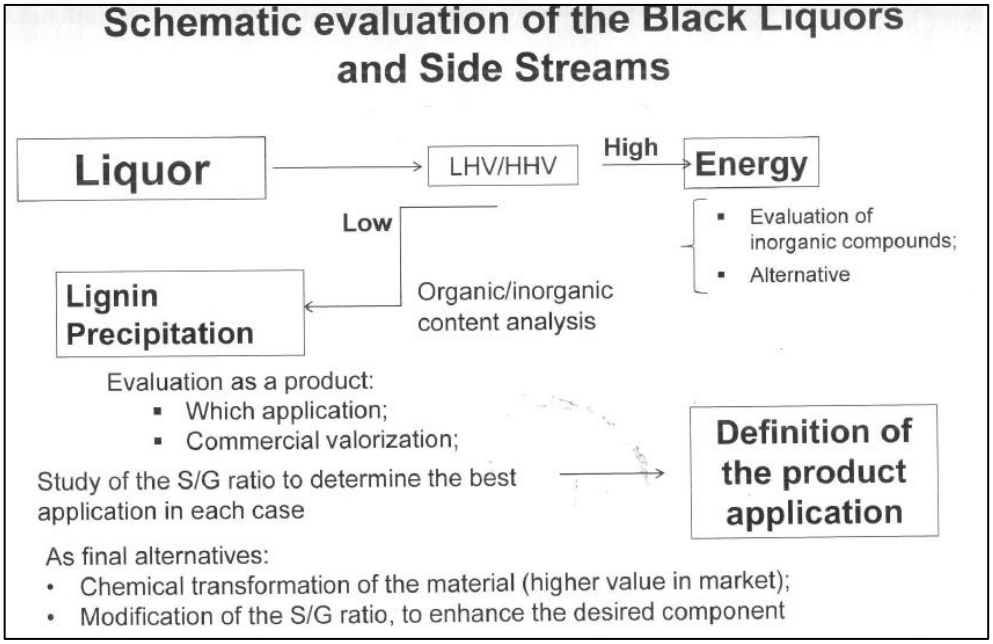
Analysis	**Methodology**
Black liquor chloride content	Tappi T699 om-87
Black liquor residual alkali	Tappi T625 cm-85
Black liquor sodium content	Tappi T25 cm-85 (adaptation)
Black liquor sulphur content	Tappi 625 cm-85
Black liquor chloride contente	Tappi T699 om-87
Black liquor residual álcali	Tappi T625 cm-85
Ash	Tappi T211
SiO2	Tappi T 244
Metals	SCAN CM 38:96
Wood/black liquor potassium content	Atomic Absorption Spectroscopy
Wood/black liquor high heating value	Tappi T684 om-97
Wood/black liquor elemental analysis	Direct measurement in CHNO equipment - dry samples

Based on the selected process, we defined which side streams would be important to be analyzed. The criterion utilized was the amount of the side stream produced and relevance in the process. Also, as a comparison, a characterization of the Suzano kraft liquor and kraft lignin was done. The main reason for this choice was to have a good reference of a consolidated process. So was chosen a side stream that is well known and very much utilized.

Selected material:

- ✓ Liquor NaOH+O_2, *globulus*, kappa 35
- ✓ Soda-AQ Liquor, Elephant Grass, kappa 20
- ✓ Kraft Liquor, Elephant Grass, kappa 20
- ✓ Organosolv Lignin
- ✓ Suzano kraft Lignin, *eucalyptus*, kappa 17
- ✓ Suzano Kraft Liquor, *eucalyptus*, kappa 17

Figure 56: Schematic evaluation of the Side streams: the strategy utilized in the project.



The figure 56 summarizes the way analyzed to define the best use and application for each stream. The division for each use was defined based on the heating value of each sample: if the sample provides a high heating value, it could be a good option for energy purposes. A more complete analysis was done to evaluate the inorganic content. This is important to understand any possible impact in the boiler operation, for instance, as chloride and potassium, which were known for its bad effect at boilers. Otherwise, if the high heating value is not high enough, a detailed analysis of the organic compounds was done. With that, could be understood more its characteristics and the value of the side stream as a product.

To have a full evaluation of the processes, a complete analysis of the compounds of each side stream was done either way: even if the liquor or lignin is good for energy, would be important to evaluate all the main elements and understand if any could cause a bad impact in a boiler, for instance,.

A deeper analysis is the study of the S/G ratio of the lignin, and with that could be defined the product application. As Suzano experience, the S/G ratio could determine the possible application and the value of the final product.

The understanding of the main characteristics of each side stream, and it main destination, as a product development or for energy purpose already fulfill the requirements of the Lignodeco project and complete the task and the deliverables related to this subject. It was also understood that the complete understand of the process was done, and all impacts were considered. With all the information provided by the Lignodeco study, would be possible to recommend the best process available for each purpose without doubt.

Table 39: High Heating Value of the selected Liquor samples. Lignin content in this sample

Sample Code		**High Heating Value, MJ/kg**	**Lignin, %**
Suzano liquor – kraft process	A	14,7	37,5
	B	14,5	38,1
Average		**14,6**	**37,8**
Black Liquor EG Kraft Kappa 20	A	14,8	38,9
	B	14,9	38,4
Average		**14,9**	**38,7**
Black Liquor EG Soda-AQ Kappa 20	A	16,8	42,1
	B	17,1	41,8
Average		**16,9**	**42,0**
Black Liquor NaOH + O_2 kappa 35	A	11,6	30,8
	B	11,6	30,2
Average		**11,6**	**30,5**

The best liquor for energy purpose, considering the high heating value, is the EG Soda-AQ. This could be explained for the high lignin content, which provides a better HHV. The black liquor from the kraft process, for both materials, Elephant grass and *eucalyptus* from Suzano, results in almost the same HHV. Also the lignin content is very similar, with a slightly higher lignin content for the EG.
The kraft liquor is a consolidated product to generate energy. The use of EG for this purpose in not a reality already, but it is shown that is a good possibility.

Table 40: Solid, Organic and Inorganic content of Liquor samples.

Liquor Samples	Solid Content, %	Organic Content, %	Inorganic Content, %
NaOH+O_2, *globulus*	11,7	45,8	54,2
Suzano Kraft	14,5	51,2	48,8

The lower solid content and higher inorganic content of the NaOH+O_2 explains its lower high heating value, in MJ/kg. This also would impact in higher recovery and recausticizing areas in a mill running in a NaOH+O_2 process.

Kraft Liquor with low kappa number is more suitable to be concentrated and burned then liquors from NaOH + O_2 process. This also confirms the high heating values from the table 5. So at this point also the Kraft Liquor is more suitable for energy purposes than NaOH+O_2 process.

Table 41: High and Low heating value of lignin samples.

Lignin Samples	LHV, MJ/kg	HHV, MJ/kg
Organosolv	23,3	24,8
Suzano Kraft	22,3	23,3

Results for already concentrated lignins. The Organosolv lignin presents a higher HHV than the kraft lignin. Comparing to the concentrated kraft liquor, which HHV is 14,5 MJ/kg, the lignin HHV is very good, with 60% higher number. We should not forget that 14,5 MJ/kg is already considered a good and viable number, as the Kraft process is a reality in large scale.
Considering only the comparison between numbers, this Organosolv lignin is better than the kraft one for energy purposes. Considering also the production cost of the lignin, the Organosolv process is more expensive than the kraft process, so this one is more suitable to be burnt.

Table 42: Elemental Analysis

Sample code	**Elemental Analysis, % BLS**						
	C	**H**	**N**	**O**	**S**		
					Total	**Unbound Sulfur**	**Bound Sulfur**
Black Liquor EG Kraft Kappa 20	37,1	3,7	0,7	36,0	2,9	1,95	0,95
	37,3	3,9	0,7	36,2	3,0	1,91	1,09
Average	**37,2**	**3,8**	**0,7**	**36,1**	**3,0**	**1,93**	**1,02**
Black Liquor EG Soda-AQ Kappa 20	39,0	3,9	0,4	37,5	1,0	0,52	0,48
	39,0	4,0	0,3	37,4	1,1	0,55	0,55
Average	**39,0**	**4,0**	**0,4**	**37,5**	**1,1**	**0,53**	**0,52**
Organosolv Lignin	67,9	5,6	0,2	25,2	0,1	0,03	0,07
	67,7	5,6	0,2	25,4	0,1	0,04	0,06
Average	**67,8**	**5,6**	**0,2**	**25,3**	**0,1**	**0,03**	**0,07**

Organosolv process gives a lignin with low sulfur content. If we compare with kraft process, it would have more sulfur, as sulfur is one of the raw materials applied in the digester.

Considering the elemental analysis of the black liquor, they are very similar for the kraft and soda-AQ process. The main difference is the sulfur content, which obviously is present in the kraft black liquor in a higher amount. he comparison between black liquor and lignin is unfair: a concentrated material would present a HHV than a diluted one.

Table 43: SG ratio of the lignin samples

Lignin Samples	SG ratio
Organosolv	2,54
Suzano Kraft	2,55

Considering only the comparison between numbers, this Organosolv lignin is better than the kraft one for energy purposes. Considering also the production cost of the lignin, the Organosolv process is more expensive than the kraft process, so this one is more suitable to be burnt. More results were done, but none in particular were shown to be a problem in the process of the side streams. The complete results are presented in the Appendix.

Conclusions

Cellulose crystallinity was not shown to correlate with hydrolysability of LGF and alkaline pulps. Other factors, *e.g.* lignin and xylan content seem to affect more. Structural changes in lignin revealed that also in LGF cooking the syringyl type units are more reactive, and condensation increases during cooking. PA is essential in these reactions. In alkaline cooks, the residual lignin of Soda-O2 pulp was less phenolic, which may restrict the reactivity in following bleaching stages if aimed at paper pulp. Also in this respect the method is more suitable for the bioethanol production. Most phenolic syringyl units were formed in Soda-AQ cooking, suggesting higher reactivity of the pulp lignin. In all the alkaline cooks, the condensation reactions were most extensive at the end of the cook.

The surface distribution of xylan was not even in the fibers of alkaline pulp, and higher xylan contents could be detected by labelling especially in damaged fibres, fibrillated fines and around pits. After reprecipitation of EG xylan, the overall labelling of bulk fibres was increased, and especially in fine fibrils and fibre defects. Some fibres were very evenly and heavily labelled.
The new material was evaluated in the kraft and Soda-AQ process. The results were good in both processes. For the kraft process, the *Eucalyptus globulus* presented a good screened yield, but a higher amount of alkali was necessary, comparing with the previous globulus analyzed. Also for the previous material the screened yield was significantly higher. For the Soda-AQ process, also a higher alkali charge was required to reach the same kappa level, comparing with the previous wood analyzed. The screened yield was lower than expected, with a difference of 4% in some cases, comparing the same kappa number for both wood. With the results obtained, we conclude that this *Eucalyptus globulus* are good to continue the research and finalize the Lignodeco project, even though the previous material performed in a better way. As additional comment, we highlight that we didn't have as much sample as necessary to complete the optimization of these processes. So maybe producing pulp with this *Eucalyptus globulus* in batch process could be optimized and better results will appear.

The delignification curve for this European *Eucalytpus globulus*, to generate data and pulp sample to the partners to continue the study. This was not planned at the beginning, and it was decided to do more trials due to problems with preservation of the wood chips. The side streams were analyzed, giving special attention to the lignin produced. All the materials could be considered for energy purposes, even though some of them present better high heating value, giving a higher energy production. The characterization of the organic and inorganic content of each side stream didn't present any material that should be a concern or could hinder the use and application. Only when we think in a high scale process in continuous looping and closed system the inorganic content could hinder the production and jeopardize the investment.Lignin could be used in several applications, and its sustainable source is only one of the good aspects of it as a product. Also its properties provide several applications and can have a good value as a commercial product. A further study would be the chemical transformation of the material. For instance, the modification of the S/G ratio, in order to enhance the desired component and customize the side stream as a desired product. An approach like this was not done, but stay as a suggestion for further developments.

Different pulping processes and raw materials result in black liquors with distinct chemical compositions that must be considered upon their utilization. The main findings on black liquor characterization were: (1) the black liquor sulfur content is lower for the pulp produced at kappa 20 than at kappa 15; (2) the black liquor gross heating value is superior for pulps produced at kappa 20 than at kappa 15; (3) the gross heating value of black liquor derived from Soda-AQ process is higher than that derived from kraft process; (4) the black liquor derived from soda-AQ pulping of elephant grass has lower heating value than that from wood at a given kappa number; (5) Black liquor derived from pulping of elephant grass has lower carbon content and unusually high nitrogen content when compared to wood black liquor; (6) when cooking eucalyptus wood to kappa 20, a small but significant amount (8-10%) of xylans of reasonable MW remains in the black liquor; (7) recovery of such xylans (~1.5% on pulp weight) is possible by adding black liquor to the oxygen delignification stage (O-stage) resulting in yield gain of

the same proportion; (8) Such practice impairs the O-stage performance only slightly but impairs bleaching significantly (+2 kg ClO_2/odt); (9) yield and pulp quality gains must be weighed against increased ClO_2 demand for pulp bleaching.

3.1.4. Progress on WP4. Tie between pre-treatment and industrial use of lignocellulosics

Task4.1: Pulp characteristics and papermaking evaluation

Morphological characteristics of the bleached eucalyptus pulps were studied in order to determine which eucalyptus pulp had the best morphological characteristics and to determine the influence of bleaching after the different cooking processes. Cooking process (Soda-AQ or Kraft) and kappa number of unbleached pulp (20 or 15) did not seem to have impact on mean area-weighted fibre length, mean fibre curl index, broken fibres content, fines content of the bleached pulps. On the other hand, vessels content was lower after Soda-AQ cooking than after Kraft cooking and a higher delignification of unbleached pulp (reduction of kappa number 20 to 15) induced (i) a reduction of mean fibre width, the hydrogen bonding potential,(ii) the increase in fibrillation of the bleached fibres and (iii) reduction of vessels content in the bleached pulp.
Consequently, Soda-AQ process seemed to be the best process for the production of special pulp, due to reduction of vessels content in the pulp which could reduce speckles and picking problems during the printing of the paper. However, it was more difficult to determine the best kappa number of the unbleached pulp because the reduction of fibre width, the hydrogen bonding potential should have a negative impact on mechanical properties of the final paper. However, the increase in fibrillation should improve mechanical properties and the reduction of vessels content should decrease speckles and picking problems. *E. globulus* seems to be the best eucalyptus species for pulp manufacture because this pulp contained the longest fibres (this pulp should have the best mechanical properties) and fewer vessels (probably less speckles and picking problems).

After bleaching, morphological characteristics of elephant grass EG1 pulps (such as fibre length, relative bonded area index, fines content and vessels content) were equivalent for each pulp. On the other hand, the reduction of kappa number during cooking reduced the flexibility of the bleached fibres. Consequently, Soda-AQ process seemed as interesting as Kraft process for the production of elephant grass pulp. Bleached pulp manufactured at kappa number 20 should have better mechanical properties than pulp at kappa number 15.
The bleaching of the pulp reduced the fibre length, broken fibres content, the hydrogen bonding potential, flexibility of the fibres, vessels content and increased fines content.

Enzymatic modification of unconventional eucalyptus kraft and Soda-AQ

A general premise of LignoDeco is the use of unconventional means to produce papermaking fiber from eucalyptus wood. Although favorable from the environmental and/or sustainability standpoint, unconventional “deconstruction” of lignocellulose generally produces inferior papermaking fiber. To enable unconventional deconstruction, enzymatic treatments of the resultant fiber may recover desirable papermaking qualities. Moreover, the unconventionally deconstructed fiber may exhibit altered sensitivity/amenability to enzymatic modification relative to the standard fiber.

14 sets of deconstructed materials were provided for quantitative evaluation of sensitivity towards enzymatic treatments by applying both proven fiber modifying cellulases as well as experimental enzymes. The enzymatic treatments were carried out at pH 7 using a 40 mM Britton & Robinson buffer at 50°C for 2 hours at 5% consistency, followed by an inactivation at 80°C for 30 min. All handsheets were prepared and tested according to Tappi Standard procedure. The results from the physical testing of the handsheets was normalized against their respective controls and can be viewed in Figure 3-59 and the freeness values for the enzymatically treated pulps can be viewed in Figure 60.

Most of the deconstructed materials were readily modified by the cellulase treatments. The prepared handsheets showed improvement in tensile strength in practically every case. Of all eucalyptus species and deconstruction techniques analyzed in these trials, the triple hybrid G1 x UGL, pulped to kappa 15, appeared to be most susceptible to cellulase enhanced tensile strengthening and showed increases from 25-35%. A reduction in the strengthening effect of the cellulases is observed on the handsheets prepared from the kappa 20 pulps which may be caused by a reduced margin for improvements as these pulps had higher initial tensile strength. There is a clear difference between the Cel45A (Fibercare R and U) and Cel7b (NS16081) endoglucanase families: while the increase in tensile strength came at the expense of tear strength for the Cel45A treatments, the Cel7b treatment surprisingly increased both tear and tensile strength of the handsheets.

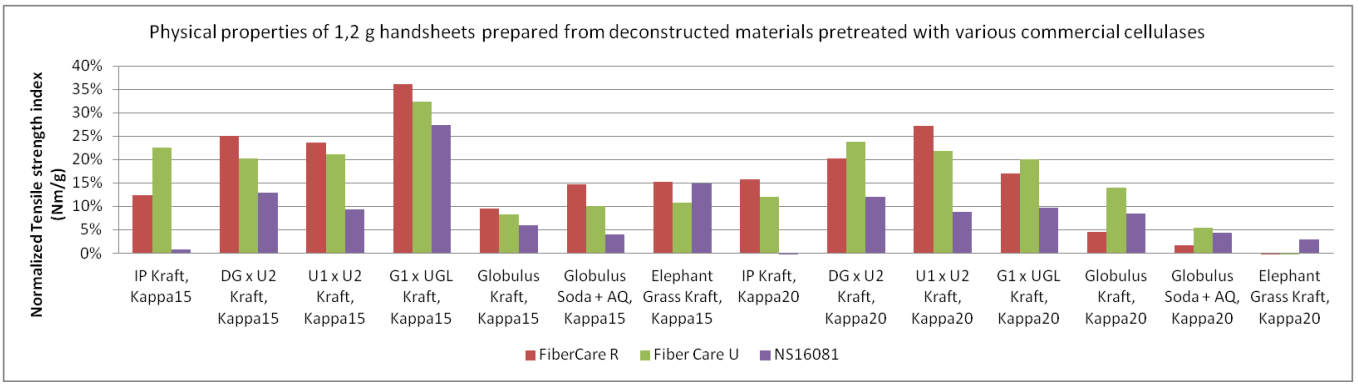


Figure 57**.** Normalized tensile strength data obtained from Tappi standard handsheets prepared from several different types of materials (eucalyptus spp and elephant grass), deconstructed via several different techniques to static Kappa levels of 15 and 20. All data has been normalized against the untreated control for each separate material.

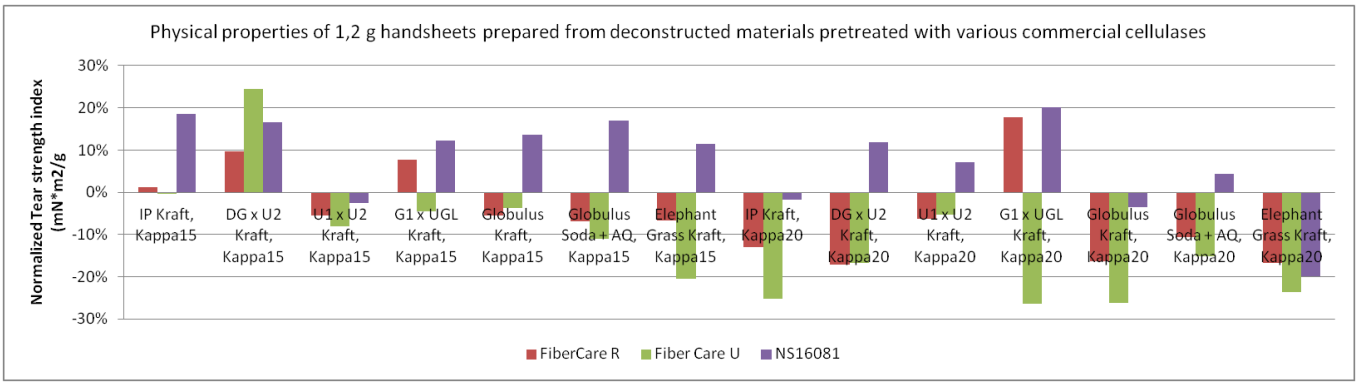


Figure 58. Normalized tear strength data obtained from Tappi standard handsheets prepared from several different types of materials (eucalyptus spp and elephant grass), deconstructed via several different techniques to static Kappa levels of 15 and 20. All data has been normalized against the untreated control for each separate material.

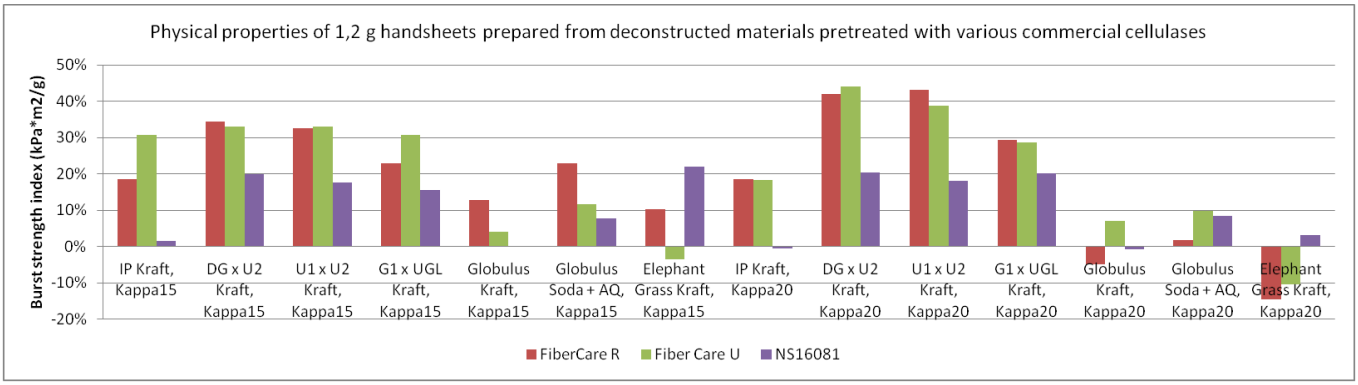


Figure 59. Normalized burst strength data obtained from Tappi standard handsheets prepared from several different types of materials (eucalyptus spp and elephant grass), deconstructed via several different techniques to static Kappa levels of 15 and 20. All data has been normalized against the untreated control for each separate material.

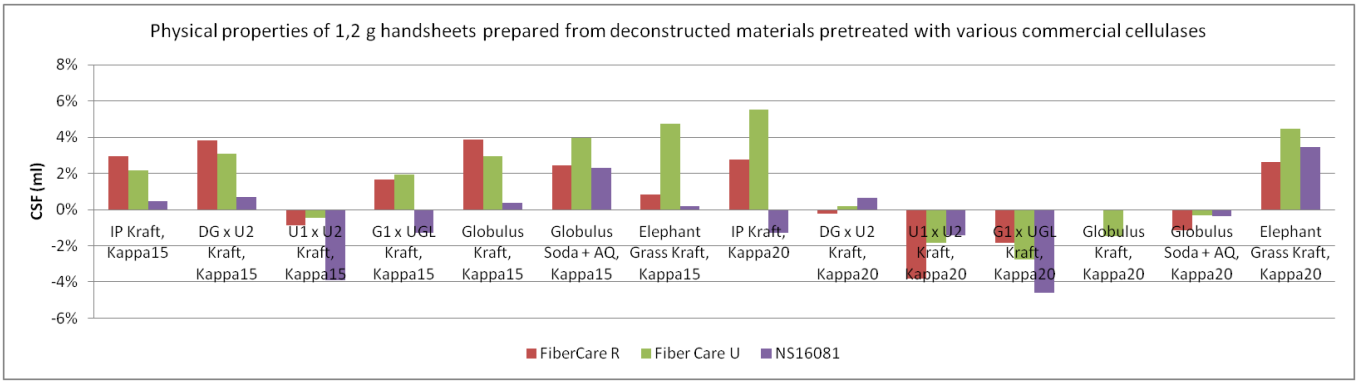


Figure 60**.** Normalized freeness data obtained from several different types of materials (eucalyptus spp and elephant grass), deconstructed via several different techniques to static Kappa levels of 15 and 20 after incubation with commercial cellulases. All data has been normalized against the untreated control for each separate material.

When comparing the different deconstruction techniques (i.e. the kraft and the Soda-AQprocess), it becomes evident that the physical properties of the kraft fiber are superior to those from the Soda-AQprocess with regard to the *E. globulus* (Figure 61). However the enzymatic treatment with Cel45A recovers sufficient strength from the Soda-AQprocess fiber to compete equally with the otherwise stronger kraft fiber.

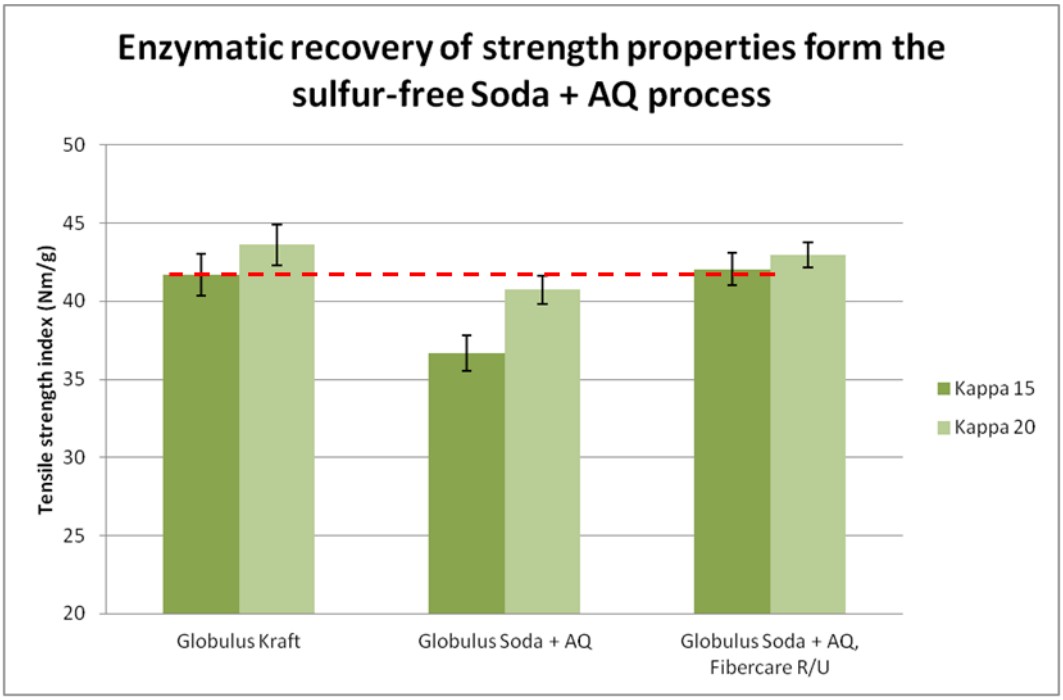


Figure 61. Comparison of tensile strength for the two different pulping processes and the enzymatic recovery of strength properties.

The following describes the evaluation of beatability and enzymatic strengthening during refining for the selected *E. globulus* Soda-AQkappa 20 pulp, where the kappa 15 pulp of same species was also included in order to evaluate which degree of delignification would be most favourable in this context.

The investigated pulps were diluted to 5% consistency (30 odg pulp total) in 40 mM Britton-Robinson buffer pH 7 and added to 1000 ml Lab-O-Mat beakers. Enzyme additions were made and the beaker were sealed and incubated for 120 min at 50°C while rotating in a Lab-O-Mat. After the incubation the enzymes were inactivated at 80°C for 30 min, followed by dilution to 2L and disintegrated for 10.000 revolutions. The pulp was filtrated and diluted to 10% consistency (assuming 30 odg transfer between steps) and distributed equally in PFI mill. Besides the non-refined controls, the pulps were refined for 1000 rev, 2000 rev or 3000 rev. After refining the samples were further diluted to 0,5% consistency and disintegrated for 10.000 revolutions and 60 g/m^2 handsheets were prepared according to Standard Tappi methods according to T-205 sp-95. Freeness measurements were carried out on a Mütek DFR-05 and all physical testing were carried out according to Standard Tappi methods, with a minor deviation in the prescribed climate conditioning of the samples.

The refining curve for the two pulps can be viewed in Figure 8 and shows the °SR freeness as a function of PFI revolutions. From this it is clear the two pulps behave rather similar with respect to increasing refining except at 3000 PFI revolutions.

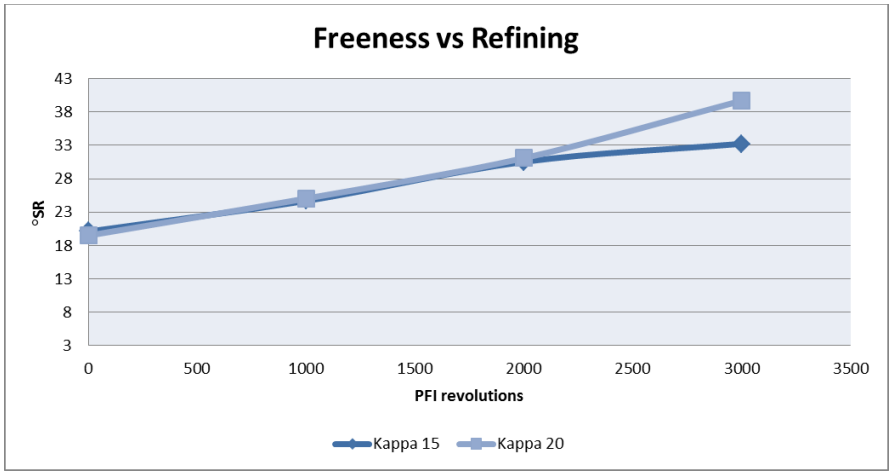


Figure 62. °SR freeness as a function of refining for *E. globulus* Soda-AQpulps at kappa 15 and 20.

The treatment of the pulps with a Cel45A (Fibercare U) gave rise to an improvement in pulp behaviour during the refining, where the pulp drainability is developed rather fast compared to the control. As can be seen in Figure 9 the °SR of the enzymatically treated pulp after 1000 rev corresponds to roughly 2200 rev for the control pulp, and would lead to decreased refining time/energy giving the other pulp properties follows. The major factor influencing the drainability of the pulp is external fibre fibrillation and is caused by the cellulase action on the surface of the fibre, which cuts the cellulose chains without liberating them and hence gives rise to increased fibrillation upon refining for the enzymatically treated pulps. The same behaviour can be seen when the kappa 15 pulp was subjected to refining as can be seen in Figure 10 where an increase of 95% in °SR can be seen after 3000 PFI rev.

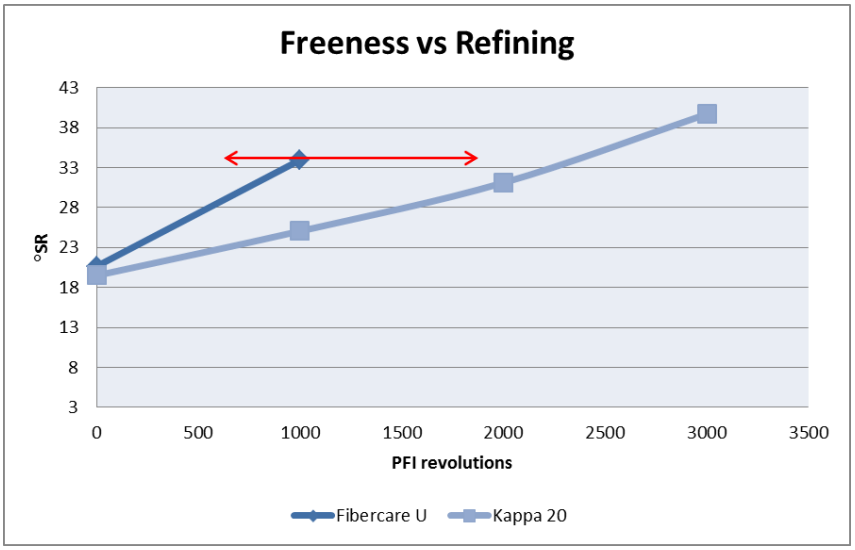


Figure 63. °SR as a function of refining for *E. globulus* Soda-AQpulp at kappa 20 compared to enzymatically treated pulp.

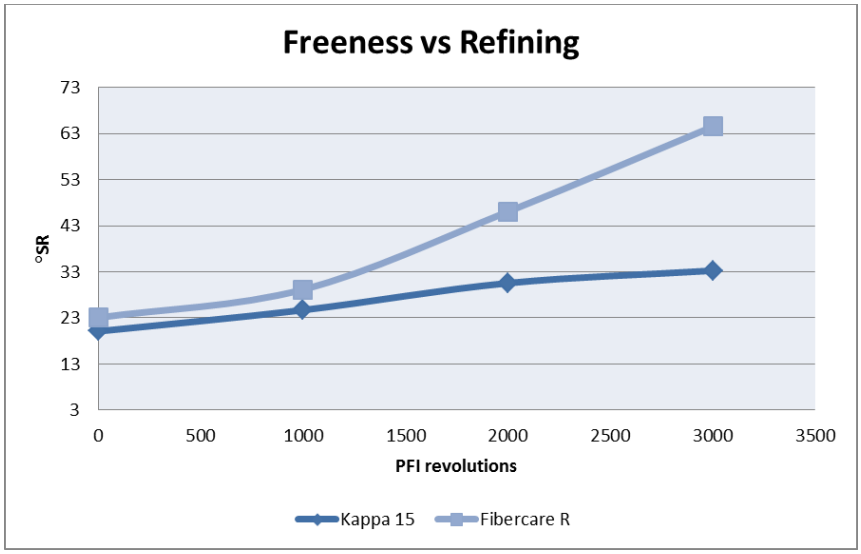


Figure 64. °SR as a function of refining for *E. globulus* Soda-AQpulp at kappa 15 compared to enzymatically treated pulp.

With regards to the physical properties of the refined samples, there does not seem to be any significant difference between kappa 15 and kappa 20 as can be viewed in Figure 11, although the kappa 20 pulp can be refined to obtain a slightly higher tensile strength after 300 PFI rev.

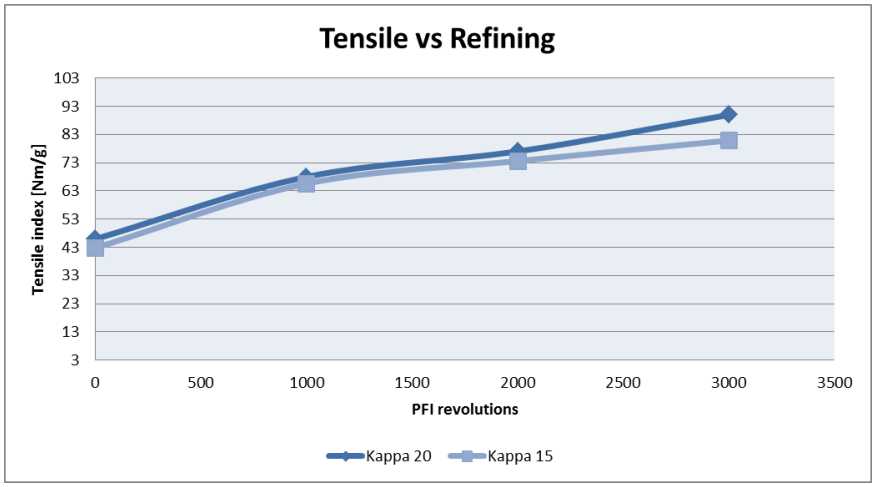


Figure 65. Tensile strength as a function of PFI revolutions for kappa 15 and 20 pulp.

The enzymatic treatment of the kappa 20 pulp revealed slight improvements with regards to tensile strengths of the prepared handsheets as can be seen in Figure 12, both before and after refining at 1000 PFI rev. This increase again indicates increase surface fibrillation which leads to increased fiber-fiber bonding and thus an increase in tensile strength is observed.

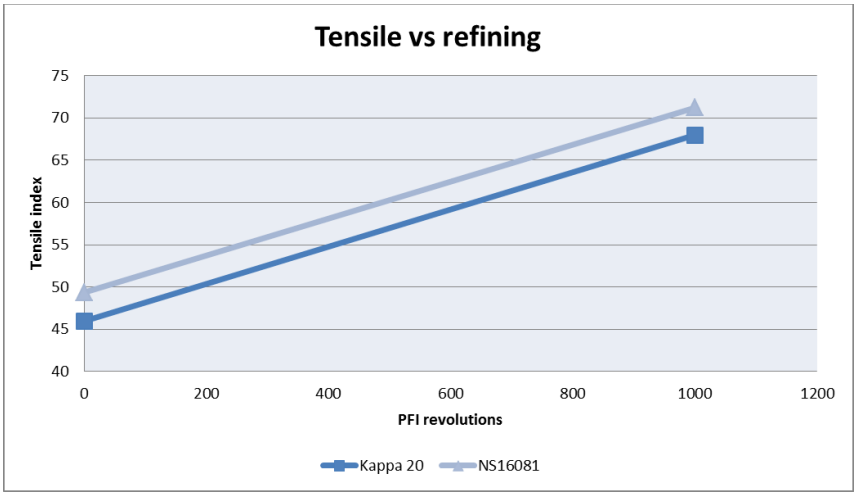


Figure 66**.** Tensile strength improvements as a result of enzymatic treatment with NS16081

The tear strength of the two pulps can be seen in Figure 13 as a function of pulp freeness. When comparing the two pulps there is a clear difference in the behaviour of the pulp during the refining, where the kappa 20 pulp exhibits the highest tear strength of the two at the same freeness level. One would expect to see the curve to drop, giving lower tear strength after a relatively low amount of refining, however this does not seem to be the case here, but may be a result of insufficient refining.

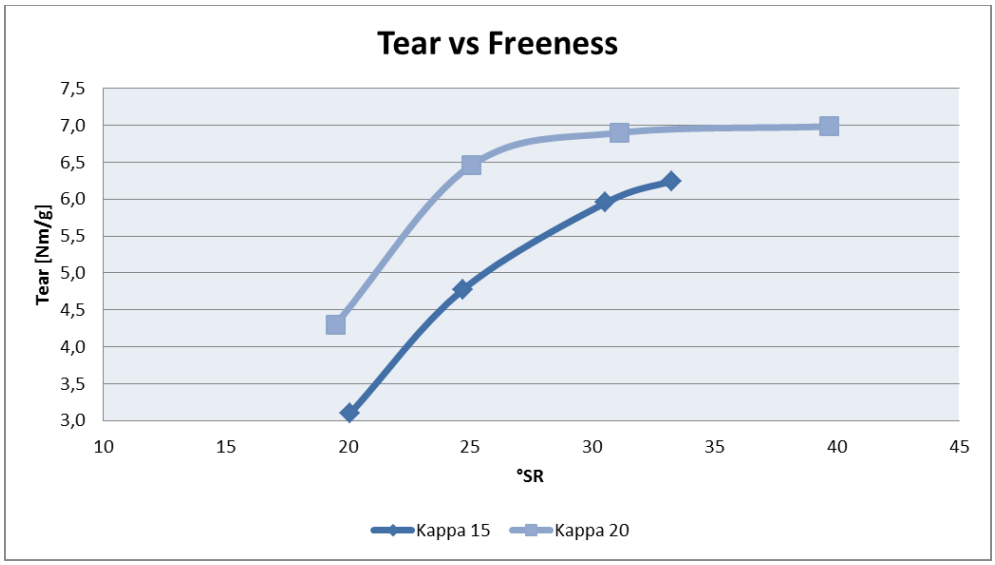


Figure 67. Tear strength as a function of freeness for kappa 15 and kappa 20 pulps.

Normally the enzymatic strengthening effect obtained by cellulase treatments of the pulps on the tensile strength of the prepared handsheets comes at the expense of lower tear strength similar to what increased refining would have. However this does not seem to be case when treating the kappa 20 pulp with NS16081 (Cel7b), where an increase in both tensile strength and tear strength is obtained without refining of the pulp as can be seen in Figure 14. However the tear strength seems to be impaired by the refining at 1000 rev giving lower tear strength for the enzymatically treated pulp compared to the control.

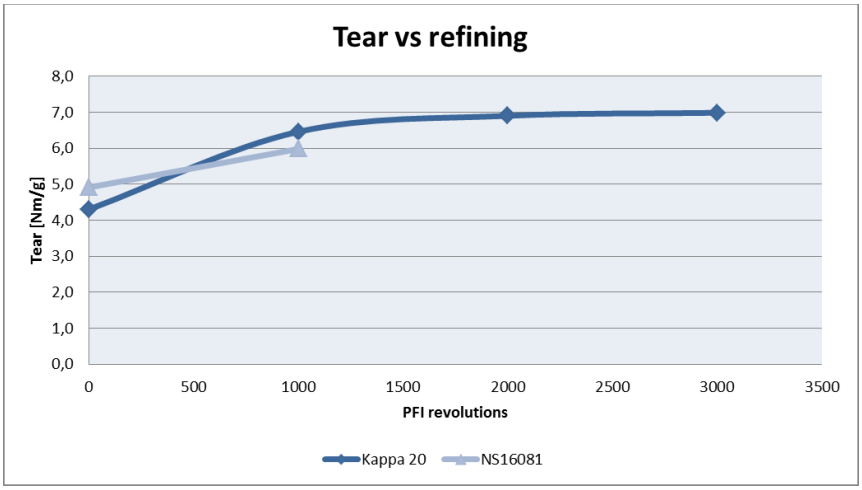


Figure 68. Tear as a function of refining for the kappa 20 pulp and the enzymatically treated pulp.

The above results generally does not indicate any benefits from pulping down to kappa 15, rather the opposite when looking at the higher tear strength of the kappa 20 pulp compared to kappa 15. The data also shows that this unconventional Soda-AQpulp is susceptible to enzymatic modifications which are most evident when looking at the development of freeness during the refining, where the enzymatically treated pulp (kappa 20) shows the same freeness after 1000 rev as the corresponding control at approximately 2200 rev. With regards to the strengthening effect both the tensile and tear strength are improved by the enzymatic treatment, however the effect is lower than would be expected when comparing to conventional *Eucalyptus* kraft pulps, but this is likely due to lack of optimization of the enzymatically treatment itself, and should be further investigated in order to obtain the maximum benefit from the treatments. It is believed that the enzymatic treatment has been too severe on the fibers especially when being refined. Also the development of the tear strength throughout the refining suggests that the revolutions should be further investigated in order to substantiate the results.

Enzymatic delignification using oxidative enzymes (laccase mediator systems)

In T4.1 Novozymes is also tasked with the evaluation of enzymatic delignification technologies based on oxidative enzymes including laccase-mediator systems. Preliminary work has been done within this task including set-up of a medium-throughput small scale bleaching assay which enables the investigation of several oxidoreductases and mediator systems on the deconstructed materials.

Investigations have been conducted in order to evaluate the unconventional Soda-AQpulp to enzymatic delignification with regards to brightness increase. Two different laccases has been investigated with various mediators. A low redox-potential laccase from *Myceliophthora thermophila* (MtL 0,1 g/L) and a high redox potential laccase from *Polyporus pinsitus* (PpL 0,1 g/L) were incubated with and without the methyl syringate (MES, 1mM) (for MTL) and violuric acid (2 mM) and 1-hydroxybenzotriazole (1 mM) (for PpL) followed by an alkaline/peroxide extraction in order to evaluate the bleaching effect of these laccase-mediator systems.

As can be seen in Figure 15 the MtL alone is able to increase the brightness with 3 units, as does the MES itself, and a total of 6 units increase is obtained using the combined treatments, so there does not seem to be any synergistic effect under these conditions. However there is a clear synergistic effect when combining the high-redox laccase with violuric acid, which by themselves does not seem to improve the brightness, but together there is a drastic increase in brightness of 16 units. Keeping in mind that these are preliminary investigations and no optimization has been conducted with regard to concentrations, temperature or time, this is a very promising enzymatic treatment, and shows that the *E. globulus* kappa 20 pulp is very much susceptible to enzymatic delignification by the PpL-violuric acid system and also outperformed the PpL-1-hydroxybenzotriazole treatment (data not shown).

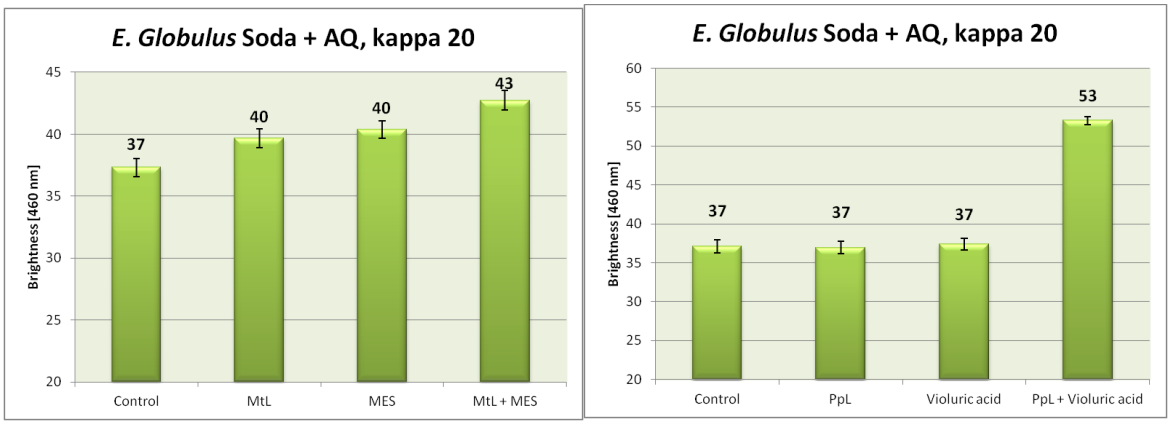


Figure 69. Brightness of the prepared mini-pads after enzymatic bleaching with MtL-Mes and PpL-violuric acid on *E. globulus* kappa 20 pulp. The pulps (60 mg dw in 4 ml) were incubated at pH 6 (50mM phosphate buffer) (MtL) and pH 4,5 (50 mM acetate buffer) (PpL) for 60 min at 50°C with O_2 added via tube, followed by thorough centrifugation and washing. All samples were extracted with 1,1 g/L NaOH and 0,9 g/L H_2O_2. Brightness is measured at 460nm using a Macbeth Color-Eye 7000 Remissions spec.

Another interesting result was made when kappa 15 and kappa 20 pulps were bleached by the PpL-violuric acid system . The enzymatic bleaching system responds equally well on both the kappa 15 and kappa 20 pulps, meaning the absolute values after bleaching and extraction are the same for the two pulps. This validates the further use of the high kappa 20 pulp versus the kappa 15, where one would obtain an increase in pulp yield and cost savings on chemicals as a result.

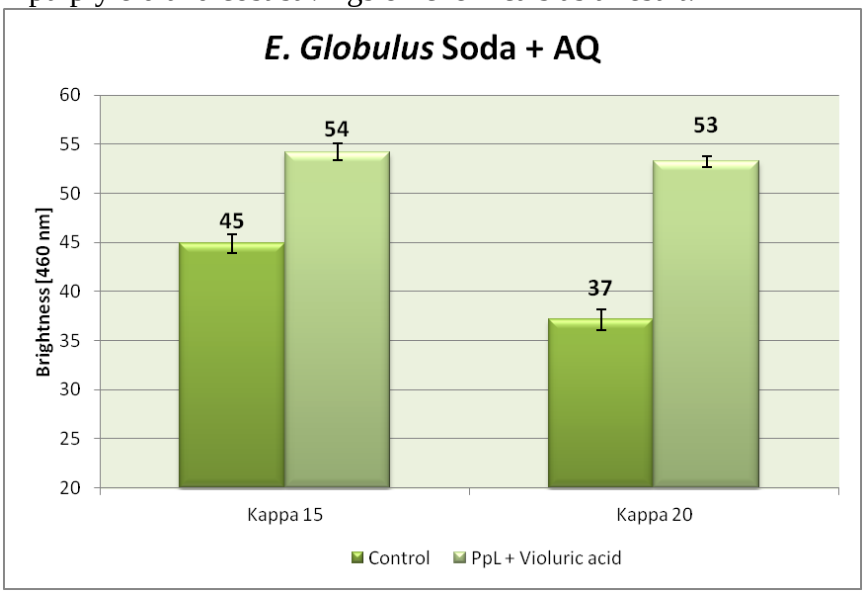


Figure 70. Comparison of brightness for the kappa 15 and kappa 20 pulps after enzymatic bleaching.

The Figure 70 data clearly shows that the unconventional Soda-AQpulps are very much susceptible to enzymatic delignification giving rise to rather large brightness increases. Also the fact that the laccase-mediator system results in the same brightness for the kappa 15 and kappa 20 pulps makes this substrate and the pulping process very interesting in terms of enzymatic bleaching.

The dosages used in the before mentioned experiments carried out with the PpL + violuric acid system were however rather high for this system to be commercially relevant, however interesting from an academic point of view. A dosage/response profile was therefore created in order to evaluate the commercial validity of this system. Figure **17** shows the brightness increase as a function of violuric acid concentration during the enzymatic delignification, and it can be seen that the optimal dosage lies between 3,5 mM and 5 mM violuric acid, which unfortunately is still rather high for an commercial application of this system.

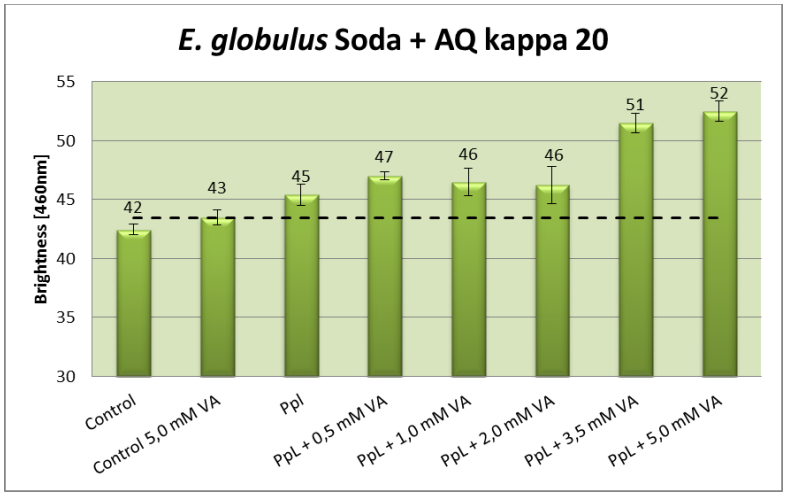


Figure 71. Dose response profile for the PpL + violuric acid system. Error bars represent standard deviations. The black dotted line depicts the high violuric acid control.

Another oxidoreductase + mediator system was investigated which hopefully could perform equally well at lower concentrations. A peroxidase (isolated from *Coprinus cinereus(CiP)*) was chosen for this investigation due to the fact that it had shown promising results in other applications, where it performed very well at low concentrations of both hydrogen peroxide and violuric acid. The results of these investigations can be seen in Figure 18 and compared to the PpL + violuric acid system clearly shows a good performance at very low dosages of both mediator and peroxide. A 6 unit brightness increase is obtained at 0,3 mM violuric acid and 0,5 mM H_2O_2 and an enzyme loading at 170 mg ep/kg dm.

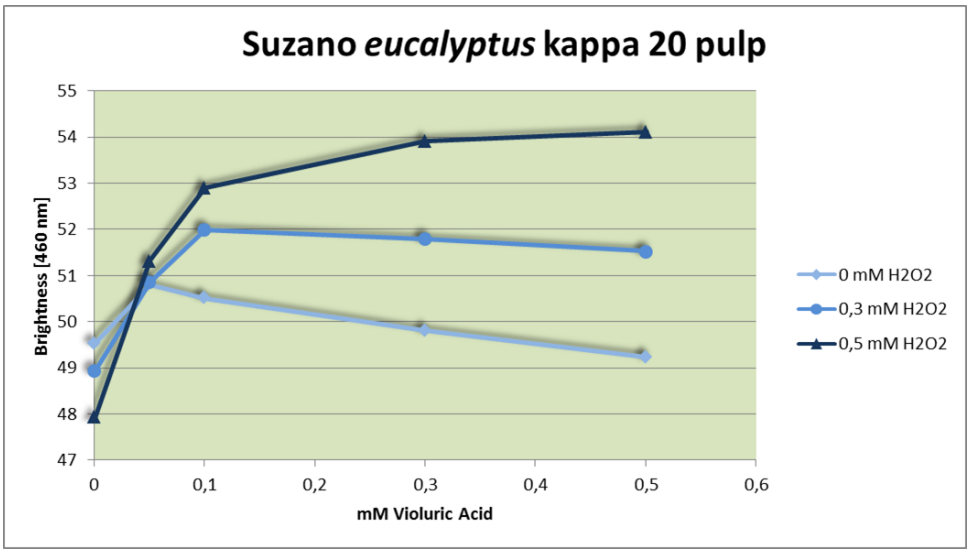


Figure 72. Dose/response of *Coprinus cinereus* peroxidase combined with violuric acid at varying concentrations and at various hydrogen peroxide levels. It should be noted that this experiment is carried out on conventional kraft pulp, and not the soda + AQ.

Similarly the performance of this CiP + violuric acid system was evaluated on the unconventional Soda-AQpulp. Again here the system performed very well at low concentrations as can be seen in Figure 19, where a brightness increase of 7 units is obtained at 0,3 mM violuric acid and 0,5 mM H_2O_2 and an enzyme loading at 170 mg ep/kg dm.

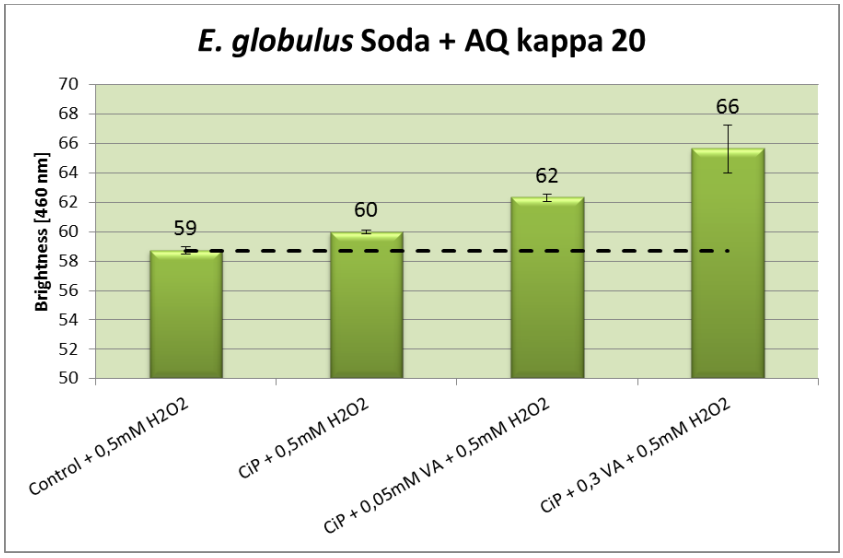


Figure 73. Performance of the peroxidase + violuric acid system on Soda-AQkappa 20 pulp. Error bars represent standard deviations.

Specialty grade pulps; enzymatic benefits in dissolving pulp

Dissolving-grade pulps have specific quality requirements such as high cellulose content, low hemicellulose content and only trace amounts of residual lignin, extractives and inorganics. The increased market demand for dissolving pulp in recent years has result in much interest from many paper-grade pulp manufacturers into the conversion of their production sites into dissolving pulp.

As most paper-grade pulp manufacturers use kraft pulping technology, the pre-hydrolysis step is needed for the production of dissolving pulps. However, opportunities to convert conventional bleached kraft pulp with little effort have emerged via enzymatic treatment of the pulp. Taken into account the research conducted within LignoDeco on the bleachability of the selected eucalypt pulp, we have identified it as a promising substrate for the further processing into a high-value specialty grade pulp.

The paper-grade bleached eucalypt kraft pulp (BEKP) from Suzano was treated with xylanase Pulpzyme HC® (X-stage), also combined with cellulase FiberCare R® (FCR), and followed by either a cold caustic extraction (CCE-stage) or hot-caustic extraction (HCE-stage). As determinant market properties of dissolving pulps, their alkali solubilities at 18% (S18) and 10% (S10) NaOH as well as their intrinsic viscosities were measured according to TAPPI T 235 and ISO 5351 standard procedures, respectivelly.

The results presented in Figure 20 show that the xylanase treatment (BEKP) increased the pulp S18 value (ca. 9% increase). This can be explained higher alkaline solubility of the residual xylan left in the pulp after the xylanase treatment. This residual xylan after the xylanase treatment likely became more degraded (lower molecular weight) and more vulnerable to alkali solubilization thus increasing S18 (1).

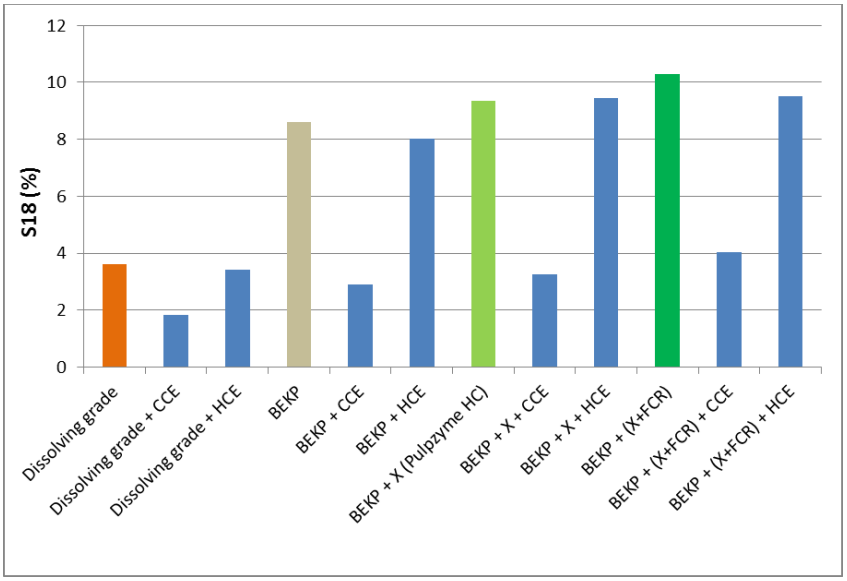


Figure 74. Alkali solubility of pulp at 18% NaOH (S18). X-stage: 0.10% odp Pulpzyme HC (0.05% odp FiberCare R); 60 ºC; pH 7; 120 min. CCE-stage: 70 g/L; 30 ºC; 30 min. HCE-stage: 4 g/L; 90 ºC; 60 min.Trials run at 10% consistency in sealed polyethylene bags imersed in a water bath.

Only after the introduction of a CCE-stage it is possible to reach the same S18-level of a standard dissolving grade pulp being the values slightly higher after the X-stage. However, if more xylan is dissolved there is the potential benefit on pulp reactivity and filterability of the dope in the viscose production process.

As regards the S10-values presented in Figure 21, the same trend is obtained with higher solubilities obtained when a xylanase treatment is included, being the pulp more soluble when combined with a cellulase treatment. The S10 determination comprises the dissolution of hemicelluloses and degraded cellulose being the S10-S18 usually an estimate of low molecular weight (LMW) cellulose (2). The solubility difference reveals that while the cellulase treated pulp have the highest amount of LMW cellulose the xylanase (monocomponent) treated pulps have the lowest after alkaline extraction (X-CCE).

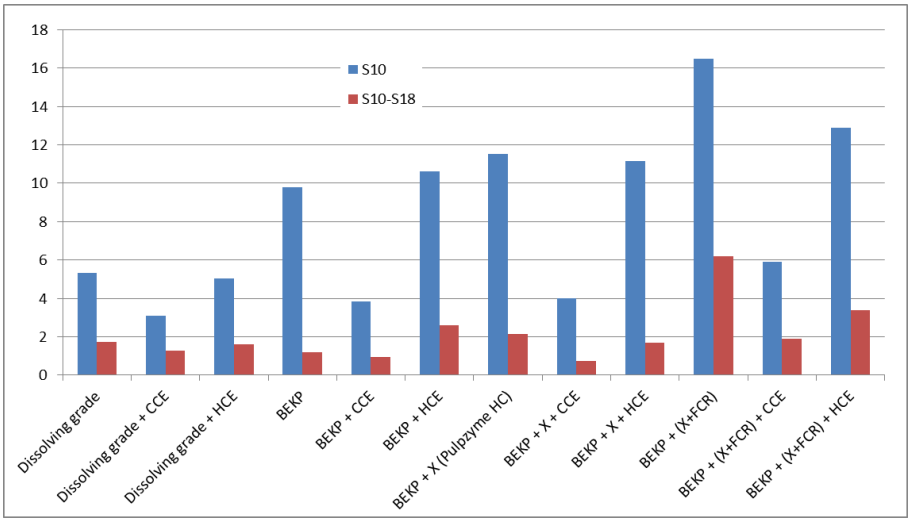


Figure 75. Alkali solubility of pulp at 10% NaOH (S10) and S10-S18 values.

Using a higher dosage of xylanase the same increased alkali solubility (S18) is obtained before and after the CCE-stage with different NaOH dosages (20-70 g/L), as depicted in Figure 22. On the other hand, the intrinsic viscosity values of the xylanase treated pulps are always somewhat higher than the controls

without enzyme. Therefore, the xylanase treatment up-shifted the average degree of polymerization. This indicates that the X-CCE pulps have a lower amount of xylan and that this degraded remainning amount is more soluble at 18% NaOH (higher S18).

To convert a paper-grade pulp into dissolving grade pulp, the intrinsic viscosity shall be reduced. The use of monocomponent endoglucanase preparations have shown in previous studies to be able to adjust the final intrinsic viscosity and at the same time increasing the reactivity of the dissolving pulp for viscose application (1,3,4). Three different monocomponent cellulases (endoglucanases) products were tested regarding the control of pulp intrinsic viscosity. The results shown in Figure 23 reveal that FiberCare R and FiberCare U decreased the intrinisc viscosity to a similar degree while Novozym 613 hardly had any effect even using a higher dosage.

A higher dosage of enzyme leads to higher decrease of pulp viscosity. This decrease is more pronounced with the more pure dissolving pulp: ca. - 200 vs. - 300 mL/kg.

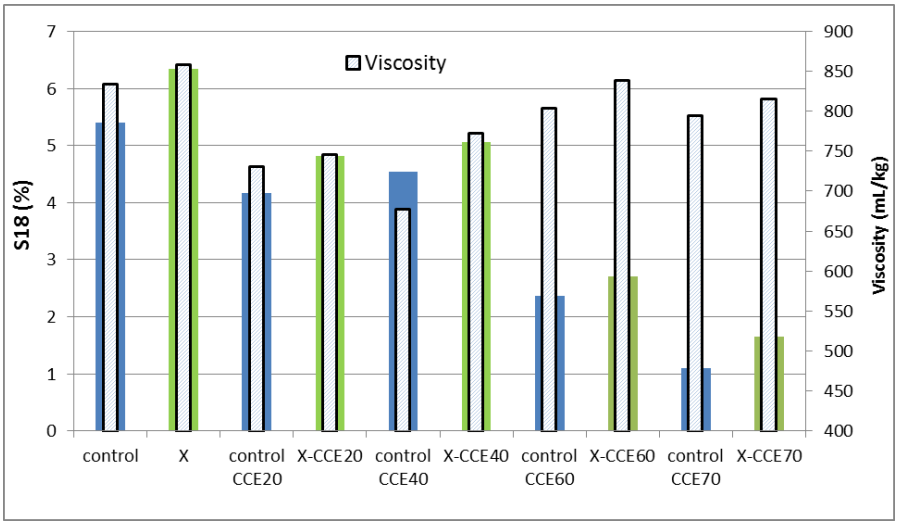


Figure 74. Intrinsic viscosity and alkali solubility of pulp at 18% NaOH (S18). X-stage: 0.30% odp Pulpzyme HC (0.05% odp FiberCare R); 60 ºC; pH 7; 120 min. CCE-stage: 20-70 g/L; 30 ºC; 30 min. Trials run at 10% consistency in sealed polyethylene bags imsersed in a water bath.

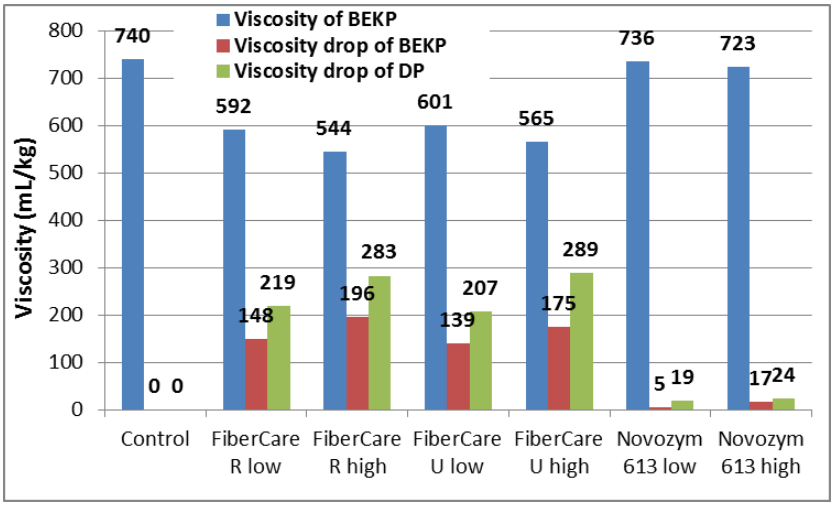


Figure 75. Intrinsic viscosity of pulps. Cellulase treatments at low and high dosage of enzyme (0.02-0.20% odp); pH 7; 60 ºC; 120 min; Trials run at 10% consistency in sealed polyethylene bags imersed in a water bath.

Bleaching of the pretreated materials obtained from alkaline deconstruction:

The elephant grass and eucalypt pulps derived from the alkaline deconstruction at kappa number 15 and 20 were bleached by the elemental chlorine free sequence (ECF) O-D-P-D to 90% ISO brightness. Table 44 presents the characterization of materials after the alkaline deconstruction. The oxygen delignification (O-stage) was run at 10% consistency, 100°C, 60 min, 700 kPa pressure, 20 kg NaOH/odt pulp and 20 kg O_2/odt pulp. The first chlorine dioxide stages (D) were carried out at 10% consistency, end pH 3.5, 85ºC, 120 min, with kappa factor of 0.20 for pulps with kappa number 15 and 0.24 for pulps with kappa number 20. The P stages were run at 10% consistency, end pH 10.5, 85ºC, 120 min, with hydrogen peroxide doses of 0.5% on pulp weight. The second chlorine dioxide stages (D) were carried out at 10% consistency, end pH 5.5, 70ºC and 120 min, with variable chlorine dioxide charges to achieve the desired brightness. The pulp bleachabilities were determined on the basis of the total active chlorine consumption per kappa unit removed across bleaching to a target brightness of 90% ISO. The bleached pulps were evaluated for their brightness, brightness stability and viscosity. All bleaching stages were controlled on the basis of end pH and chemical consumption across the stage so that a minimum oxidant residual was maintained but no excess residuals were accepted.

Table 44. Characteristics of the elephant grass and eucalypt pulps obtained by the Kraft and Soda-AQ processes at kappa 15 and 20, according to task T2.1

		Kappa 15						Kappa 20					
		Bright-ness, % ISO	Visco-sity, dm^3/kg	Glucans	Xylans	Uronic Acids, %	HexA, mmol/kg	Bright-ness, % ISO	Visco-sity, dm^3/kg	Glucans	Xylans	Uronic Acids, %	HexA, mmol/kg
Kraft	U1xU2	33.6	1032	82	13.9	0.6	39.8	31.7	1073	81.2	14.2	1.3	48.8
	G1xUGL	33	1144	81.6	15.4	0.7	41.8	32.2	1193	80.8	15.8	1.2	48.6
	DGxU2	34.2	892	82.2	13.4	0.7	39.1	31.3	1054	81.2	14	1.3	48.9
	IP	34.1	939	81.9	14	0.6	39.5	31.0	1100	80.6	14.9	1.3	46.4
	IB	34.6	990	79.5	17.4	0.8	39.1	31.6	1064	79.3	17	1.1	37.3
	E. Grass	32.9	1100	77.6	18.9	0.7	13.2	31.1	1359	77.7	18.5	0.8	11.1
Soda-AQ	U1xU2	33.3	875	88.1	8.5	0.2	15.8	30.8	919	84.9	11.3	0.5	40.3
	G1xUGL	34	748	86.2	10.4	0.3	21.2	32.1	917	83.9	12.2	0.7	48.2
	DGxU2	34	951	88	8.8	0.3	15.9	30.8	1020	84.2	11.8	0.6	43.4
	IP	33.9	972	87.1	9.6	0.3	218	29.6	1028	84.5	11.2	0.6	45.7
	IB	35.5	990	83.9	12.9	0.7	29.4	34.1	1064	82.1	14.1	1.3	41.6
	E. Grass	31.6	875	80.3	16.5	0.4	15.15	28.5	883	78.1	17.6	0.6	16.3

***Oxygen Delignification Results**:* The pulps produced by two processes (kraft and Soda-AQ) and two kappa numbers (15 and 20) were submitted to oxygen delignification. Table 45 shows the oxygen delignification (O-stage) results. The overall oxygen delignification stage performance was measured by the kappa drop and brightness gain across the O-stage. It was observed that the kappa drop decreased with increasing kappa number, a result likely explained by the decreased content of lignin containing free phenolic hydroxyl groups and increased HexA content with increasing kappa (COLODETTE et al., 2007). Oxygen delignification performance is hampered by the lack of free phenolic hydroxyl groups in the pulp since such groups are the main sites of oxygen reactions during O-stage. On the other hand, HexA groups are resistant to oxygen reactions and little of it is actually removed in the O-stage. Hence, pulps containing large HexA amounts comprising its kappa number will respond poorly to the O-stage (VENTORIM et al, 2006; EIRAS, 2003). This fact explains why the elephant grass pulps showed better performance in the O-stage than the eucalypt ones, as measured by the kappa drop across the stage. The same rationale can be applied to explain why the Soda-AQ pulps performed better in the O-stage than the Kraft ones (Table 45). Another relevant point that helps to explain the higher efficiency of the pulp

obtained by the Soda-AQ process is a possible higher content of free phenolic hydroxyl groups (ZONG LAI (1999), which are the main sites for oxygen reactions (COLODETTE et al., 2007). It is known that in Soda-AQ pulping, AQ oxidizes the reducing end groups of carbohydrates, thus stabilizing them towards peeling reactions in alkaline media. The reduced form, AHQ, cleaves part of the β-aryl ether linkages in lignin. Thus, the molecular mass of the residual lignin is reduced and new phenolic hydroxyl groups are formed. Both effects render the lignin more soluble (KLEEN et al., 2002). Since phenolic hydroxyl groups are essential to lignin dissolution in alkali, a higher content of this functional group in the residual lignin may be partly related to the residual lignin being more condensed. For woody samples, in his work ZONG LAI (1999) showed that the tendency of alkaline lignin condensation reactions would increase in the order of: high sulfidity < kraft < Soda-AQ < soda cooks.

Table 45. Oxygen delignification performance for eucalypt and elephant grass Kraft and Soda-AQ pulps of kappa 15 and 20

		Kappa 15		Kappa 20	
		Kappa drop, %	Brightness gain, % ISO	Kappa drop, %	Brightness gain, % ISO
Kraft	U1xU2	48.4	19.6	46.1	17.7
	G1xUGL	46.2	18.1	46.1	16.5
	DGxU2	46.6	18.4	46.2	17.7
	IP	47.4	20.1	43.8	16.2
	IB	44	19.3	46.3	17.9
	E. Grass	61.9	12	50.9	7.5
Soda-AQ	U1xU2	57.5	18.1	50.5	17.1
	G1xUGL	57	19.8	45.5	15.8
	DGxU2	57.7	18.2	47.8	15.1
	IP	54	18.3	47.3	16.6
	IB	53.3	18	47.4	16.7
	E. Grass	73.7	14.5	69.4	14.7

Bleachability Results: The pulps were bleached aiming brightness of 90% ISO as described in the methodology. For a fair analysis of all pulps, the parameter bleachability was used to compare the behavior of the various pulps across bleaching. In this work bleachability has been defined as the ratio between kappa drop across the bleaching sequence and total active chlorine (TAC) required for attaining the target brightness of 90% ISO. Total active chlorine was defined by the following equation:

$$\mathbf{TAC=[(ClO_2\times 2.63)+(H_2O_2\times 2.09)]}$$

In the equation above, the factors 2.63 and 2.09 are simple conversions of ClO_2 and H_2O_2 into active Cl_2 based on their oxidation equivalents. The parameters TAC, ClO_2 and H_2O_2 are expressed in kg/odt pulp.
For the eucalypt pulps (Fig.76), the highest bleachabilities were achieved with the Kraft pulps of kappa 20 whereas the worst ones were seen for the Soda-AQ pulps of kappa 15. The same trend was observed for the elephant grass pulps. In general, the elephant grass pulps showed lower bleachabilities in relation to the eucalypt ones, a result probably explained by the more condensed nature of the grass residual lignin and their higher transition metal contents in relation to the eucalypt ones.
Among the eucalypt pulps, the highest bleachabilities of the kraft pulps at kappa 20 (0.223 kappa unit/ kg TAC) and 15 (0.193 kappa unit/ kg TAC) were achieved with the G1xUGL clone. On the other hand, for the Soda-AQ pulps the highest bleachabilities at kappa 20 (0.172 kappa unit/ kg TAC) and 15 (0.136

kappa unit/ kg TAC) were attained with the clones IP and G1xUGL, respectively (Figure 2). For the elephant grass, the bleachabilities of the kraft pulps at kappa number 20 and 15, were 0.154 and 0.112 kappa unit/ kg TAC, respectively. On the other hand, bleachabilities values of 0.119 and 0.107 kappa unit/ kg TAC, respectively, were obtained for the Soda-AQ pulps at kappa 20 and 15 (Fig. 77). It is worth noting that the elephant grass used in this was not genetically improved as was the case for the eucalypts. Potential genetical improvements could be achieved aiming at the pulp production, so that the TAC values could be reduced. For example, the transition metal contents of the elephant grass pulps were higher than desired.

It is worth noting that Soda-AQ pulps of kappa 20 produced showed bleachabilities somewhat similar to Kraft pulps of kappa 15. Thus, cooking at kappa 20 could be a practical alternative to implement the Soda-AQ process give its many advantages compared to the Kraft counterpart, particularly in the light of the biorefinery prospects. The Soda-AQ process is highly desirable when the use of black liquor for biorefinery purposes is at stake. The absence of sulfur compounds in the Soda-AQ black liquor enormously facilitates its further fractionation into valuable components.

The total active chlorine (TAC) demands to bleach the pulps to 90% ISO are shown in Figures 78. Although the bleachability of the eucalypt and elephant grass kappa 20 kraft pulps were the highest, their TAC were not the lowest due to the effect of the kappa number value. Actually the lowest TAC was achieved for the eucalypt kraft pulp at kraft kappa 15. In general the highest values of TAC were seen for the Soda-AQ pulps of kappa 20.

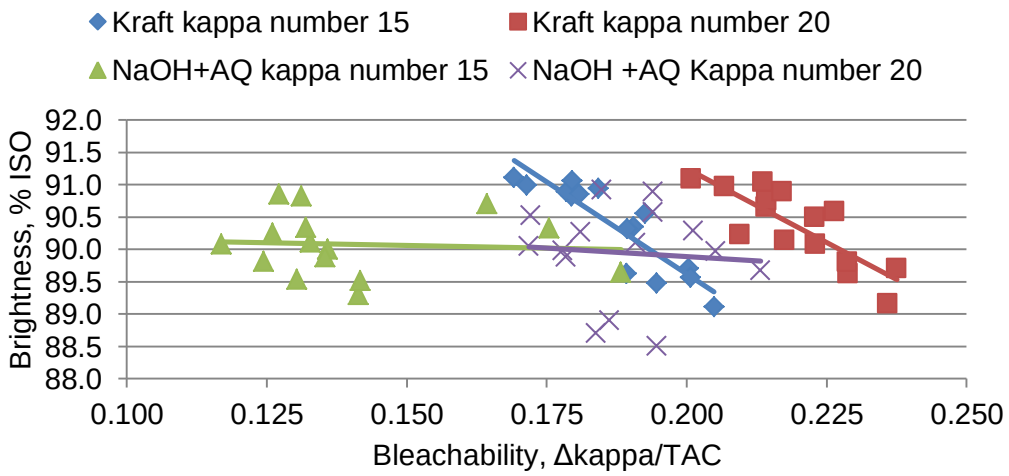


Figure 76. Bleachability (kappa unit/ kg TAC) of eucalypt kraft and Soda-AQ pulps obtained at kappa 15 and 20.

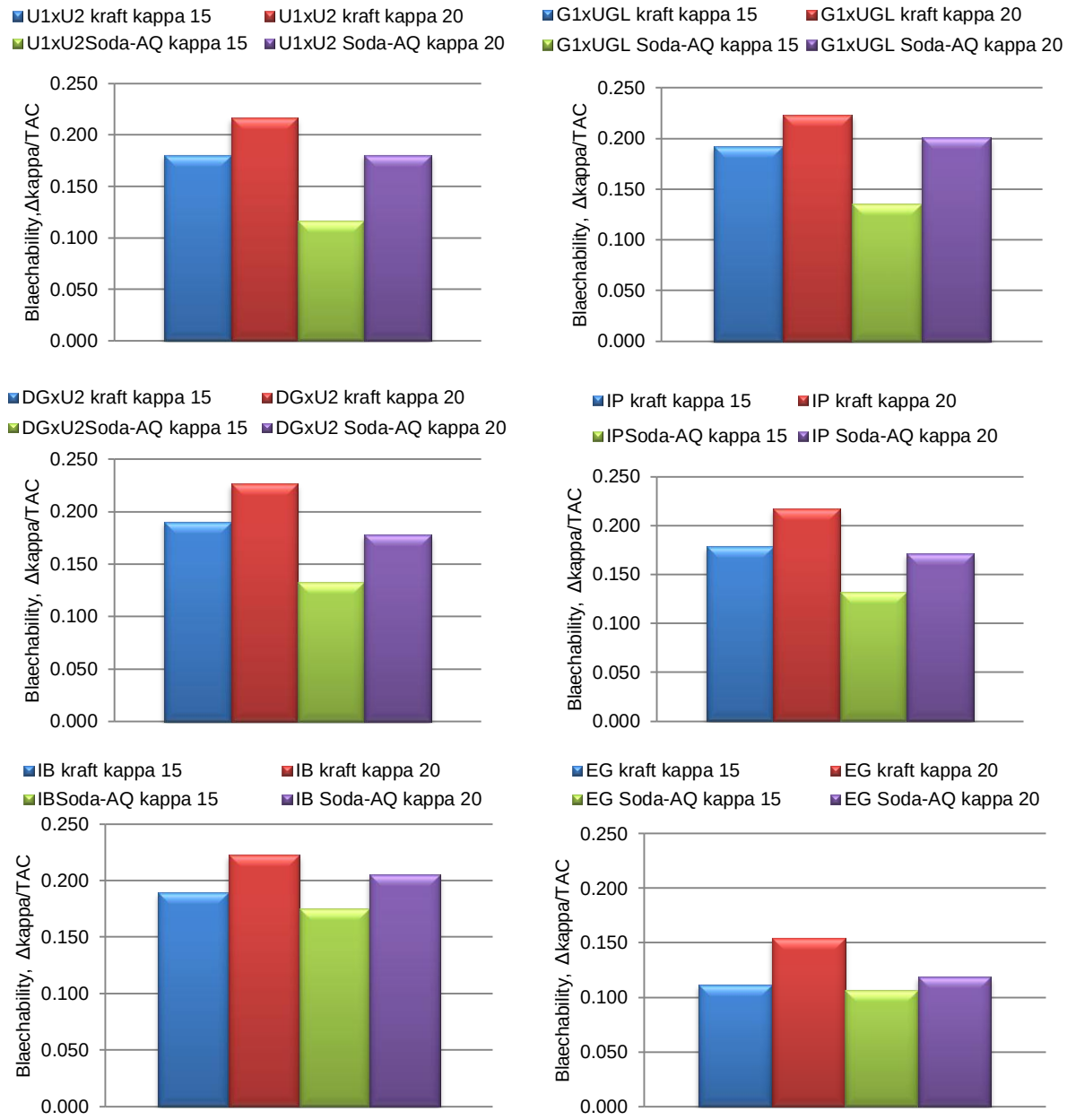


Figure 77. Bleachability (kappa unit/ kg TAC) of bleached pulps from eucalypts and elephant grass obtained by the Kraft and Soda-AQ processes at kappa 20 and 15.

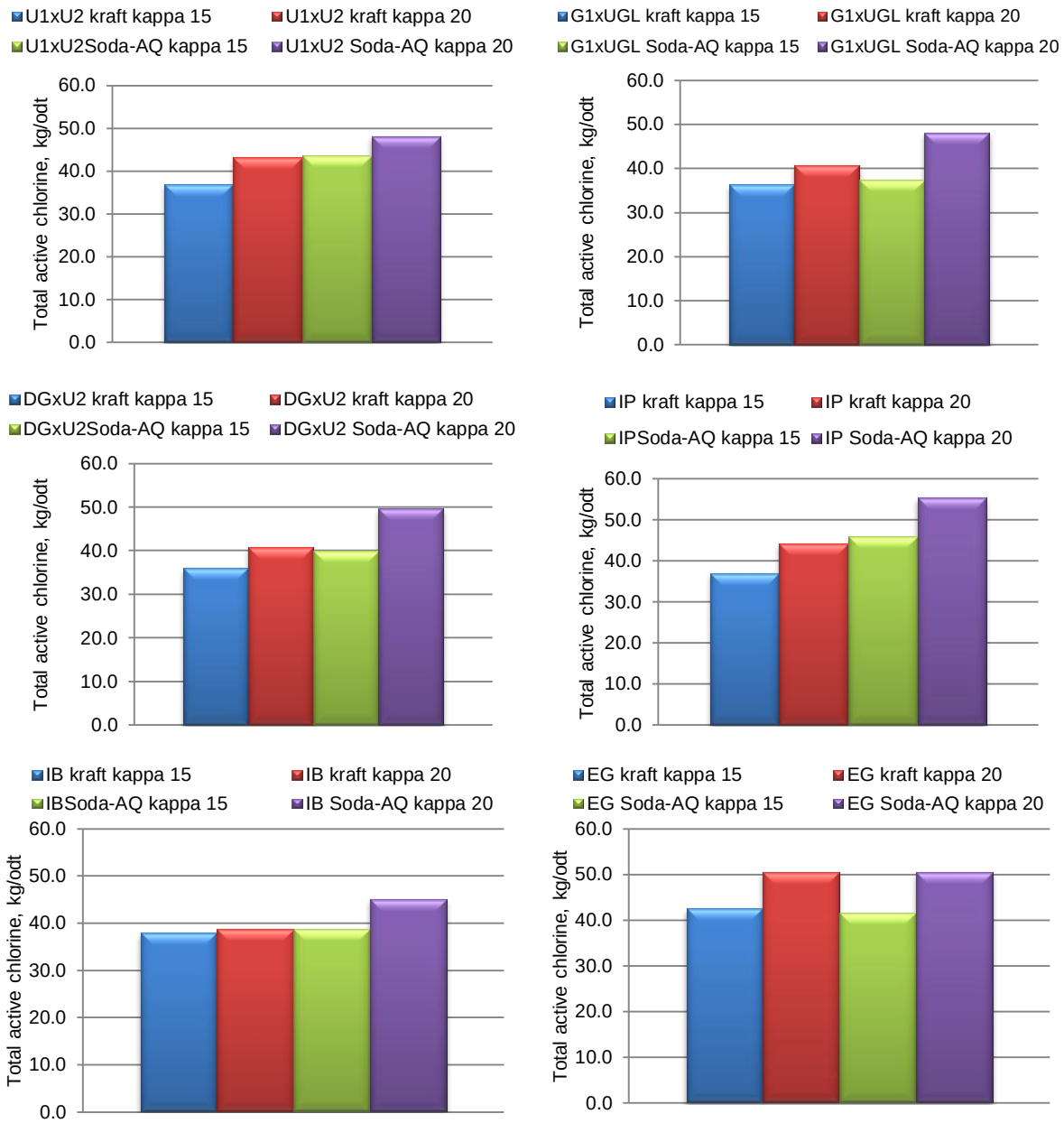


Figure 78. Total active chlorine used in the bleaching of eucalypt and elephant grass kraft and Soda-AQ pulps obtained at kappa 15 and 20.

***Bleached Pulp Viscosity and Brightness Stability*:** Figure 79 shows viscosity results for eucalypt and elephant grass bleached pulps. Among the eucalypt pulps, the kraft kappa 20 IP bleached pulp showed he highest value (760 dm^3/kg). The lowest viscosity value was obtained for the IP Soda-AQ at kappa number 15 showed the lowest viscosity value (427 dm^3/kg), but still sufficient high for applications in most paper grade pulp products. In regard to viscosity among all samples studied, the elephant grass kraft Kappa 20 bleached pulp showed the highest value (886 dm^3/kg), a result explained by the low xylan content of the elephant grass pulp. In spite of the kraft process to present the higher values to viscosity, the Soda-AQ process resulted in quite acceptable viscosity for IB (European E. globulus). As stated previously, the Soda-AQ process is the most indicated for a more efficient use of biomass in biorefinery applications, so among the eucalypt clones the IB seem to be the most desired raw material

for this project. On the other hand the elephant grass also presented a quite acceptable viscosity by Soda-AQ process at kappa 20.

Brightness stability of the bleached pulps was expressed as post color number for both elephant grass and eucalypt pulps. The brightness stability increased with increasing kappa number (increasing TAC) as tendency previously reported (EIRAS, 2001), this result is shown in Figure 80. However, no significant and logical effects of pulping process and biomass raw materials were observed on brightness stability.

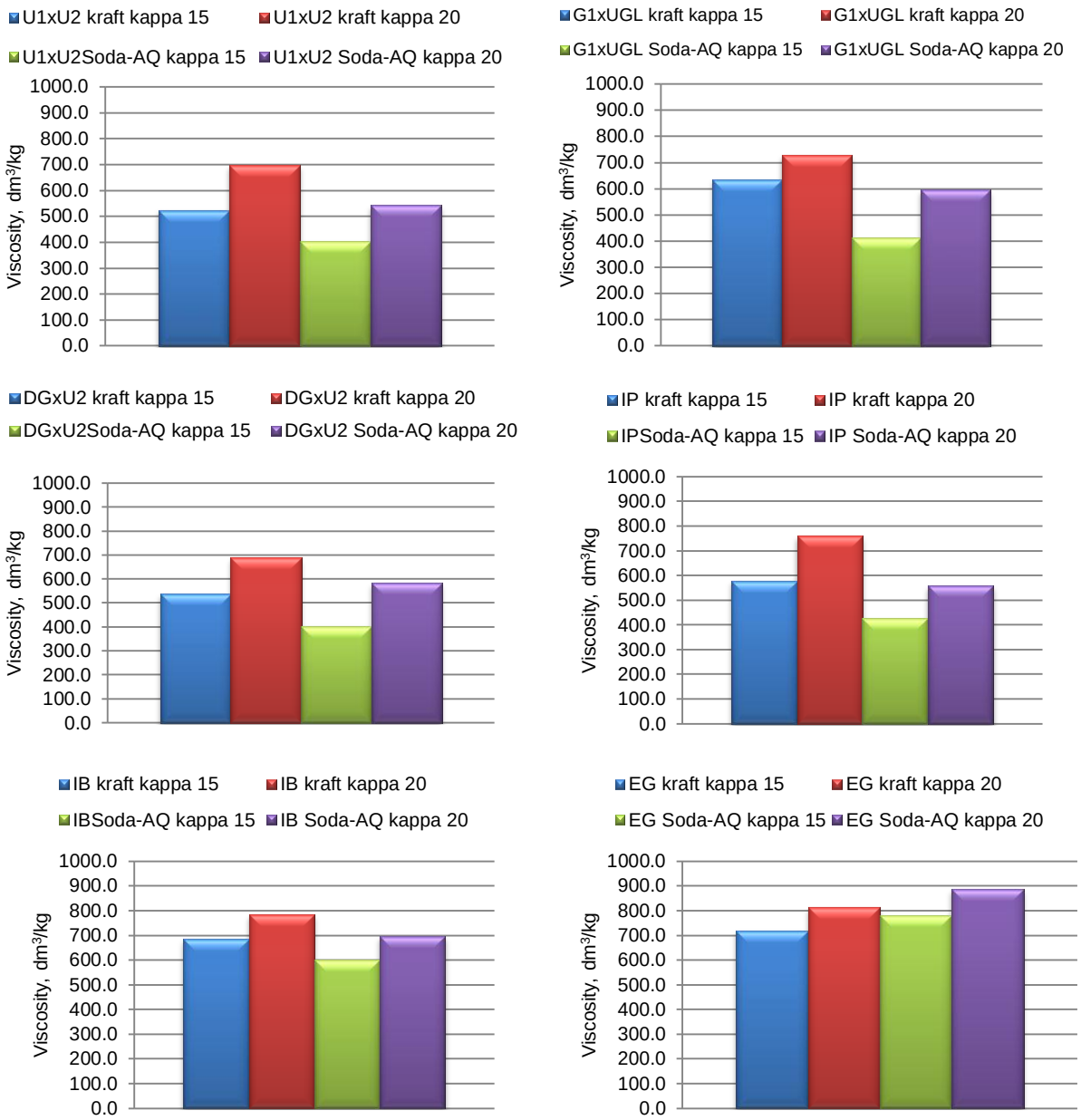


Figure 79. Viscosity of bleached pulps from eucalypt and elephant grass obtained by the Kraft and Soda-AQ processes at kappa 20 and 15.

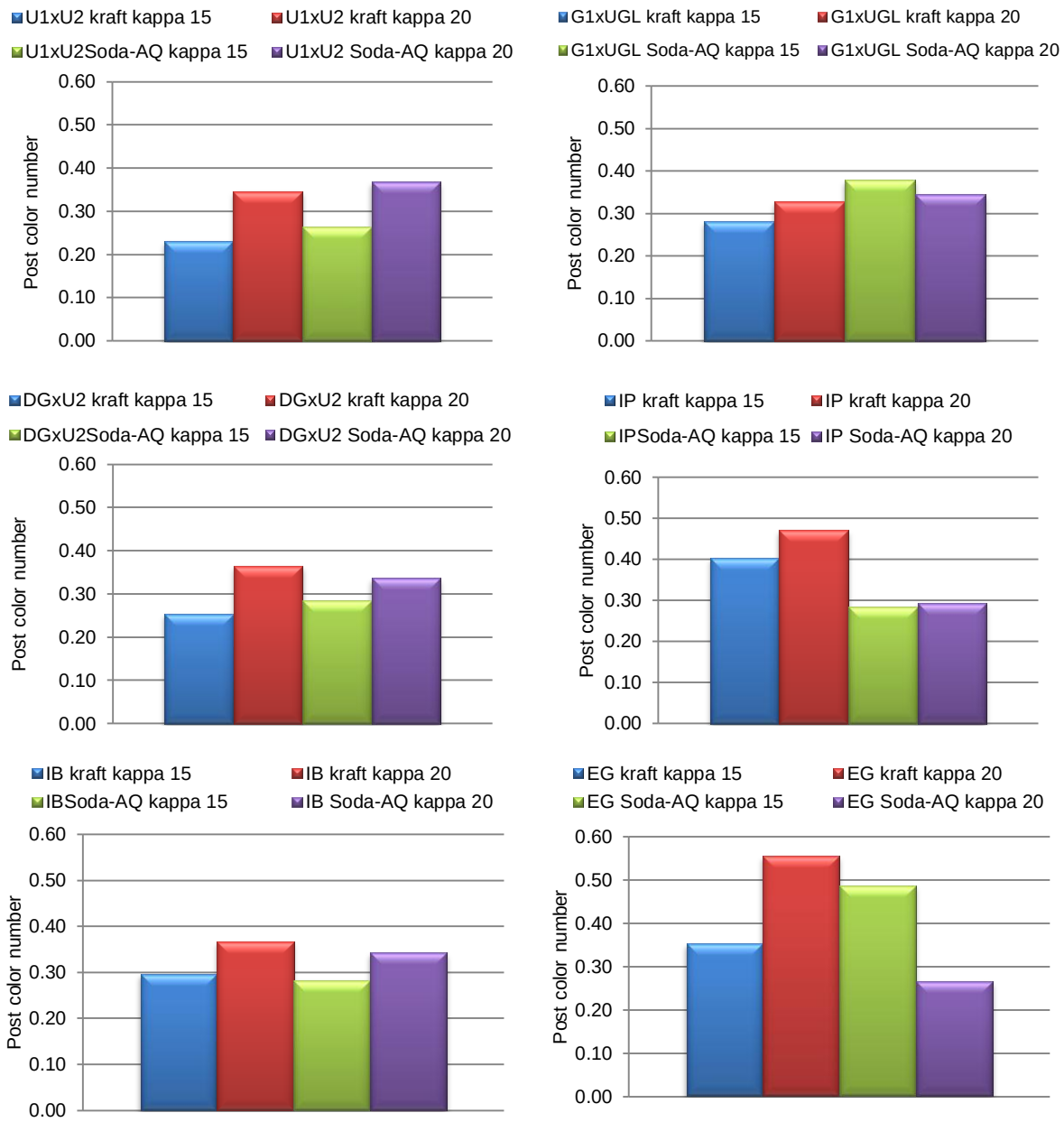


Figure 80. Post color number of bleached pulps from eucalypts and elephant grass obtained by the Kraft and Soda-AQ processes at kappa 20 and 15

Task 4.2 Biofuel potential of pre-treated materials and residues

Methods

In most cases, the enzymatic hydrolysability was evaluated according to sugar release (DNS). The washed pulp samples were suspended into 100 mM sodium citrate buffer, pH 5, 45ºC temperature at 1% consistency. Enzymatic hydrolysis was started by adding commercial Novozyme's cellulase mixture (Celluclast 1.5 FP) at the dosage of 10 FPU/g dry weight and β-glucosidase (Novozym 188) at the dosage of 500 nkat/g dry weight. The suspensions were incubated at 45ºC with magnetic stirring for 72 hours, and the content of released sugars was followed as a function of time. The 2,4-dinitrosalicylic acid (DNS) assay was used for the determination of reducing sugars. In some cases, the glucose, xylose, mannose and galactose concentrations were analysed from the supernatant by HPLC (without additional acid hydrolysis). The Aminex HPX-87H column (Bio Rad) was used with 0.6 ml/min flow of 5 mM H_2SO_4 as an eluent. To improve the separation of the monosaccharides, HPLC Fast Acid Analysis Column (Bio Rad) was used before Aminex HPX-87H column. In order to remove impurities from the samples, Cation-H Refill Cartridges (Bio Rad) was added as a pre-column.

Fermentations were carried out in erlenmeyer flasks (25 ml) in an incubator at 10% consistency. Same enzyme loading was used as in hydrolysis experiments (100 FPU/g Celluclast 1.5L and 500 nkat/g Novozyme 188). After 6h prehydrolysis at 45°C, the yeast (Red Star) was added with an OD_{600} of 3.5 ≈1g/l to the flasks to start the SSF and the temperature was lowered to 30°C with slow shaking (100rpm). Theoretical yield in fermentation is 0.51g ethanol/g glucose.

Selection of optimal LGF cooking time in respect of bioethanol production

As suggested on the basis of the chemical compositions (WP2), the shorter cooking times of 16-20h were sufficient for enzymatic hydrolysis of eucalyptus. Already during 16-20h cooking, the fiber structure was opened up and delignified enough for efficient hydrolysis (Figure 81), and highest proportion of the original feedstock could be released as sugars. The same was detected with both *G1xUGL* and *Suzano* clones. Cooking time of 20h was thus selected for the pilot demonstration performed in WP5.

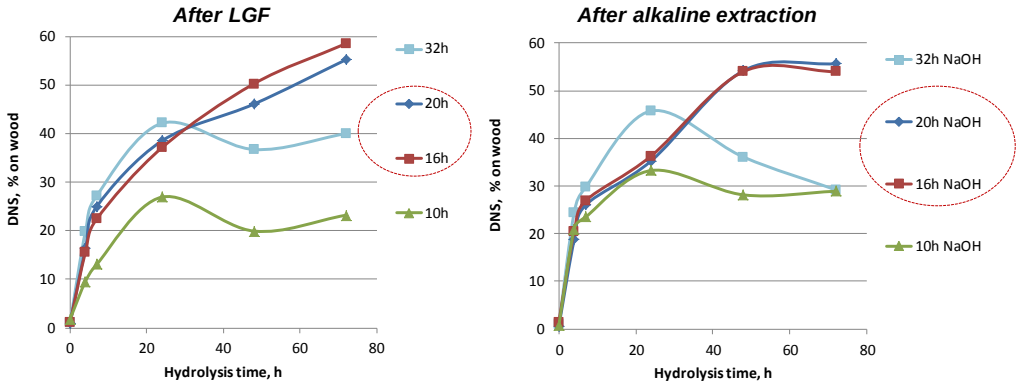


Figure 81. Effect of cooking time on sugar release in enzymatic hydrolysis of organosolv pulps (3.5% H_3PO_2, 130°C, 15% water content) for Suzano's eucalyptus clone. The results are corrected with pulp yields and shown as % on original wood raw material.

Bioethanol production potential of selected feedstocks after LGF cooking and alkaline extraction

As reported previously, the alkaline extraction improves LGF pulp hydrolysability because of enhanced lignin and xylan removal. In most cases, the hydrolysis time of 48h was required for the maximum sugar release. After LGF cooking and alkaline extraction, the *DGxU2* hybrid gave highest sugar release as could be expected based on highest cellulose yield reported in WP2. Despite the relatively good cellulose yield and better delignification efficiency compared to the other eucalyptus clones, the *E.globulus* unexpectedly gave lowest sugar release. As reported previously, the hydrolysis yield is to some extent dependent on cooking time, and optimal cooking time may vary between the clones. For *E.globulus* with lower initial lignin content the 20h cooking time may have been too long. No actual fermentation trials were performed for the LGF lab scale pulps.

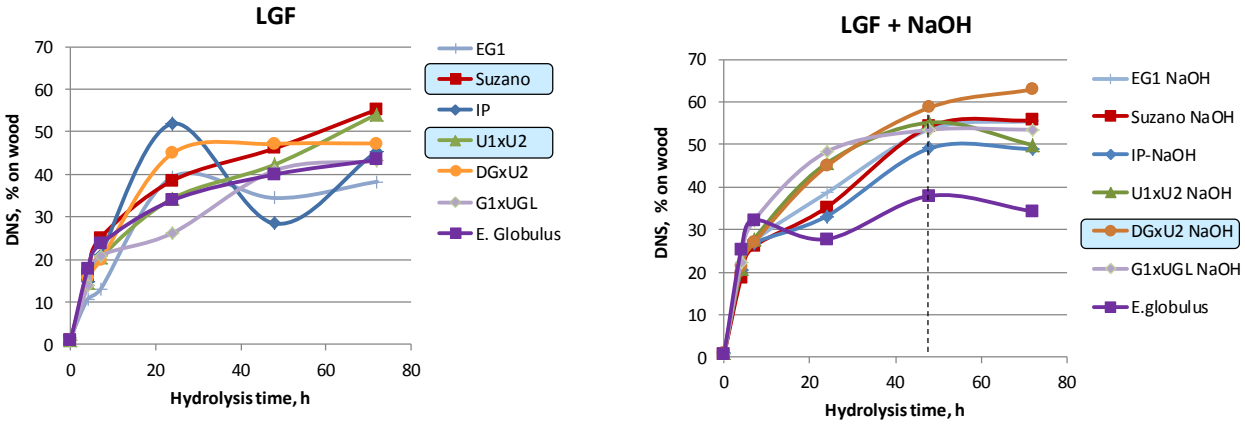


Figure 82. Effect of feedstock on sugar release in enzymatic hydrolysis of organosolv pulps (3.5% H_3PO_2, 130°C, 15% water content, 20h).The results are corrected with pulp yields and shown as % on original wood raw material .

Comparison of pretreatment methods

Effect of alkaline deconstruction on hydrolysis and fermentation efficiency

Alkaline oxidation (NaOH-O_2) and soda-anthraquinone (Soda-AQ) treatments were tested as potential alkaline deconstruction methods for bioethanol production. The alkaline treatments were performed in Suzano for elephant grass (EG1) and *G1xUGL* eucalyptus hybrid. In all cases, pulps at kappa levels 50, 30 and 15 were prepared.

In all cases, the *G1xUGL* eucalyptus provided higher ethanol yield than elephant grass. Cooking to low kappa level of 15 was necessary with both alkaline cooking methods (Soda-AQ, NaOH-O_2) to provide reasonable ethanol production with *EG*. With the eucalyptus, the NaOH-O_2 pretreatment was more suitable for bioethanol production than Soda-AQ. The Soda-AQ treatment required low kappa levels of 15, whereas after NaOH-O_2 treatment kappa levels of 35-50 provided well hydrolysable pulp for fermentation. The NaOH-O_2 treatment probably opens up the fiber ultrastructure better at the same lignin content, and even at high kappa levels the reject can be hydrolysed more efficiently.

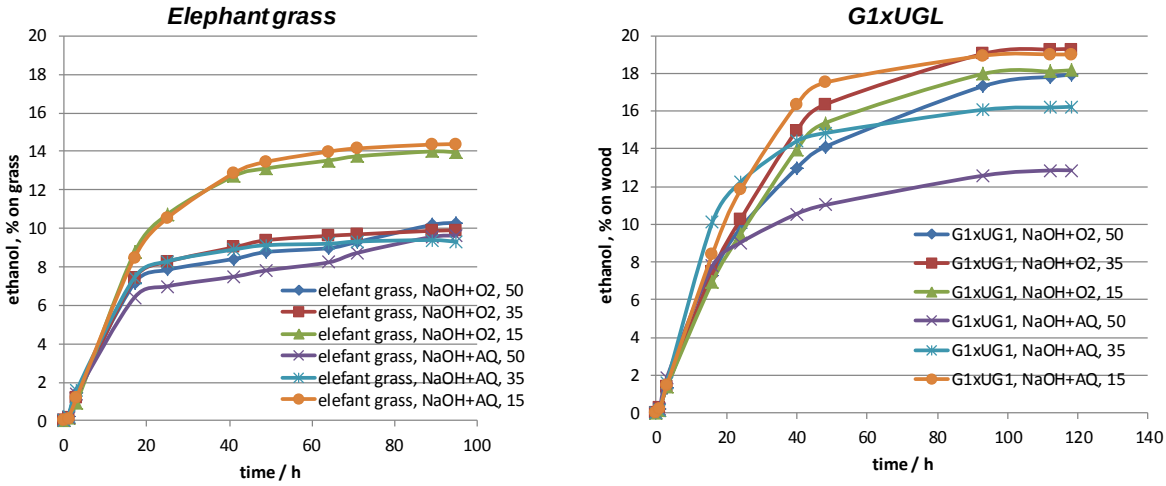


Figure 83. Effect of alkaline pretreatments (NaOH+O_2, Soda-AQ) on ethanol production from EG and G1xUGL pulps at kappa levels of 50, 30 and 15. The results are corrected with pulp yields and shown as % on original wood raw material.

Effect of pressafiner and hydrolase pretreatment on bioethanol production

Hydrolytic enzymes and laccase, were tested in CSIC and IRNAS for deconstruction of lignocellulosics for bioethanol production. After screening at lab scale, the elephant grass was treated in CTP by pressafiner (pilot scale) followed by hydrolytic enzyme treatment. After this pretreatment, only very limited ethanol production was detected unlike at lab scale. This is probably due to the insufficient biomass deconstruction and thereby hindered hydrolysability. As shown in Figure 30, the pressafiner had no significant mechanical crushing effect on EG. In previous lab scale experiments, the samples were ball milled, and higher ethanol yield was reported even for the reference without any enzymatic pretreatment (Fig. 86).

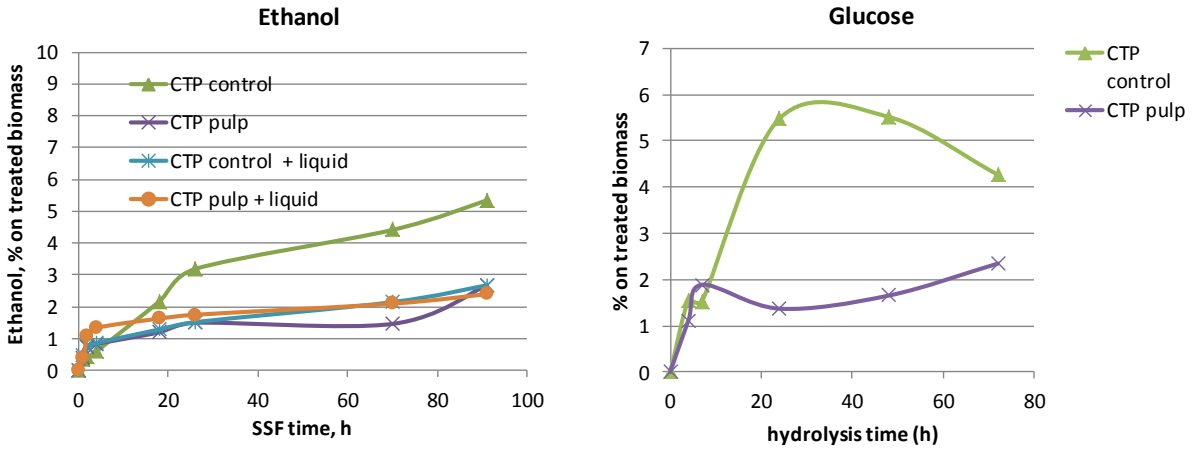


Figure 84. Effect of pressafiner and hydrolytic enzyme pretreatment on ethanol production from elephant grass.

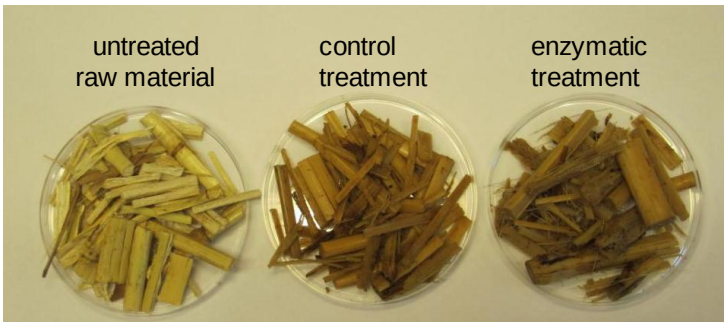


Figure 85. Effect of pressafiner (control) and following hydrolytic enzyme pretreatment on deconstruction of elephant grass.

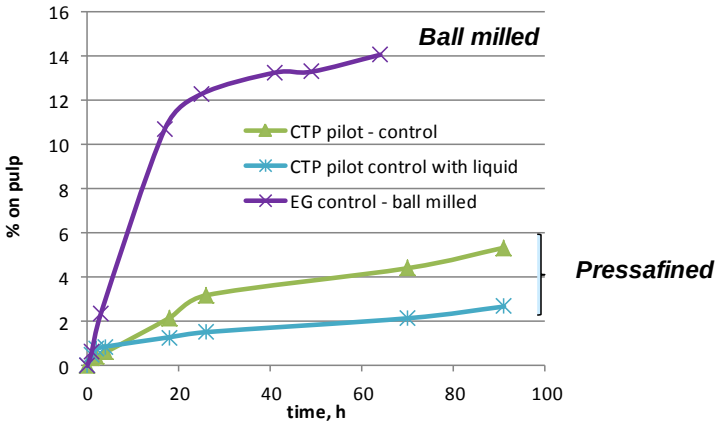


Figure 86. Effect of mechanical treatment on ethanol yield.

Effect of laccase treatment on hydrolysis and fermentation efficiency

The effect of laccase pretreatments performed at lab scale in IRNAS for *E. globulus* and elephant grass was investigated by enzymatic hydrolysis and fermentation experiments. As shown in Figure 87, the laccase treatment with HBT mediator (**L HBT)** clearly improved the enzymatic hydrolysability of elephant grass and *E. globulus*, and the effect was more pronounced for eucalyptus. The laccase treatment without mediator (**L)** had practically no effect, and hydrolysability was equal with the control sample. Probably due to the lower density and higher amorphicity, the elephant grass was much easier than *E. globulus* to hydrolyse even without any enzymatic pretreatment.

The actual bioethanol yields were well in line with the hydrolysability results (Fig. 88). The elephant grass provided better ethanol yield compared to *E. globulus*, and laccase with HBT mediator was better than laccase without any mediator. The effect of HBT mediator was more pronounced with *E. globulus*. In all cases, the ethanol yield was relatively low even though the samples were ball milled before the enzymatic treatments.

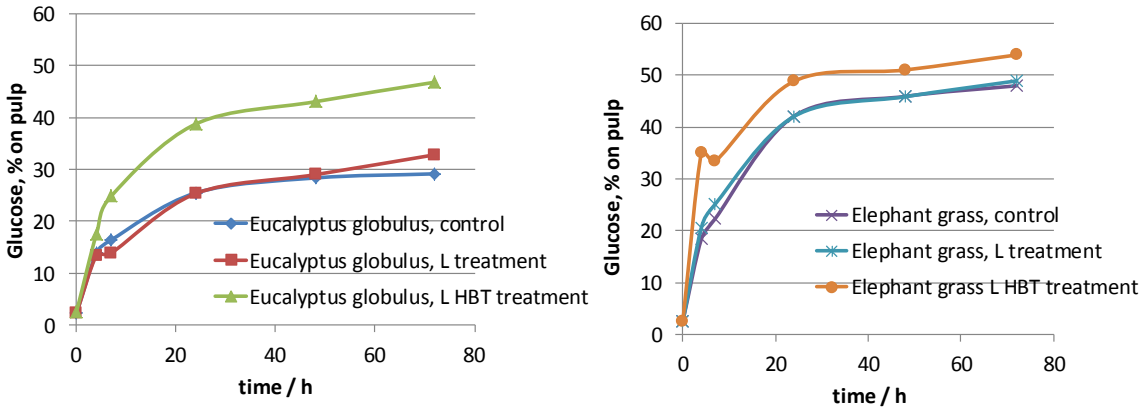


Figure 87. Effect of laccase treatment on glucose release in enzymatic hydrolysis. The results are not corrected with pulp yields.

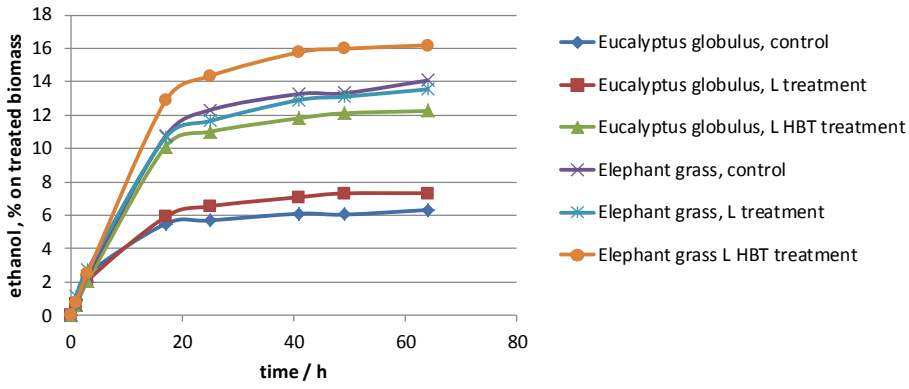


Figure 88. Effect of laccase treatment on ethanol yield.

At pilot scale, the same laccase treatments were performed for refined *E. globulus* at CTP. In this case, the ethanol production was very limited probably due to the insufficient biomass deconstruction and hydrolysis. The effect of ball milling on enzymatic hydrolysability was probably thus higher in previous lab scale experiments than the effect of laccase treatment.

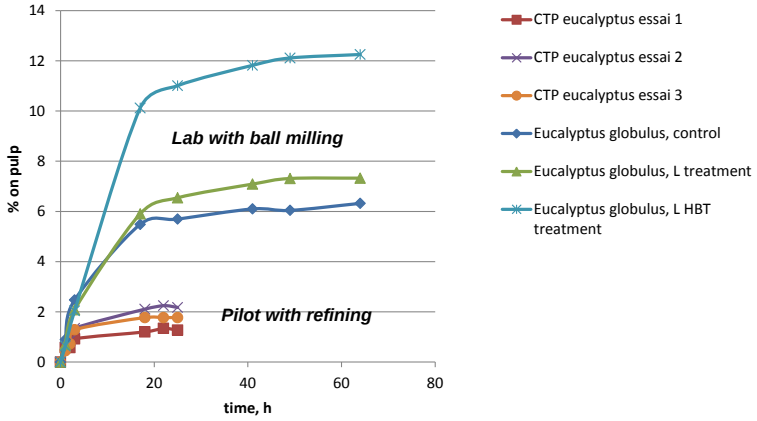


Figure 89. Effect of ball milling (lab scale) and refining (pilot scale) prior to the laccase treatment on bioethanol production yield.

Optimisation of enzymatic hydrolysis for the pilot pulps

To ensure reliable comparison, the enzymatic hydrolysis was optimised according to the raw material and pretreatment method. The optimisation was performed for the LGF pilot pulps (*E. globulus*) produced in WP5, and Soda-O2 pulp (*E. globulus*, kappa 35) showing the best bioethanol production potential.

Optimization of enzymatic hydrolysis was done using the Response Surface Methodology (RSM), which describes the relationship between several explanatory variables, i.e. different enzymes used in various loadings, and the response variable, i.e. the reducing sugars obtained, by a second degree polynomial. Additionally to the enzymes Celluclast 1.5 and Novozym 188, which were used in the screening experiments, purified xylanases with 3.2mg protein per ml (purified from Ecopulp, Ab Enzymes) was used. The dosages were chosen to be in the range of 5-15FPU/g_dm for Celluclast, 50-200nkat/g_dm for Novozym and 0.05-0.25mg protein/g_dm for the purified xylanases. In pretests, an addition of purified mannanases in the range of 0.05 -0.2mg protein/g·dm was found to have no statistically significant effect. Design of Experiment (DoE) of choise was a Central Composit Face Centered design (CCF) with the three enzymes as factors and reducing sugars as response variable. In the CCF design, each enzyme is used in three different dosages, the lowest, highest and mid point, in various combinations with the other enzymes.

The response surfaces obtained for all three pulps are shownin Figure 90. Xylanases were kept constant at 0.15mg protein per g·dm. Optimal reducing sugar concentration after 48h of hyrolysis were found to be 9.5 g/l at an enzyme loading of 13.9 FPU/g_dm and 130.3 nkat/gdm for LGF pulp, 11.3 g/l sugars at 11.3 FPU/g_dm and 160 nkat/gdm for LGF-OH pulp and 10.1g/l at 10.7 FPU/g_dm and 259.7 nkat/gdm for Suzano pulp. Compared to the constant charges used in sample comparisons (10FPU Celluclast, 500 nkat/g Novozym 188), the optimum Cellulcast dosage was slightly higher, but the Novozym charges could be reduced significantly.

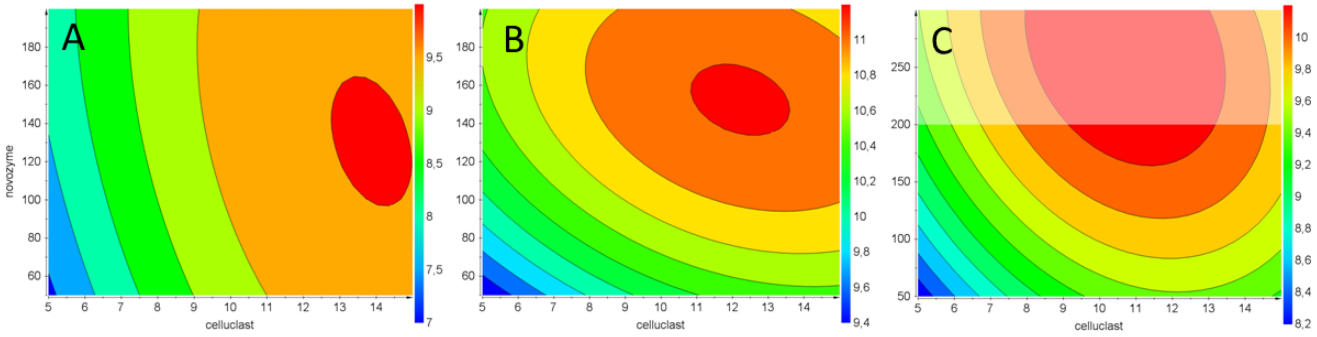


Figure 90. Response surfaces obtained from the optimization experiments. A: LGF; B: LGF-OH; C: Suzano.

As expected, the enzyme mixtures Celluclast and Novozym gave the highest variability in the response surface model. Xylanases only contributed minor variability (data not shown). The Celluclast preparation already contains traces of xylanases activity, and the pulps contain only small amounts of xylan (below 10%), therefore the addition of purified xylanases has only little effect on the resulting sugar concentrations.
A bench-top procedure was developed to gauge the biogas potential of several raw feedstocks and deconstructed materials from the LignoDeco partners. 4 different raw eucalyptus species and the elephant grass were milled to < 0,5 mm with a Wiley knife mill. An amount of wood powder or elephant grass, equivalent to 0,9 grams of cellulose (based on the amount of volatile solids, VS), was added to three 1 L laboratory digesters (see Figure 24) for each sample. The deconstructed materials i.e. various kraft eucalyptus clone pulps (kappa 20) and Soda-AQeucalyptus globulus pulp (kappa 20) were homogenized in a food-processor for 15 min, and an equivalent amount based on VS were added to the bioreactors. As a positive control, 0,9 grams of pure crystalline cellulose (Avicel) was added to 3 separate digesters. A third set of three digesters was reserved for the seeding sludge only, in order to determine the background biogas produced by the inoculum itself.

Seeding sludge was provided by Snertinge, Denmark, which was allowed to degas for 3 days at 49°C in order to minimize the background biogas production. 100 g of inoculum was added to the digesters. This results in a ratio of 0,7/0,3 for the inoculum and substrate/cellulose, respectively, based on the amount of volatile substrate. Each digester was then flushed with N_2 for 1 min, and sealed with OxiTop Control AN 6/AN 12 (WTW) and incubated anaerobically for 14 days at 49 °C. The biogas produced results in increased pressure within the digester which is automatically measured by the OxiTop.

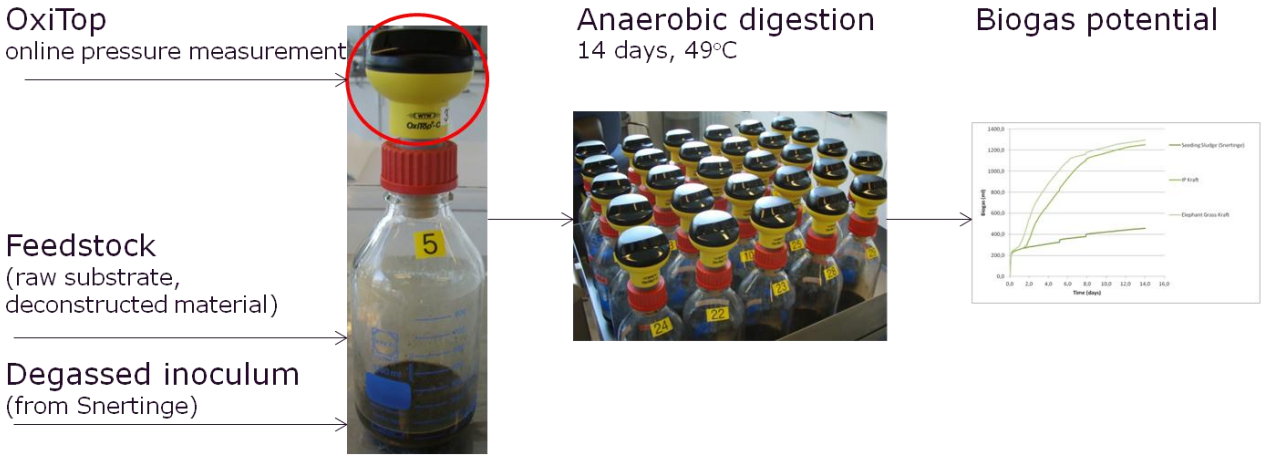


Figure 91. Outline of the procedure developed to gauge the biogas potential of the raw feedstocks and deconstructed materials.

The biogas development over the 14 days of anaerobic digestion at 49°C can be viewed in Figure 25. As is immediately obvious, raw eucalyptus wood presents a rather recalcitrant feedstock for anaerobic digestion. The absolute biogas potential after the 14 days of incubation corresponds to a theoretical carbohydrate conversion between 7-9% for the eucalyptus species, when using the compositional data from the 6th months technical report for these species. This low conversion may be caused by either the presence inhibitory compounds from the lignin fraction of the wood which hinders the microbial degradation of the substrate and/or more probable that the carbohydrate fraction is simply inaccessible to the degradative mechanisms of the microbes. The raw elephant grass did however show to be a suitable substrate for biogas production where the theoretical carbohydrate conversion is 71% and thus appears to be decidedly more bioavaible than the raw eucalyptus wood.

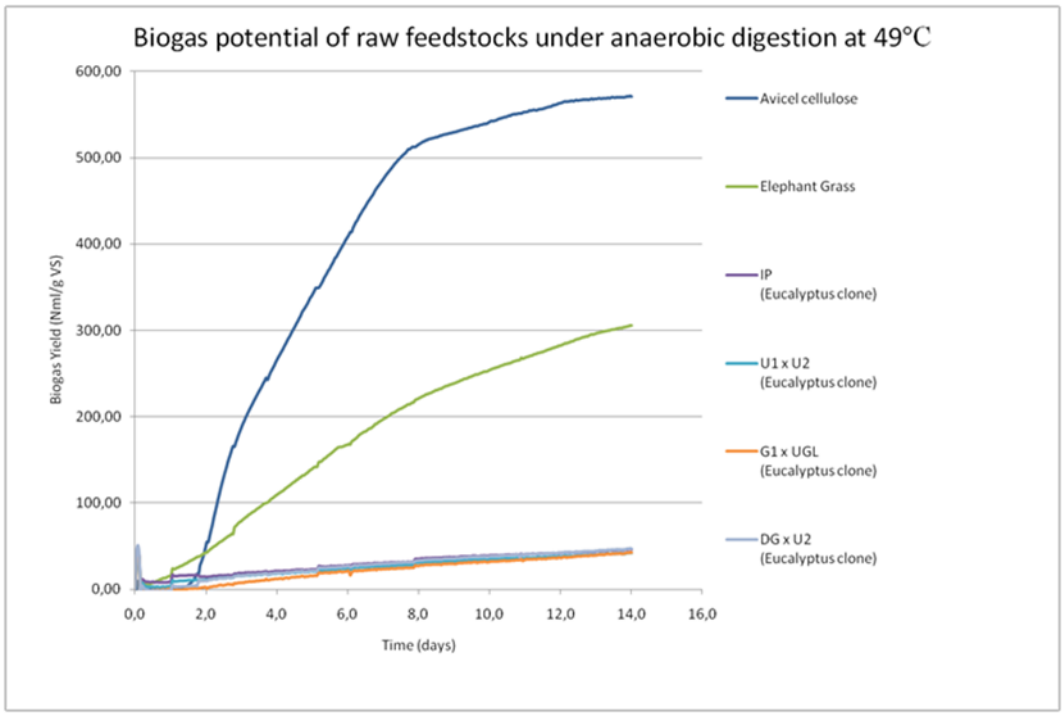


Figure 92. Biogas development over 14 days of anaerobic digestion at 49°C for the raw feedstocks including the positive cellulose control.

The total biogas yield after 14 days of anaerobic digestion at 49°C for the deconstructed materials can be viewed in Figure 26. The pulps investigated were deconstructed to kappa 20 and were mainly kraft pulps. However, *E. glubulus* Soda-AQ(kappa 20) pulp was included in order to identify possible differences between in biogas yield between the two pulping processes. Generally, when comparing the biogas yield of the kraft pulps to the raw feedstocks, it is clear that the deconstruction has had a positive impact on the digestibility. This may be a result of several factors including making the carbohydrate fraction much more accessible for the microbes. Moreover the removal of a large part of the lignin fraction may also have a positive impact on the digestibility of the substrate. Giving that compositional data for these deconstructed materials has yet to be provided, a theoretical carbohydrate conversion cannot be calculated at this point. However when comparing the absolute values for the biogas yield of these deconstructed materials (580-690 Nml/g VS for the eucalyptus species) to the positive cellulose control (570 Nml/g VS) it is clear that the substrates are easily digested. However the higher biogas yield for the pulps may be a result of other volatile gasses (sulfur compounds) released from the pulps and may not represent methane and carbon dioxide arising from the anaerobic digestion of carbohydrates.

A minor contribution from the digestion of protein and fats in the feedstock is also included in the total biogas yield measured, but is considered to be negligible in the conversion calculation due to the small amounts present in the samples compared to the carbohydrate content. The theoretical biogas yield arising from carbohydrate conversion is 750 Nml/g VS and results in 50% methane and 50% carbon dioxide v/v.

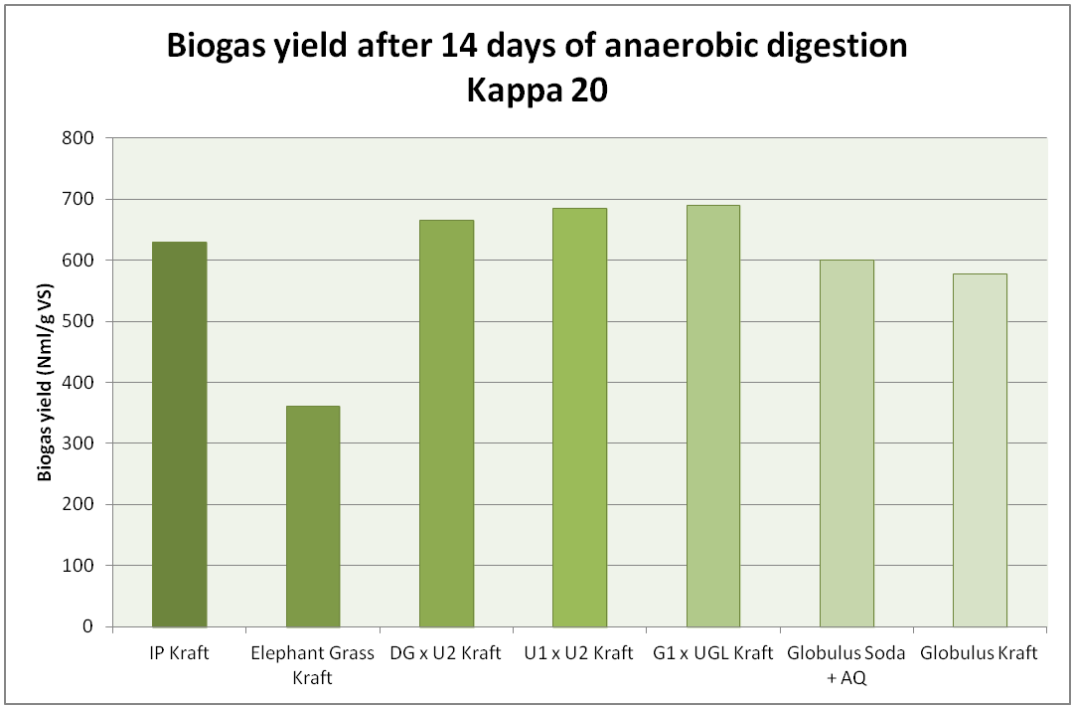


Figure 93. Biogas yield for the deconstructed materials after 14 days of anaerobic digestion at 49°C. All investigated pulps were kraft kappa 20 pulps but also the Soda-AQ*E. globules* kappa 20 pulp were included.

The biogas potential of mill sludge effluent was also investigated. However due to delays of samples (see 3.2. Deviations from the Work Plan) these investigations have not come to an end yet due to the long incubation times associated with these experiments (incubation alone is 14 days). Initial results have however been obtained after two days of incubation, which is clearly not enough to get a proper estimation of the total biogas potential of the mill sludge. Some of the biogas reactors containing the mill sludge and inoculum were also treated with a multicomponent cellulase mixture, a xylanase multicomponent mixture and a protease (and their respective inactivated mixtures). The idea with these treatments is to get a faster release of fermentable sugars from the substrate (and the inoculum itself) to increase the rate of biogas production. The data presented in Figure 27 shows that in this instance there is no effect of the enzymes on the initial biogas yield. This is can be caused by presence of enzyme inhibitors in the effluent or simply that these are not the optimal blend of enzymes for this specific mill sludge substrate. Another possibility could be to treat the mill sludge separately with for example an laccase to remove aromatic substances which may be inhibitory for the inoculum itself and thereby boost the biogas production.

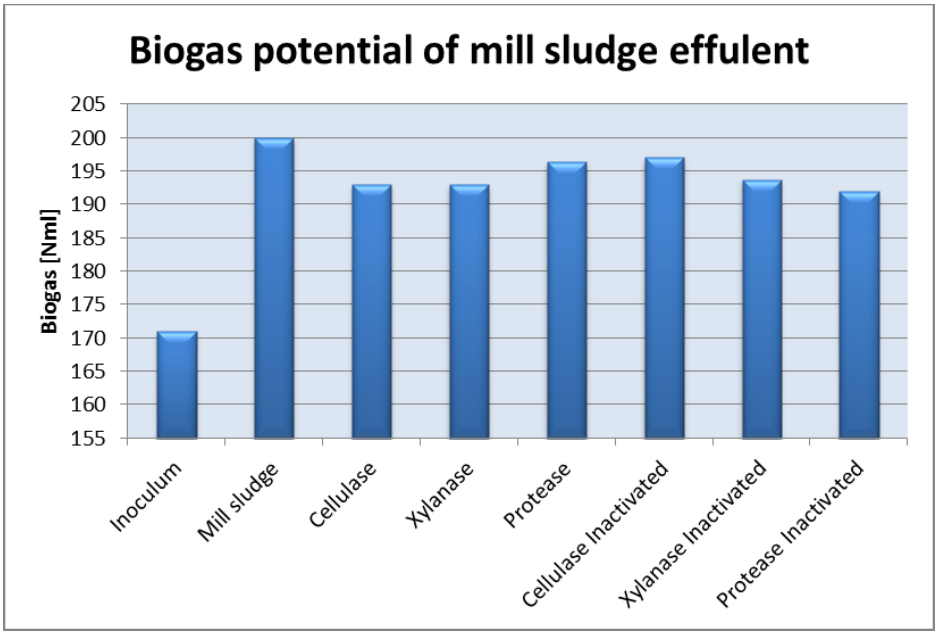


Figure 94. Biogas potential after two days of anaerobic incubation at 49°C. Mill sludge samples were treated with a multicomponent cellulase product, multicomponent xylanase product, a protease and their inactivated versions (1hour incubation at 90°C)

Task 4.3: Upgrading xylans as paper grade pulp additives

This task is divided into two main parts, namely: (I) production of special printing and writing paper grade pulps by using xylan as additive; (II) Production of special tissue grade pulps by partial xylan removal.

Part I: production of special printing and writing paper grade pulps by using xylan as additive

The use of non-woody xylan (obtained by extraction pre-treatment in WP2) as additive for eucalypt paper grade pulps were investigated. In this study three novelties were introduced: (1) the ideal conditions for xylans extraction, (2) the resorption of xylans during the oxygen delignification phase of the pulp bleaching process, (3) the production of special tissue grade pulp by partial removal of its xylans. The advantage of resorbing xylans during the oxygen delignification phase of the process is that the extracted xylan color coming from lignin and extractive impurities becomes irrelevant since they will go through subsequent bleaching. Therefore, much less purification of the extracted xylan is required. Eucalyptus fibers are largely used for printing & writing (P&W) paper grades. Pulps for P&W papers require significant refining and can benefit from a high content of xylans, which purportedly facilitate this operation. High quality printing and writing paper grades require pulps of high tensile strength to withstand the forces the paper undergoes during manufacturing and use in high speed printing machines. In this way two different pulps were compared regarding their behavior during papermaking: (1) reference pulp without xylan addition; (2) xylan treated pulp, in which xylans extracted from elephant grass were redeposited onto the *E. globulus* pulp during the oxygen delignification stage. On the other hand it was investigated an application for the pulps which the xylans were extracted (xylan depleted pulps) aiming at special tissue grade pulp production.

The ideal conditions for xylans extraction: For the xylans extraction it was used the Cold Caustic Extraction technology (CCE) under well optimized conditions of alkali charge, time and reaction consistency at room temperature. The studies were carried using a kappa 20 elephant grass brown pulp produced by the Soda-AQ processes. Figure 6 shows the effect of the alkali charge on xylan removal for experiments carried out at 10% consistency and 60 minutes reaction time at room temperature (Figure 95). The dosage of 550kg NaOH/odt pulp was considered the optimal value to run the CCE stage (10%

consistency, 60 min, room temperature) since charges above this value had only small effects on xylans removal. On the other hand, charges lower than this would not remove the targeted 7% of xylans established as a minimum in our previous studies. The value of 7% is the number that can actually be redeposited onto fibers.

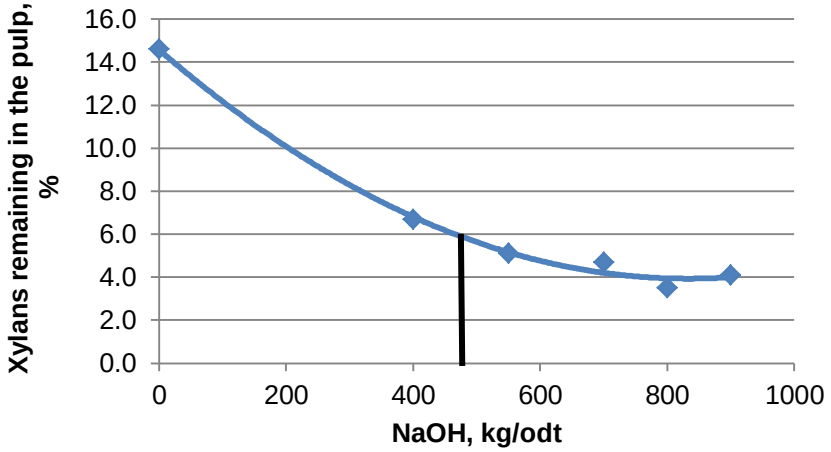


Figure 95. Effect of NaOH charge on xylan removal during the CCE treatment run at room temperature, 10% consistency and 60 minutes.

The effects of reaction time and consistency on CCE performance were evaluated at two alkali charges, namely 400 and 550 kg NaOH/odt pulp. The consistency affects the CCE performance because it influences de alkali concentration in the system and the alkali concentration is the main variable affecting xylan removal in the CCE stage. Figure 96 shows experiments run at room temperature and 15 min reaction where a more efficient xylan removal is visible at the higher consistency values, in the range of 10-20%, for a given alkali charge; the xylans extracted with 400 kg NaOH/odt at 15% consistency is actually higher than that extracted with 550 kg NaOH/odt at 10% consistency. The effect is very significant when the consistency is increased from 10 to 15% and not so significant when it is increased from 15 to 20%. Therefore, the 15% consistency is likely the most desirable value to run the CCE stage. On the other hand, one needs to pay attention to the consistency since too high a consistency may cause conversion of cellulose I into cellulose II, which makes the pulp useless for paper applications. Regarding reaction time, the results in Figure 97 show that 15 min suffices for efficient CCE stage. Increasing reaction time to 60 min resulted only slight xylan removal improvement.

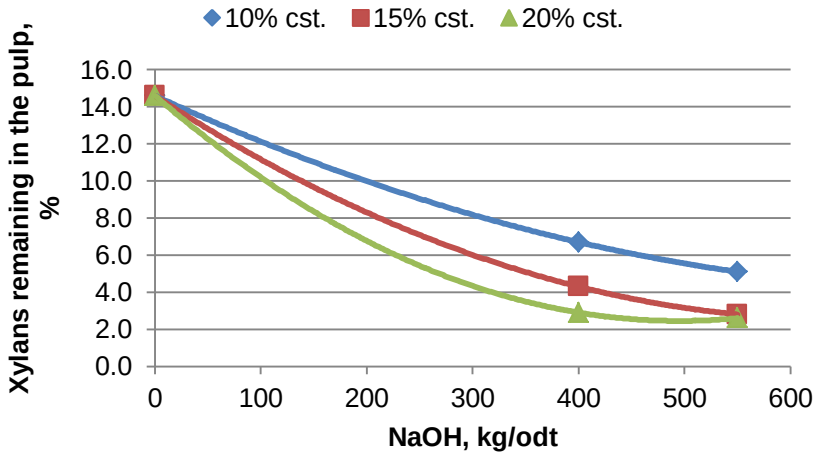


Figure 96. Effect of reaction consistency on xylan removal in the CCE stage run at room temperature and 15 minutes with different alkali charges.

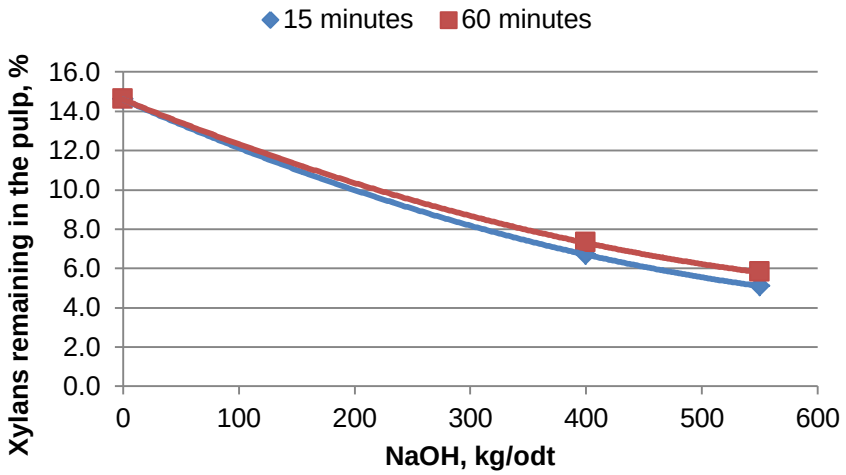


Figure 97. Effect of reaction time on xylan removal in the CCE stage run at room temperature and 15% consistency with different alkali charges.

Impact of xylans deposition on oxygen delignification performance: Xylans were extracted from elephant grass brown pulp by cold caustic extraction (CCE) and were added to *E. globulus* kappa 20 brown pulp (produced by the Soda-AQ pulping processes) during the oxygen delignification stage. Table 46 summarizes the results of the xylan assisted oxygen delignification. The xylan contents of the reference and xylan assisted processes after the oxygen delignification stage were 14.4% and 17.3%, respectively. The xylan addition onto pulp caused an increase of 2.9% of gravimetric yield. It was observed a slight effect of the xylan deposition on the oxygen stage end pH, which was lower than the reference, indicating the need for increased alkali addition in the O-stage, when xylan is added to this stage. The oxygen delignification efficiency was affected by the presence of lignin in the xylan extract, since the pulp rich in xylan showed higher kappa number and lower brightness than that of the reference treatment. This additional lignin consumed part of the alkali and oxygen required for the reactions during the oxygen delignification. The xylan deposition resulted in decreased pulp viscosity, which can be explained due to the low molecular weight of these hemicelluloses in relation to cellulose.

Table 46. Results of oxygen delignification carried out under reference conditions and assisted with xylans addition.

Conditions and Results	**Reference Process**	**Process assisted with xylans***
Consistency, %	10	10
Temperature, ^{o}C	100	100
Time, min.	60	60
Pressure, kPa	700	700
O_2, kg/odt	18	18
NaOH, kg/odt	20	20
Final pH	12.1	11.5
Brightness, %ISO	49.2	48.1
Viscosity, dm^3/kg	1011	951
Kappa number	12.7	13.2
Hexenuronic Acid, mmol/kg	64.4	68.3
Xylans, %	**14.4**	**17.3**
Yield, %	**98.1**	**101.1**

CCE stage was done in a non-woody pulp after O_2 delignification. The xylans were precipitated from the CCE liquor by ethanol addition followed by centrifugation. Xylans, were dissolved in water to a known concentration (70 g L^{-1}) and added to the brown pulp during the oxygen delignification at 7.0% of dry basis weight of pulp.

Impact of O-stage xylans deposition on bleaching performance: The reference pulp and xylan enriched pulp during the oxygen delignification were subsequently bleached with the D(EP)D sequence under conditions shown in Table 47. As expected the pulp rich in xylan showed a lower brightness than the reference, which can be explained by the sizable amount of lignin existing in the xylan extract, which contaminated the pulp during the oxygen delignification stage to a point that the post-oxygen kappa number was 0.5 unit higher for this sample than for the reference. The additional lignin and hexenuronic acid in the pulp rich in xylans consumed part of the bleaching reagents and decreased pulp brightness and pulp brightness stability. Note that the issues of lower brightness and brightness stability are easily solvable by adding additional amounts of chlorine dioxide during bleaching. The xylan deposition resulted in decreased bleached pulp viscosity, which can be explained by the low molecular weight of these hemicelluloses in relation to cellulose.

The deposited xylans were stable across the bleaching process. The xylans losses across bleaching for the reference and xylans enriched pulps were similar. After bleaching, the xylans content were 13.7% and 16.8% for the reference and xylans enriched pulps, respectively. The xylans deposition produced a total yield gain of 3.1% across the whole process, including oxygen delignification and bleaching.

Table 47: Conditions and results of the bleaching process for reference and xylans enriched pups.

Pulp Bleaching Conditions			
Conditions	**D**	**P**	**D**
Consistency, %	10	10	10
Temperature, ^{o}C	85	85	70
Time, min.	120	120	120
ClO_2 , kg/odt	8	-	6.5
H_2O_2, kg/odt	-	6.5	-
NaOH, kg/odt	-	8.0	-
Final pH	3.5	10.5	5.5
Pulp characteristics after DPD bleaching			
Samples	**Reference Pulp**	**Xylans Enriched Pulp**	
Brightness, %ISO	89.6	88.7	
Post Color Number	0.17	0.44	
Viscosity, dm^3/kg	792	704	
Kappa number	0.4	0.9	
Hexenuronic Acid, mmol/kg	4.9	10.5	
Xylans, %	13.7	16.8	
Δ xylans across bleaching	**-0.7**	**-0.5**	
Yield across bleaching, %	**96.4**	**99.5**	

Impact of xylans deposition on pulp properties: The stability of the deposited xylans and their effect on pulp beatability and properties was evaluated after the bleaching and in papers sheets with a schopper riegler degree around 35, which is typical for printing and writing paper grades for eucalypt. The deposited xylans resisted well to the bleaching operation and to the mechanical forces during beating (Figure 98). So, xylans deposition during oxygen delignification showed to be a good alternative for increasing fiber line yield.

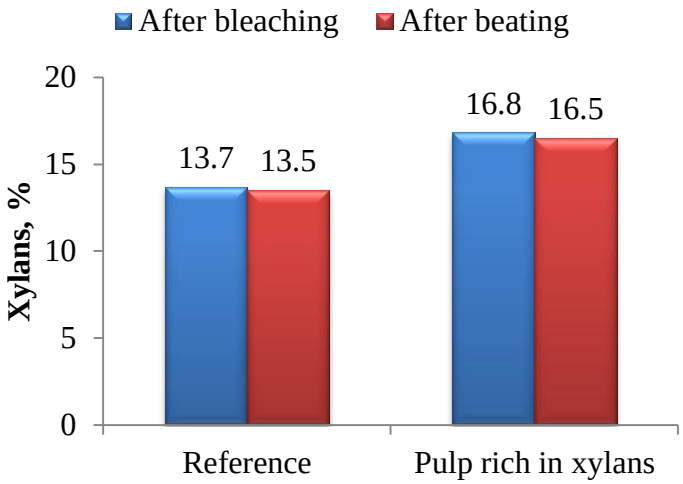


Figure 98: Xylans behavior after bleaching and beating for the reference and xylans assisted oxygen delignification process.

For the determination of pulp physical and mechanical properties, the samples were refined in the laboratory using PFI mill method at 10% of pulp consistency. Physical and mechanical tests were performed using laboratory hand sheets according to TAPPI test methods (Table 48) after pulp conditioning for 24 h in a room at 50 ± 2% of relative humidity and temperature of 23 ± 1^{o}C. Tensile tests were done according to TAPPI T494 om-96 using INSTRON tester (model 4204 computer controlled) under the following conditions: Cross head speed=25 mm/min.; Load cell capacity=1.0 KN; specimen dimension=160x15 mm; Grip distance=100 mm.

Table 48: Test methods for pulp beating and strength property evaluations.

Laboratory beating of pulp	T248 sp-08
Forming Hand sheets for Physical Tests of Pulp	T205 sp-06
Physical Testing of Pulp Hand sheets	T220 sp-06
Internal Tearing Resistance of Paper (Elmendorf)	T414 om-04
Tensile Breaking Properties of Paper	T494 om-06

High quality printing and writing paper grades require pulps of high tensile strength to withstand the forces the paper undergoes during manufacturing and use in high speed machines. The pulps were evaluated in papers with a schopper riegler degree around 35, which is industrially used to printing and writing paper grades. The results are shown in Figure 99. The xylans enriched pulp tended to form papers of higher tensile energy absorption, specific elastic modulus, tensile index, burst index and air resistance permeance, when compared to the reference pulp. However, the xylan enrichment resulted pulp of lower tear index and bulk compared to the reference. When the results of properties at a given beating energy consumption were analyzed, all properties evaluated showed higher values for the xylan treated pulp with much less energy beating energy demand. The only exception occurred for the tear index. This is explained by the lower amount of cellulose chains in the xylans enriched samples compared to the reference; the tear strength property benefits from cellulose rich pulps.

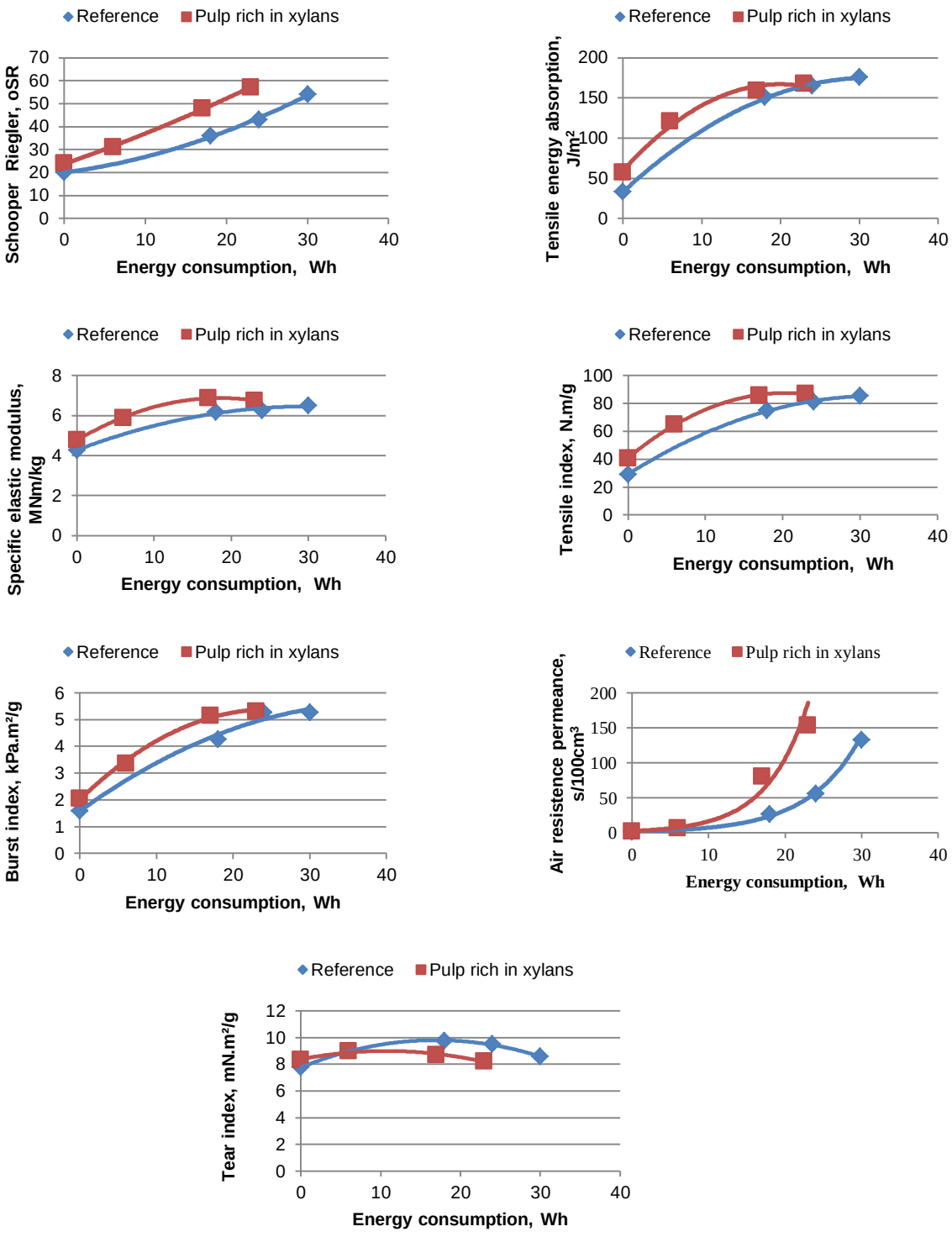


Figure 99: Energy consumption versus pulp properties for the reference and xylan enriched pulp samples.

Part II: Production of special Tissue Grade Pulps by Partial Xylan Removal

Tissue papers are responsible for a significant portion in the total paper manufacture (8-10%). Among the requirements of pulps quality for these papers, their level of xylan stands out, which has been a controversial matter. The aim of this study was evaluating the influence of eucalypt pulp xylan content on its tissue grade properties. A brown eucalyptus kraft pulp was treated with variable alkali charges in a CCE stage in order to obtain materials with different xylans content. The resulting samples were bleached by the ODPD sequence and evaluated for their main properties as applicable to tissue grade

pulps. For this study, it was uses an industrial *Eucalyptus urograndis* kraft pulp was used: kappa number = 16.1, viscosity = 1226 dm^3/kg, brightness = 42.1%ISO, xylan content = 15.6% and HexA = 61.8 mmol/kg.

Four alkali charges (10, 30, 50 and 70g/L NaOH) were used to produce pulp of different xylan contents, maintaining constant other operating conditions (30 min, 30°C, 10% consistency). This alkaline treatment, also designated as Cold Caustic Extraction (CCE) was carried out with 100 grams of pulp in polyethylene bags. The pulp sample was thoroughly mixed with sodium hydroxide and water and let stand for 30 minutes at 30°C, after which a sample of liquor was squeezed out from the pulp for analyses. Following, the pulp was washed with distilled water in excess. The filtrates collected after extraction were analysed for COD (Standard Methods 5220D). All experiments were performed in duplicate. These four CCE treated pulp samples and the reference pulp were subjected to analysis of viscosity (SCAN cm-15: 99), gravimetric yield and xylan content (HPLC - after acid hydrolysis according to TAPPI T249).

Following, they were oxygen delignified in a reactor/mixer, model Mark V (Quantum Technologies Inc), and further bleached with D-P-D sequence. The bleaching stages were carried out n polyethylene bags under the conditions shown in Table 49. By the end of each stage, the pulp was washed with the equivalent of 9 m^3 of distilled water per ton of pulp. All stages were performed in duplicate. The bleached pulps were evaluated for their kappa number (TAPPI T236cm-85), brightness (TAPPI T525 om-92), brightness reversion (TAPPI UM200 - 4h, 105±3°C) and hexenuronic acid content (TAPPI T282pm-07).

Table 49. General Bleaching Conditions

Conditions	**O**	**D**	**P**	**D**
Consistency (%)	10	10	10	10
Time (minutes)	60	120	60	120
Temperature (°C)	100	75	82	76
Pressure O_2 (kPa)	500	-	-	-
ClO_2 as Cl_2 (kg/odt)	-	kappa factor = 0.16	-	5; 10; 15
H_2O_2 (kg/odt)	-	-	3.0	-
NaOH (kg/odt)	15	-		-
End pH	-	3.0	10.8	4.5

The bleached pulps were refined in a PFI mill, according to TAPPI 248 om-00. The beating intensity was expressed in Schöpper-Riegler degrees (°SR), according to TAPPI 200 sp-01. The number of PFI revolutions was variable, in order to obtain three levels of beating, allowing the development of refining curves in the range 15 to 50°SR. The hand sheets were formed using a TAPPI former, with a grammage of approximately 60 g/m^2, according to TAPPI 205 sp-02 and TAPPI T218 sp-97. The hand sheets were placed in acclimatized room at temperature of 23 ± 1°C and relative humidity of 50 ± 2% before testing.

The pup hygroscopic properties were evaluated for water absorption capacity test - the basket test (NBR 15,004), Water Retention Value - WRV (TAPPI UM 256) and Klemm capillary (ISO 8787:86). The pulp optical properties of light scattering coefficient (ISO 2471:98) were measured in a spectrophotometer Datacolor, model Elrepho 450X. Tests relating to tensile index were made in Instron apparatus, with a distance between jaws 100 mm, test speed of 25 mm / min load cell 1000 N and computerized data acquisition and analysis (TAPPI T494 om- 01). Tests for tear strength (TAPPI T414 om-98) were performed on the Eldendorf device. The test for the specific apparent volume (bulk) followed TAPPI T220 sp-01 standard.

The results were statistically analysed using the Statistica software (version 7.0). The analysis of variance (ANOVA) was done at the 5% significance level. The hypotheses tested were: H0: all means are equal, i.e., there is no significant difference between treatments, and Ha: there is at least an average statistically distinct from the others. Since the ANOVA showed significant difference between treatments, the Tukey test was applied to find out which treatments were distinguished from each other.

In addition, it was used the Curve Expert software (version 1.4) to obtain the models from the drainability and beatability analysis of the pulp samples and their different levels of refining. The adjusted equations were compared by F test, using the identity test models at the 5% significance level, according to the methodology for linear (Regazzi, 1993) and for nonlinear models (Regazzi, 2004). The hypotheses tested were: H0: all equations are equal and can be represented by a reduced common equation, and Ha: the equations are statistically different and cannot be reduced to a common equation.

Xylan Extraction from Kraft Pulp by Alkali Treatment: The alkaline treatment aimed at producing brown pulp with different levels of xylan content. The resulting pulps were evaluated for bleachability and their properties for production of special tissue papers. The effect of the NaOH charge on the pulp characteristics are shown in Table 50.

Table 50. Effect of the NaOH concentration on brown pulp characteristics after the CCE treatment (30 min, 30°C, 10% consistency)

Results	**NaOH concentration, g/L**				
	0 (Ref.)	**10**	**30**	**50**	**70**
Xylans (%)	15.6[a]	14.5[a]	10.8[b]	8.1[c]	5.9[d]
HexA's (mmol/kg)	61.8[a]	61.4[a]	50.0[b]	34.7[c]	22.8[d]
Yield (%)	-	98.5[a]	96.7[b]	89.2[c]	85.7[d]
Kappa number	16.1[a]	15.2[b]	13.3[c]	10.5[d]	7.9[e]
ISO Brightness (%)	42.1[a]	40.1[b]	41.4[a]	43.2[c]	42.9[d]
Viscosity (dm^3/kg)	1226[a]	1204[a]	1245[a]	1278[a]	1308[b]
COD of the filtrate (kg/t)	-	6.3[a]	66.3[b]	196.4[c]	206.6[d]

As anticipated the amount of xylan remaining in the pulp decreased with increasing alkali concentration in the CCE stage, reaching 62% removal when treated with 70 g/L NaOH. This result ins in agreement with those of Wang, 2008. According some authors, xylans are soluble in alkali and water, and therefore they are easily removed in alkaline treatments (Al-Dajani, 2008; Sjostrom, 1999; Hashimoto, 1975; Scott, 1989; Cunninghan, 1986; Walton, 2010). The xylan removal had a direct effect on pulp HexA content and pulp yield. The HexA are directly linked to the xylan chain, and thus removed along with these polymers (Petit-Breuilh et al, 2004). The yield drop is caused by the xylan losses. Other materials such as degraded cellulose and lignin also contribute to yield drop.

The pulp kappa number drop across the CCE treatment indicates that lignin and HexA are partially removed. The HexA contribution to the kappa drop can be determined considering that each 10 mmol/kg of HexA lost represents one kappa unit.

The pulp brightness showed a small increase in the CCE treatment due to the partial removal of lignin chromophores present in the brown pulp. Although the viscosity is influenced by the low molecular weight carbohydrates such as hemicellulose and degraded cellulose, there was no statistical difference in the results thereof, except for the pulp treated with NaOH 70 g/L. The results for the COD in the filtrate correlated with the xylan and kappa losses across the CCE stage. An increase in the filtrate COD values was observed as the alkaline extraction became more severe.

Oxygen Delignification and D-P-D Bleaching of the Xylan Depleted Pulps: The results of the oxygen delignification are shown in Table 51. The reduction in kappa number for the alkali pre-treated pulp ranged from 31.7 to 33.6%, i.e. the decrease in xylans content had no significant impact on the efficiency of this stage. For the reference treatment the efficiency was 32.3%. It was expected that this efficiency would increase in the pulps treated with alkali, because they contained a lower content of HexA, which notoriously have a negative effect on the efficiency of the O-stage. However, this effect was masked by the fact that the alkali-treated pulps had different initial kappa numbers. It is not correct to compare the efficiency of the O-stage in pulps of different kappa numbers. As expected, the HexA

removal in the range of 2.6 to 4.6% was very low in the O-stage, since they do not react with oxygen and do not contribute significantly to kappa number reduction (Vuorinen, 1996)

Table 51. Oxygen delignification results for pulps containing variable amounts of xylans

Results	Xylans content, %				
	15.6	14.5	10.8	8.1	5.9
Kappa number	10.9[a]	10.1[a]	9.0[b]	7.1[c]	5.4[d]
HexA (mmol/kg)	59.8[a]	59.8[a]	48.1[b]	33.1[c]	22.0[d]
Viscosity (dm^3/kg)	1101[a]	1071[b]	915[c]	-	904[c]
ISO Brightness (%)	49.2[a]	53.4[b]	54.0[c]	55.2[d]	57.8[e]
Kappa reduction (%)	32.3	33.6	32.3	32.4	31.7
HexA reduction (%)	3.1	2.6	3.7	4.6	3.5
Viscosity reduction (%)	10.2	11.0	26.5	-	30.9
Brightness gain (%)	7.1	13.3	12.6	12.0	14.9

The viscosity of the pulps were lower in the samples treated with higher concentrations of NaOH (904-1071 dm^3/kg) when compared to the reference (1101dm^3/kg). The pulps treated with alkali are more susceptible to degradation since they are devoid of hemicellulose in the fiber surface, which protect the cellulose chains against the attack of radical species common in the O-stage. The brightness gain in the oxygen delignification increased with increasing concentration of the NaOH liquor, which can be explained by the lower kappa numbers achieved.

The oxygen delignified pulps were bleached under similar conditions with the sequence D-P-D, keeping a constant kappa factor (0.16) in the first chlorine dioxide stage and varying the dosage of chlorine dioxide in the second stage. All the experiments aimed at achieving final brightness of 90% ISO. The bleaching results are presented in Table 52.

Table 52. Bleaching results with the D-P-D sequence for pulps containing variable amounts of xylans

Results	Xylans content, %				
	15.6	14.5	10.8	8.1	5.9
Total active chlorine [1/] (%)	3.87[a]	3.75[a]	3.07[b]	2.27[c]	1.99[d]
Bleachability (kappa ud / % active Cl)	2.8[a]	2.7[a]	2.9[a]	3.1[a]	2.7[a]
ISO Brightness (%)	89.8[a]	89.8[a]	89.9[a]	89.8[a]	90.1[a]
ISO Brightness reversion (%)	2.7[a]	2.6[a]	2.4[a]	2.5[a]	2.2[a]
Viscosity (dm^3/kg)	926[a]	911[a]	813[b]	-	767[c]
HexA's (mmol/kg)	6.6[a]	7.3[b]	7.3[b]	9.4[c]	5.5[d]

In general, the xylan removals in the CCE treatment decrease bleaching chemical demand. The lowest value of total active chlorine demand for bleaching was achieved with the pulp containing the least xylans, and this is explained by the lower kappa number of this pulp after the oxygen delignification stage (Table 51). The results also show that the bleachability between 2.7 to 3.1 did not vary greatly among the samples. There was no statistical difference among treatments for brightness reversion. The pulp brightness reversion ranged from 2.2 to 2.7 %, with the lower values observed for the pulps containing less xylan. On the other hand, the bleached pulp HexA content varied between 5.5 and 9.4 mmol/kg, with the highest value observed in the pulp with highest xylan content. The highest brightness reversion in the pulps with lower xylan content can be explained by its lower HexA content. These components have been pointed out as the greatest responsible for the pulp brightness reversion (Vuorinen, 1996). The pulp viscosities ranged from 767 to 926 dm^3/kg, with the highest value found for the reference sample and lowest for the samples treated with higher concentration of alkali solution. The

results confirm the role of xylans in protecting the cellulose chains against degradation by oxidants during bleaching. Thus, by removing xylans, the cellulose chains become susceptible to degradation.

Properties of xylan depleted pulps: By decreasing the xylan content in the pulp, the energy consumed during beating is increased. It is well known that the xylan exerts positive influence on the swelling, hydration and fiber flexibility, which favors the refining process. Pulps that are used in the manufacture of tissue paper are slightly refined, to about 20°SR in order to produce a tensile tensile index in the range of 20Nm/g. Extensive beating is not desirable because it impairs the ability of the paper to absorbs and retain water through capillarity.
Figure 100A shows the impact of pulp xylan content on beating energy consumption to achieve the tensile index of 20 Nm/g. It is observed that by increasing the xylans content from 6 to 16%, the power consumption is decreased by 88%, which corresponds to approximately 9% energy savings per each 1% increase in xylans content. This trend has been observed by other workers (Vuorinen, 1999). Figure 100B shows the effect of xylan content on the tensile index for the energy consumed to achieve 20°SR (this value is considered a target in the most common mills). It can be concluded that pulps with lower contents of xylans have lower tensile index and consume more energy for the same Schöpper-Riegler. This implies that for a given energy consumption, pulps with lower levels of xylans will have lower tensile index.

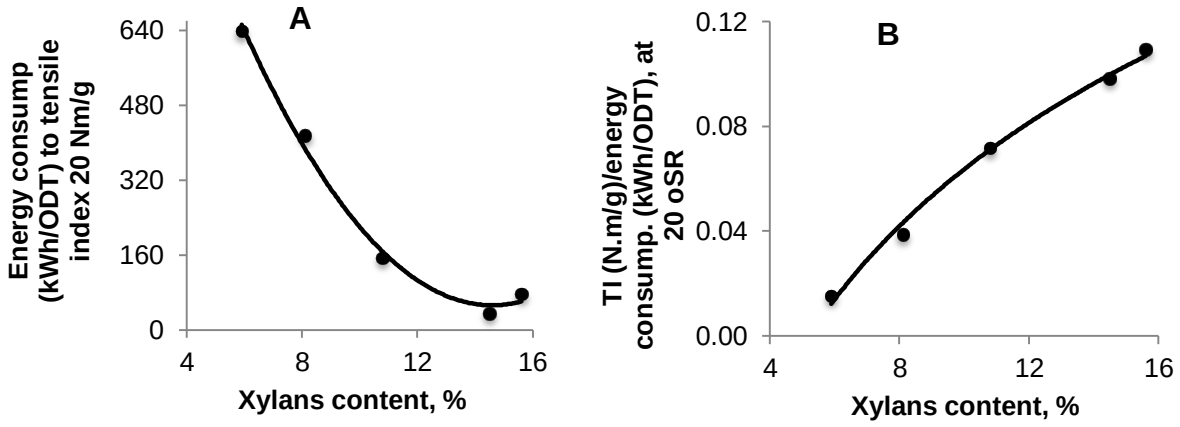


Figure 100. Effect of pulp xylan content on beating energy demand expressed two different ways: (A) at a fixed tensile of 20 N.m/g and (B) at fixed drainage resistance degree of 20°SR.

The drainability of the pulps affects the paper machine speed and its runnability. It is measured by the drainage resistance (°SR) at a given energy consumption for refining. Figure 101A shows that pulps with lower xylans content have lower drainage resistance at a tensile strength of 20 °SR. This implies that lower the xylan content in the pulp, for each unit of energy (Wh or kWh), the lower the drainage resistance of the pulp. Figure 101B also shows that resistance to drainage is highly minimized when pulp xylan content is decreased. These results confirms the action of hemicellulose in the fluid retention during drainage of the cellulosic material. Therefore, pulps of low xylan contents are desirable for tissue paper production because of their enhanced drainability, which indirectly means improved runnability.

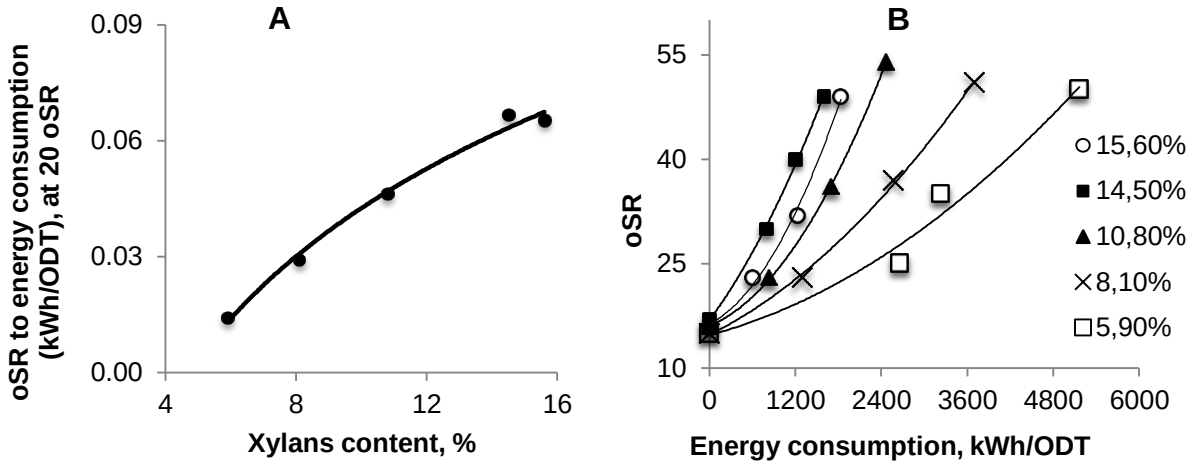


Figure 101. Effect of pulp xylan content on drainability expressed two different ways: (A) at fixed drainage resistance degree of 20°SR, (B) at various drainage resistance degrees.

The Water Retention Value (WRV) data for the five bleached pulps samples containing different amounts of xylan are shown in Table 53. There was a clear trend of decreasing WRV with decreasing pulp xylan content, with the effect being more pronounced at the very low levels of xylans. These results were anticipated since xylans are well known to be highly hydrophilic and thus their removal from the pulp tend do decrease pulp water retention capacity. The WRV is a combined property involving chemical effects (hemicellulose content) and physical effects which include fiber population, total pore volume and distribution of the capillaries (Foelkel, 2007). The properties of swelling and hydration of the fibers are very much influenced by the cooking and bleaching operations, since they affect the amount of hemicelluloses content and the cell wall integrity. The high content of hemicelluloses, associated with large number of fibers and degraded fibers (low viscosity), leads to pulps with high water retention. Therefore, it makes the pulp very difficult to drain and flow into the paper machine. These pulps even when unrefined, have high Schöpper-Riegler (°SR) and this shows that the drainability is hampered even for unrefined pulps (Foelkel, 2007). Of course, lowering the water retention value of the pulp by decreasing their xylan contents can be advantageous for runnability and positive for certain tissue paper grades.

Table 53. Effect of pulp xylan content on water retention value (unbeaten pulp).

Xylans content, %	15.6	14.5	10.8	8.1	5.9
WRV, %	162[a]	161[a]	154[b]	141[c]	131[d]

Figure 102A shows the effect of xylan content on pulp water absorption capacity at 20ºSR. It is shown that water absorption capacity increases with decreasing pulp xylan content. Water absorption capacity is one of the most fundamental properties of tissue grade pulps. The presence of xylan in the pulp creates difficulty in the movement of water molecules in the pulp fibers, thus preventing a good absorption of water therein. The pulp absorption capacities at different refining levels are presented in Figure 102B. The reference sample and the sample containing 14.5% of xylan were represented by a single curve, since they were statistically equal according to the identity test models; this similarity occurred because the xylan content of this pulp and that of the reference were somewhat similar as well. With the progress of pulp beating, the water absorption capacity of the pulps decreased. At the higher beating levels (> 30°SR) the water absorption capacity of the pulps were no longer affected by the pulp xylan content. Therefore, the pulp xylan content influences the water absorption capacity only at low beating levels, but low beating levels are typical of tissue grade pulps.

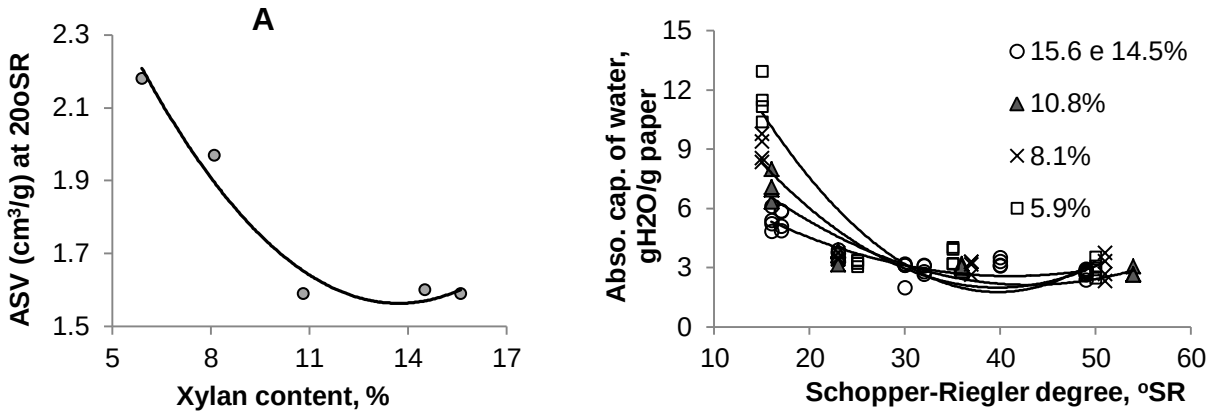


Figure 102. Effect of pulp xylan content on water retention capacity expressed two different ways: (A) at fixed drainage resistance degree of 20°SR, (B) at various drainage resistance degrees.

The Klemm capillarity is an assay that measures the rate of absorption of liquids, being a very important in test for tissue grade pulps. This property indicates the easiness by which the water is absorbed by the capillaries within the paper. Thus, pulps that present higher Klemm capillarity tend to have less bonding and greater amounts of empty spaces (Castanho, 2000). The beating aims to increase inter-fiber bonding *via* hydrogen bonding by the hydroxyl groups in the cellulose chains. The frequency of these bonds can be increased with a larger contact area between fibers. Therefore, the movement of water molecules in the fiber is restricted. As a result, the values of Klemm capillarity tends to decrease with increasing beating intensity.

It can be seen in Figure 103A that the decreases of xylan content in the pulp increased Klemm capillarity, i.e., increases the distance that the water flows into the paper structure. One explanation for this is that water that moves through the paper structure interacts greater with the hemicelluloses than with the cellulose chains. It is known that hemicelluloses are amorphous, thus hampering the displacement of water in the paper structure. Thus, papers made from pulps with lower xylan content are favourable for the production of tissue paper because they have good absorption of liquids, which is desirable during the tissue paper applications**.** Figure 103B shows the results of the Klemm capillarity according to the refining energy consumption for the papers produced from five samples of bleached pulps. One of the major effects of the refining process is the increase of pulp fibrillation which results in an increase in surface area for the beaten fibers. Besides the increase of interfiber bonding, the refining also produces fines. As a result, the movement of water molecules within the fiber network is more difficult. Then, the Klemm capillarity values tend to decrease by increasing the refining energy.

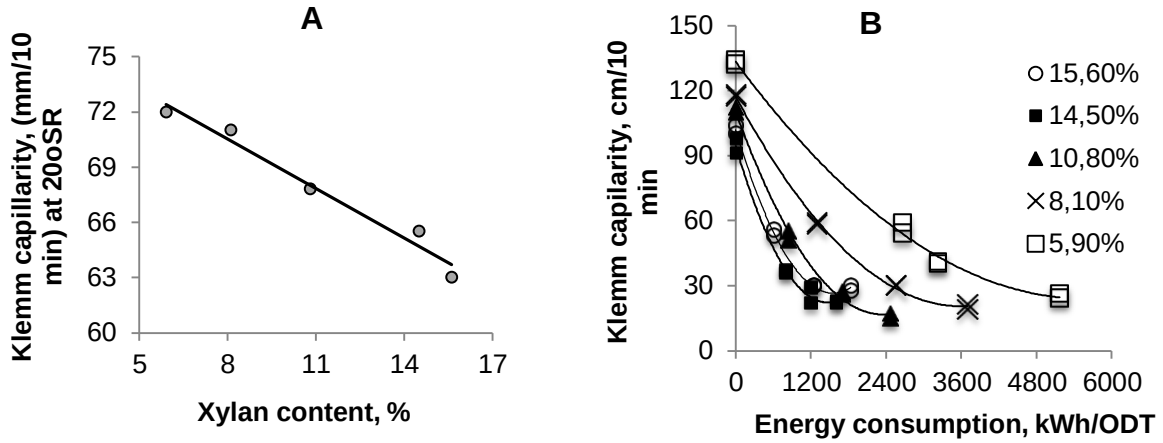


Figure 103. Effect of pulp xylan content on capillarity Klemm expressed two different ways: (A) at fixed drainage resistance degree of 20°SR, (B) at various beating energy consumptions.

It is desired that the tissue paper possess a minimum tensile strength enough to run the paper machine but not too high to a point where it becomes harmful for paper softness. It is observed in Figure 104A that xylan content negatively influences pulp tensile index. However, a pulp containing about 8% xylans possess a tensile index sufficiently high for tissue grade applications with the benefit of being softer that the reference pulp for example. This is advantageous for tissue grade pulp production. Figure 104B shows the tensile index curves for pulps produced at various refining energy consumptions. It can be seen that pulps with higher xylan contents require less energy to achieve a given tensile index.

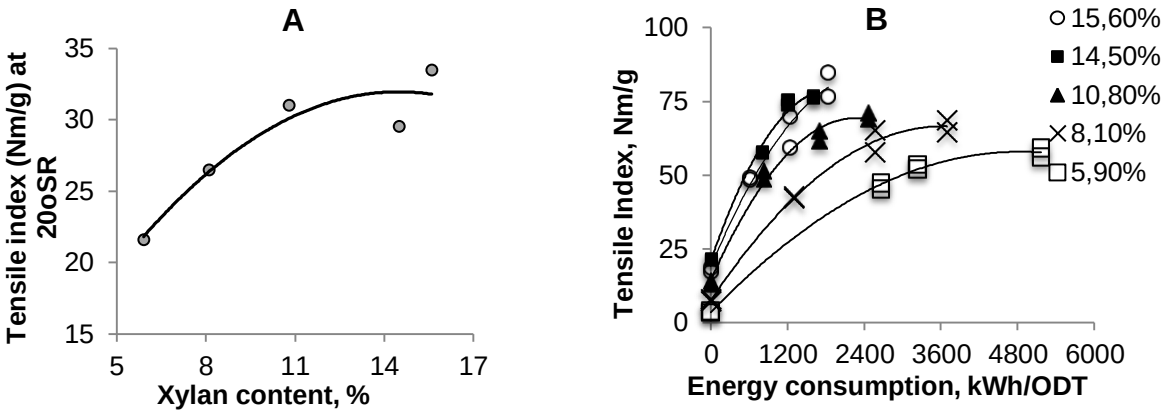


Figure 104. Effect of pulp xylan content on tensile index expressed two different ways: (A) at fixed drainage resistance degree of 20°SR, (B) at various beating energy consumptions.

Tear index is the average force required to tear a paper sheet divided by its weight. The tear resistance test is used to evaluate the resistance of papers such as bags, labels, printing and writing, tissues and other papers that requires tear strength during their use (‘DAlmeida, 1988). Figure 105A shows that that tear strength correlate positively with pulp xylan content at a 20 °SR. Tear index indirectly measures the strength of individual fibers. The results obtained for the tear index in this study is in accordance with published data (Rebuzzi, 2006), that report tear values of 3 and 9 mN.m2/g for sulphite pulp and kraft pulps containing 6% and 17% pentosans, respectively. Figure 105B shows the curve of tear index for pulps produced at various bating energy consumptions. It is observed that pulps with higher contents of xylan, at the same tear index, required less energy when compared to the pulps with lower contents of xylan. For all pulps, the tear index increased with increasing beating energy to a certain level but with the intensification of beating the tear index decreased. This beating causes damage to the fiber structure,

causing reduction of fiber length. Pulps of low xylan content tend to have low tear index due their high refining intensity requirements and consequent larger fiber damage.

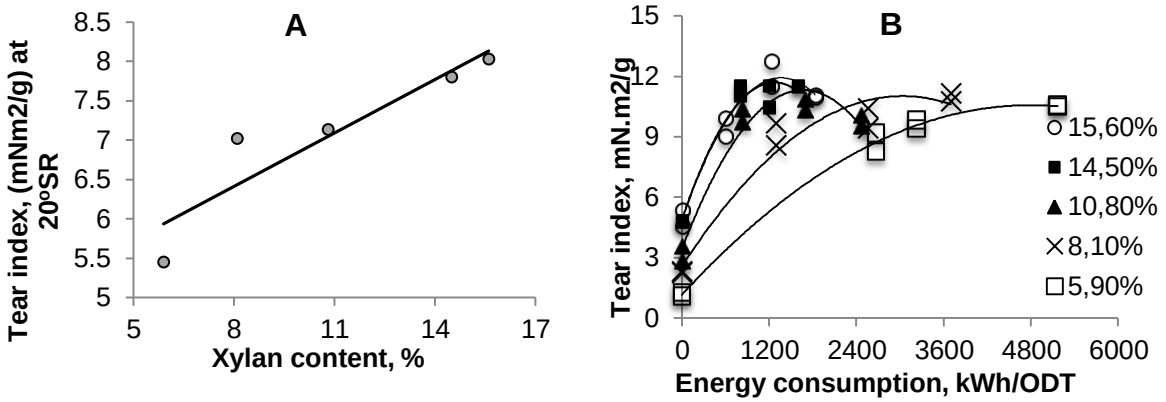


Figure 105. Effect of pulp xylan content on tear index expressed two different ways: (A) at fixed drainage resistance degree of 20°SR, (B) at various beating energy consumptions.

The apparent specific volume, also known as bulk, is calculated by dividing the thickness (µm) of the paper for its weight (g/m^2). The structural characteristics of the fibers also influence the determination of this property. Stiffer fibers produce bulkier papers due to their low ability to form a fiber mat (Howard, 1992). Figure 106A shows the effect of pulp xylan content on bulk calculated for 20°SR, and indicates that bulkier paper is obtained with pulps of lower xylan contents. Bulkier pulps are interesting for tissue grade paper manufacture because they present high internal softness. It can be observed that the reference sample and the one containing 14.5% xylans showed similar bulk, and are represented by a single curve in Figure 106B. The other samples had significant differences in their bulk values. With the increasing beating energy the bulk property tended to decrease for all samples regardless of their xylan contents, but the trend was maintained.

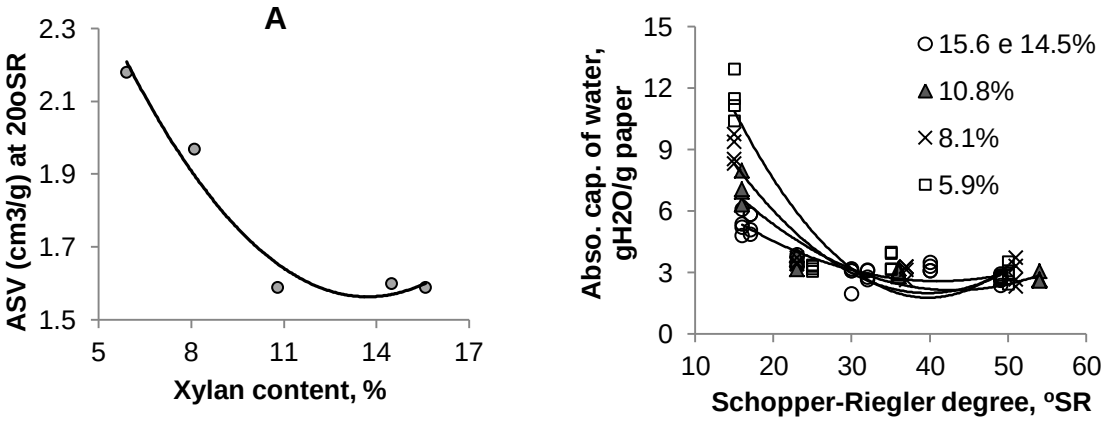


Figure 106. Effect of pulp xylan content on apparent specific volume (ASV) – bulk, expressed two different ways: (A) at fixed drainage resistance degree of 20°SR, (B) at various beating energy consumptions.

Paper optical properties are attributable to the amount of inter-fiber bonding, the number of optical surfaces and the refractive particles of light scattering potential (Carpim, 1987). Pulps with low xylans content have higher light scattering coefficients (LSC) and opacity, which can be explained by the influence of xylans in the inter-fiber bonding. The decrease in xylans reduces the interfaces fiber - air, thereby reducing the LSC of the pulp (Figure 107A). Figure 107B shows that the LSC of the pulps was influenced by the content of pulp xylans regardless of beating energy applied. Pulps with higher xylan content demanded less energy to achieve a given LSC. The intensification of refining caused a negative effect on the LSC, and its values abruptly decreased with increasing pulp xylan content.

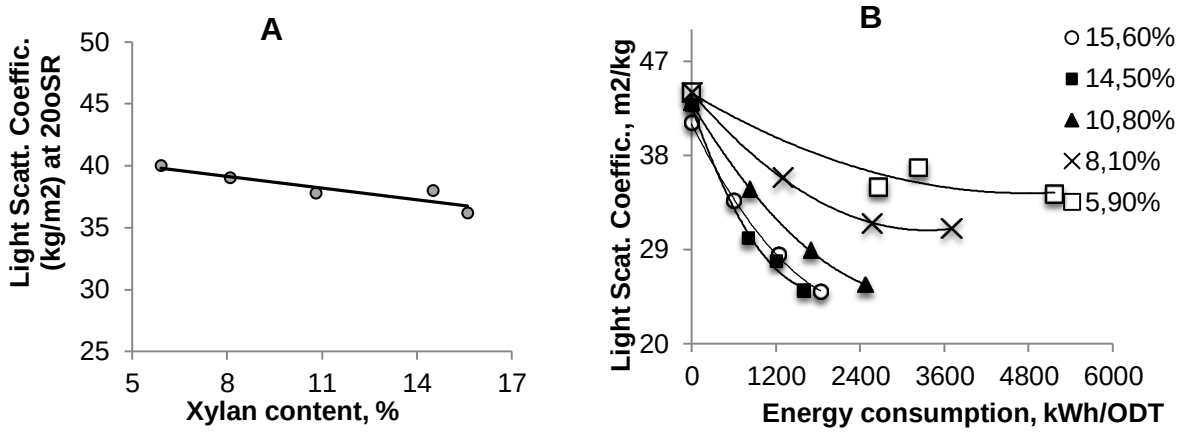


Figure 107. Effect of pulp xylan content on light scattering coefficient, expressed two different ways: (A) at fixed drainage resistance degree of 20°SR, (B) at various beating energy consumptions.

Conclusions on WP4

Several enzymatic fiber modification trials have been conducted with the unconventionally deconstructed materials. Most of the deconstructed materials were readily modified by the cellulase treatments and the prepared handsheets showed improvement in tensile strength. Specifically the Cel45A and Cel7b endoglucanase families demonstrated the ability to improve resultant handsheet physical strength properties, the latter enzyme treatment without sacrificing tear strength. Also the Soda-AQ*E. globulus* fiber (both kappa 15 and 20) showed to be susceptible to enzymatic strengthening, resulting in tensile strengths equal to the otherwise stronger kraft fiber, thus enabling a completely sulphur-free pulping process. Refining trials carried out on the selected Soda-AQ*E. Globulus* pulps did not reveal any significant difference in the physical properties between the kappa 15 and 20 pulps, except in tear strength where the kappa 20 pulp was superior. The enzymatic treatments of these pulps with both Cel45A and Cel7b revealed the need for further process optimization in order to obtain the expected benefits, although large increases in freeness developments were identified as well as increases around 10% in tensile strength after 100 PFI rev.

Furthermore these unconventional pulps were investigated with regards to their susceptibility enzymatic delignification (i.e. bleaching) by the application of oxidoreductases both alone and in combination with mediators. These trials revealed that the high redox-potential laccase from *Polyporus pinsitus* showed synergistic behaviour when combined with violuric acid, reaching a brightness increase of 16 units for the kappa 20 pulp. Also interesting was it that the same absolute brightness values were obtained when kappa 15 and kappa 20 pulps were subjected to the enzymatic bleaching, which can be translated into increased pulp yield and cost savings on chemicals. Data is presented throughout the report which continuously favours the use of this unconventional sulphur-free pulp, especially at kappa 20, with regards to enzymatic strengthening and enzymatic bleaching. The enzymatic delignification system was further optimised with the *Coprinus cinereus* peroxidase combined with violuric acid and it was shown that this system increased brightness by 7 units at a very low dosage of 0,3 mM of violuric acid and 0,5 mM of hydrogen peroxide.

It was also shown that conventional bleached kraft pulp with little effort could be converted into dissolving pulp of a proper grad via enzymatic routes. The application of xylanases and cellulase had positive influence on the solubilities (S10 and S18) and the intrinsic viscosities of the investigated pulps, thus enabeling a regular kraft mill to obtain dissolving pulp grades without the need for a pre-hydrolysis step.

The LGF organosolv cooking with ethanol solvent and phosphinic acid catalyst produced well hydrolysable pulp with high ethanol yield, and the hydrolysability was further improved by alkaline extraction of the LGF fibers. Cooking time between 16-20h was optimal at relatively low cooking temperature of 130˚C with 3.5% phosphinic acid and 15% water content. In comparison with other deconstruction methods, the oxidative alkaline pretreatment ($NaOH+O_2$) provided also well hydrolysable pulp at high kappa levels of 35-50, being another potential pretreatment method for bioethanol production from eucalyptus and elephant grass. Enzymatic laccase and hydrolytic pretreatments alone were not sufficient to open up fiber ultrastructure for efficient enzymatic hydrolysis, and thus ethanol fermentation. The investigations of the biogas potential of the various substrates used in LignoDeco revealed that the raw feedstock's themselves were rather recalcitrant and produced very little biogas, except for elephant grass which showed the highest yields. With regards to both the conventional and unconventional deconstructed materials, all showed to be good substrates for biogas production, except the elephant grass which was inferior to the other tested substrates. However it is not advised to produce biogas from these perfectly nice pulped fibres, rather to used waste streams containing these and rejects for biogas production. The investigations of the mill sludge effluent are still inconclusive giving the very short incubation time, which hardly gives off any right evaluation of the potential biogas potential.

The main objective of Task 4.3 was assessing the potential of upgrading xylans as paper grade pulp additives. The results obtained were relevant to fine tune the methodology and assess the potential of the technology. Positives result were obtained, such as: (1) the ideal conditions for xylans extraction are: 15 minutes, 15% of consistency and 400 kg/odt of NaOH; (2) Grass xylan deposition on wood pulp was successful, increasing *the* xylan content from 14.4 to 17.3%; (3) The deposited xylans are stable across bleaching and beating; (4) Bleaching yield gains of about 3% due to xylans deposition were achieved; (5) Pulps with improved strength properties and beatability were obtained; (6) The removal of the xylans from pulp using alkali treatment reduced significantly the chlorine demand in the bleaching sequence DPD at 90% ISO brightness; (7) the xylan depleted pulps derived from the xylan extraction treatment showed potential for production of special tissue grade papers with improved drainability, bulk, softness and water absorption capacity, and with acceptable tensile and tear strength.

3.1.5. Progress on WP5. Demonstration activities

T.5.1. VTT organosolv demonstrations

The upscaling of LGF organosolv process was performed using *E. globulus*. Although the *E. globulus* did not show best bioethanol production potential in lab scale comparison of feedstocks, it was selected for the pilot demo as it best represents the Eucalyptus species available also in Europe. The same raw material was also used in pilot demonstration of CTP, allowing direct comparison of the pretreatments.

The LGF cooking was performed for 20 kg (b.d.) batch of screened and dried *E. globulus* chips using a forced circulation reactor of 250 l at temperature of 130°C with 3.5% H_3PO_2 and 15% water for 20 h. During cooking, the organosolv liquor was circulated through the chips continuously. After cooking, the pulp was washed with hot ethanol:water (85:15) mixture, followed by washing with water. After LGF cooking, the alkaline extraction of the pulp was performed with 1M NaOH at 2.5% consistency over night at room temperature.

Pilot scale cooking efficiency compared to lab results

The pilot demonstration resulted in LGF pulp with 56 % yield, and 51% total yield after alkaline extraction. This was somewhat lower than at lab scale, but also the delignification efficiency was better. This is probably due to the circulation of the liquor through chip pad throughout the cooking, and more efficient washing with hot liquors. Cellulose yield was comparable to the laboratory pulp.

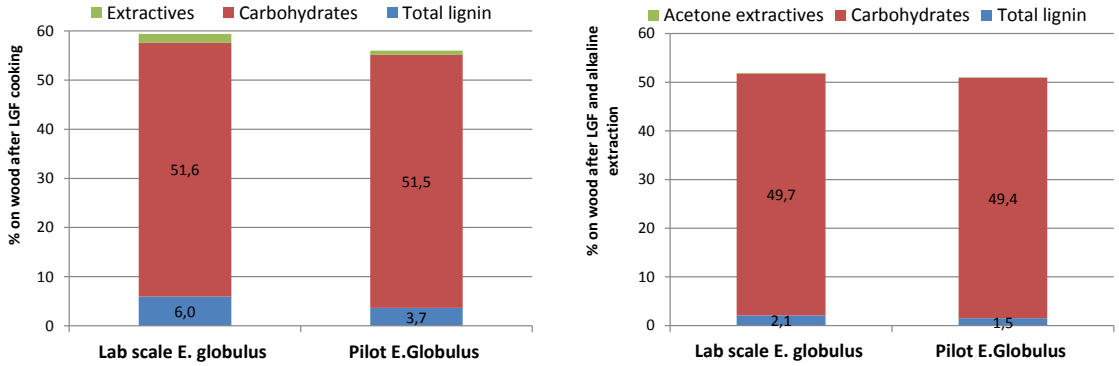


Figure 108. Chemical composition of E. globulus lab and pilot pulps after LGF cooking (left) and the following alkaline extraction (right). All the results are normalised to pulp yield.

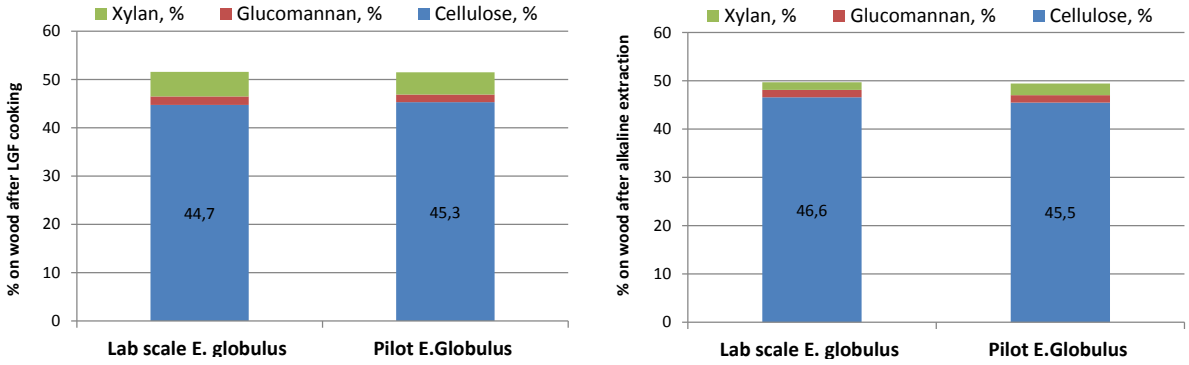


Figure 109. Carbohydrate composition of E. globulus lab and pilot pulps after LGF cooking (left) and the following alkaline extraction (right). All the results are normalised to pulp yield.

Bioethanol production potential

Despite the similar cellulose content, the hydrolysability of pilot pulp was significantly better compared to the corresponding lab pulp, or any of the LGF pulps produced at lab scale (*Suzano/DGxU2*). This is probably due to the more efficient delignification and also lower xylan content. After alkaline extraction, the maximum hydrolysis yield was reached already after 24h hydrolysis.

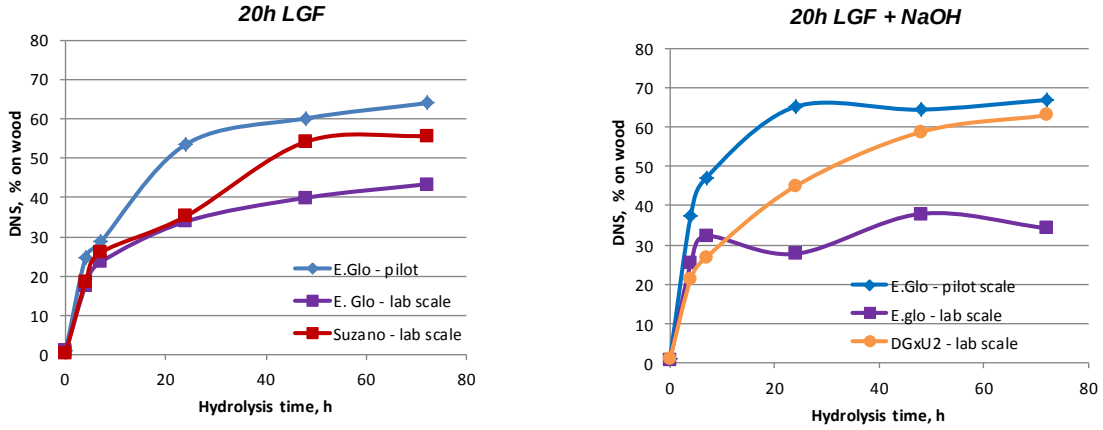


Figure 110. Enzymatic hydrolysability of the E. globulus pilot and lab pulps compared to the best pulps prepared in similar conditions at lab scale.

In comparison with the alkaline oxidation pretreatment, the LGF organosolv cooking at pilot scale produced slightly better bioethanol yield. After alkaline extraction of LGF pulp, clearly higher bioethanol production was shown compared to the Soda-O2 pulp. The better bioethanol yield of LGF pulp was in line with higher cellulose and lower xylan content compared to Soda-O_2 pulp.

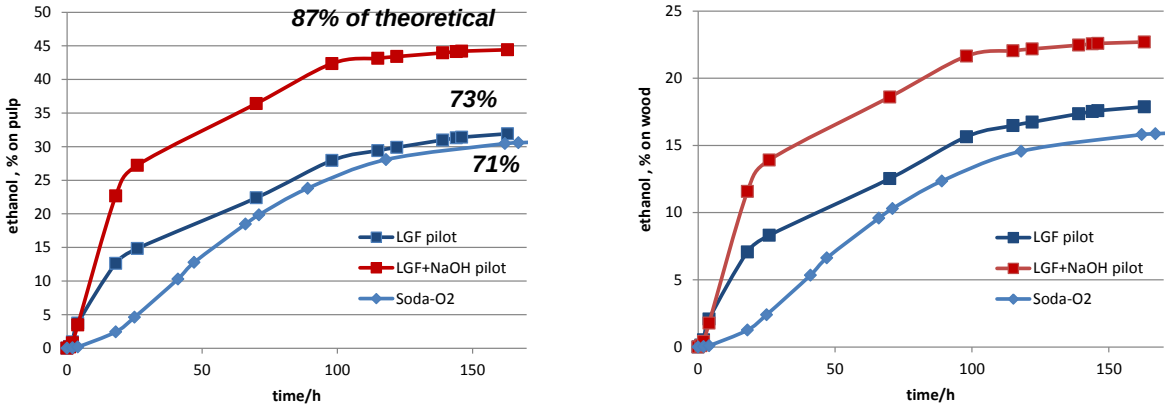


Figure 111. The bioethanol yield of LGF pilot pulps compared to the pulp after alkaline oxidation pretreatment.

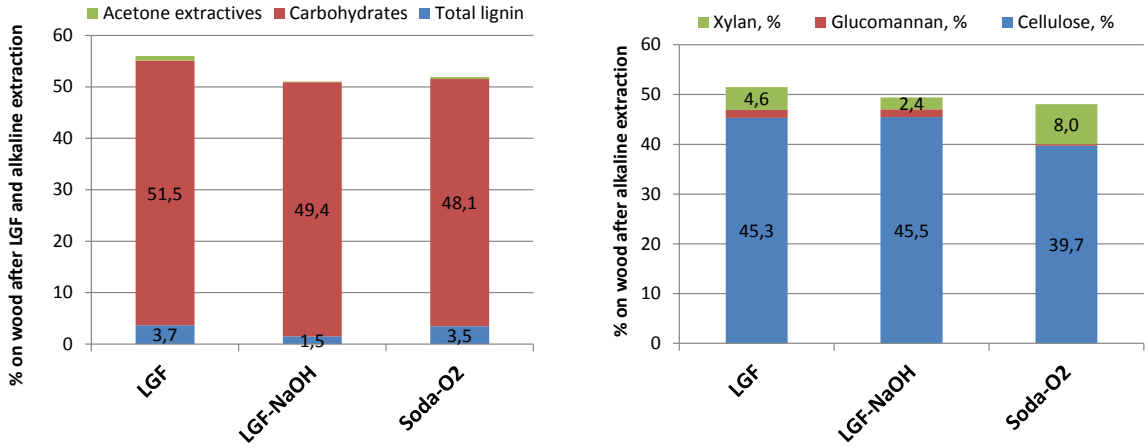


Figure 112. The chemical compositions of LGF pilot pulps compared to the pulp after alkaline oxidation pretreatment.

Task 5.2. CTP enzymatic pre-treatment and bleaching pilot plant trials

From the previous report from **CIB**, we had concluded that NS-22086 would be a suitable enzyme preparation to investigate further for incorporation into whole EG through the use of the MSD Pressafiner available at **CTP**. In order to establish some reaction parameters, the effect of pH and hydrolysis medium was investigated at **CIB**.

The solubilisation, reducing sugar release and acetic acid release from whole EG and Pressafiner pre-treated EG was investigated over a pH range of 5-7.4 (**Fig. 113**). As reported before at a single pH value, the hydrolysis of pre-treated EG by NS-22086 was slightly more extensive than the hydrolysis of the initial whole material. pH 6 was the optimum value for hydrolysis, the degree of solubilisation dropping as the pH was increased further. As this was the same for both substrates, it shows that the Pressafiner pre-treatment did not change the physico-chemical properties of EG and the optimum reflects the enzymes present in the cocktail. The reducing group levels in the hydrolysates did show differences with respect to reaction pH. While the initial material has a pH optimum of 6, the pre-treated material has a broad high level of reducing groups between pH 5 and 6.2, dropping thereafter as the pH increased. At pH 7.4, the amount of reducing groups generated through the action of the enzymes was 3 to 4-fold less than the optimum value. The differences in degree of solubilisation was not so drastic and suggests that the enzymes responsible for matrix loosening and solubilisation of poly and oligomeric material still function at the more neutral pHs, while the ability of this material to be broken down into small oligos and monosaccharides has been lost at this higher pH. The release of acetic acid from both EG substrates remained high across the pH range, but the highest level was recorded between pH 5.8 and 6.0 for the initial EG.

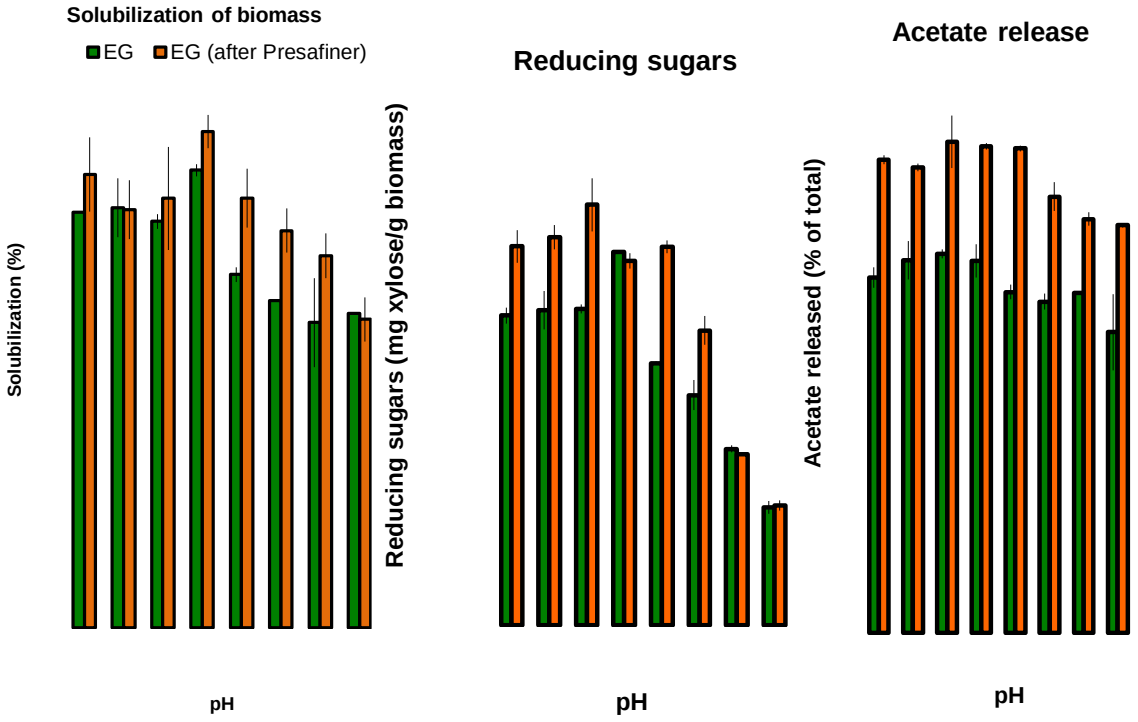


Figure 113. The effect of pH on the hydrolysis of EG by NS-22086 at 50^{o}C.

The next selection criterion was that of a suitable solvent. EG at 5% (w/v) consistency, was incubated with or without NS-22086 at 50^{o}C in the presence of water, 50 mM phosphate (pH 6.0) or 50 mM MOPS (pH 6.0). The results are shown in Figure 114.

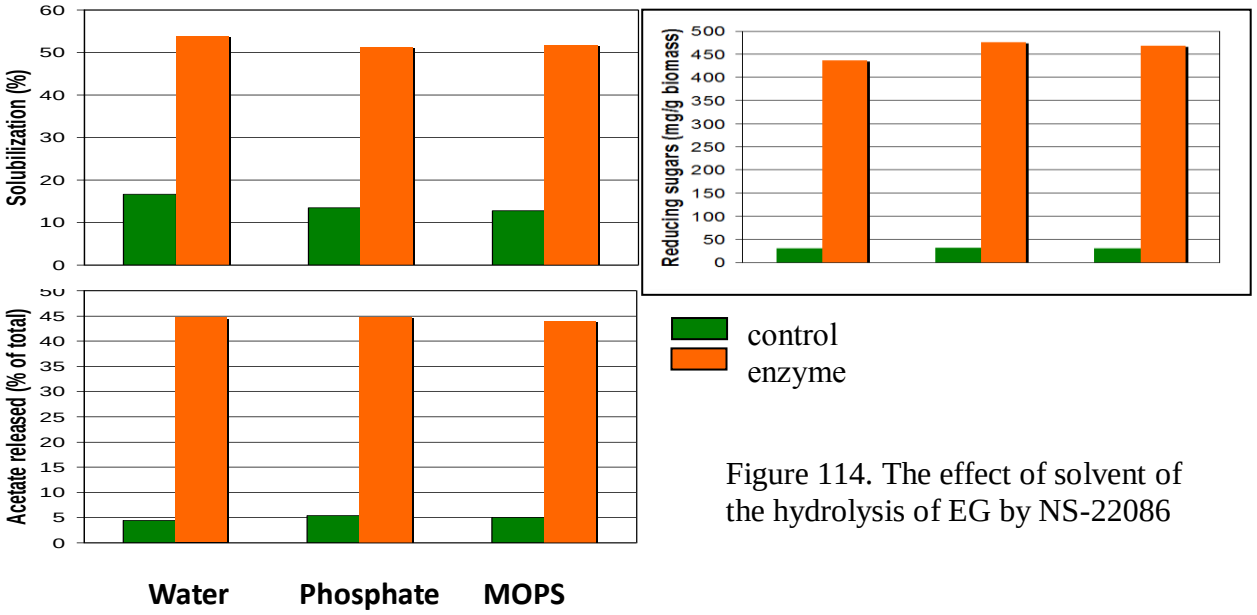


Figure 114. The effect of solvent of the hydrolysis of EG by NS-22086

There was no difference between the 3 solvents examined, with or without enzyme. So it is suggested that CTP should use the following conditions for the Pressafiner trials:

- **Enzymatic phase-1** (impregnation with cellulases using MSD-pressafiner): Elephant grass (2 kg, o.d.) passed through the MSD-pressafiner and the "extruded" material directly drop (for impregnation) on the Cellulase cocktail (Cellulase NS22086 from **Novozymes**; 100 mL/kg) (if required for mixing in phase-2, some extra water could be added to the enzymes)

- **Enzymatic phase-2**: The cellulase impregnated grass incubated at 50ºC for 24 h in storage chest or other reactor with mixing (consistency as high as possible but enabling mixing). No azide to be added.

- **Controls**: 1) Initial Elephant grass (without any treatment); and 2) Treatment (MSD-pressafiner and incubation) without enzyme

- **End**: Whole (wet) material to be sent to **VTT** (1.5 kg dry weight/each treatment) for (additional) saccharification and fermentation, and **CIB** (50 g dry weight/each treatment)

- **Materials** needed: i) Elephant grass (8 kg); and ii) Cellulase NS22086 (500 mL sent by **Novozymes**)

Task 5.3: Pulp refining pilot trials

The bleached Soda/Antraquinone and Kraft pulps were refined at pilot scale. In the project research programme, Suzano was responsible of this task. However, due to the lower quantity of *E. globulus* wood, this task was transferred to CTP. Suzano needed 80 kg (o.d) of pulp to perform refining demonstration whereas CTP needed only 5 kg (o.d) of pulp.

Refining trials were performed with a simple disc refiner (Figure 115).

Before comparing refining trial on the bleached Soda-AQ and Kraft pulps, different tests were performed in order to identify the best refining conditions.

Three refining intensities by adapting the specific edge load (0.1, 0.2 or 0.4 W.s/m) were tested in conditions described in Table 9.

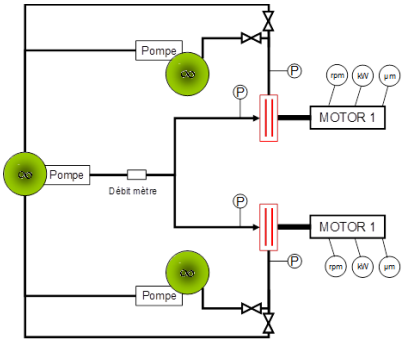


Figure 115. Simple disc refiner pilot

Temperature,°C	40
Pulp consistency,%	4
Flow,m^3/h	7.5
Rotation speed, tr/min	1500

Table 54. Refining conditions

	angle	a (1/16")	b (1/16")	c (1/16")	Length of cut blade (km/tr)	Length of cut blade at 1500 tr/mn (km/s)	Refining intensity, Ws/m
Rotor	5	2	2	3	0.82	20.50	0,4
Stator	5	2	2	3	0.82	20.51	0,4
Rotor	5	1.5	1.5	2	1.77	44.25	0.2
Stator	5	1.5	1.5	2	1.77	44.25	0.2
Rotor	15	1	2	5	3.80	95.50	0.1
Stator	15	1	2	5	3.80	95.50	0.1

Table 55. Refining disc characteristic

The Figure 116 present mechanical properties of bleached Soda-AQ pulp refined with different refining intensities versus energy consumption. Properties were also examined in annex 1 versus drainage index.

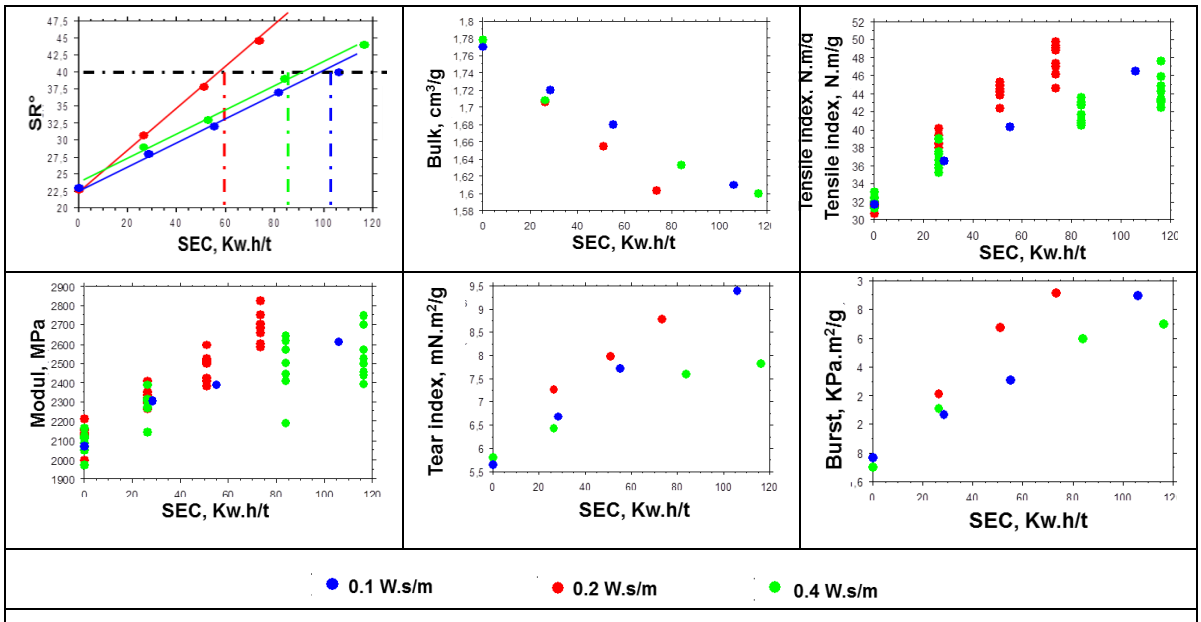


Figure 116 : Mechanical properties of bleached Soda/AQ pulp refined with different refining intensities.

For similar energy consumption, the best mechanical properties were obtained with 0.2 Ws/m refining intensity, because the eucalyptus chemical pulp fibres were quite amenable to fibrillation and therefore to refining.

The Figure 117 present mechanical properties of bleached Kraft pulp refined with different refining intensities versus energy consumption. Properties were also examined in annex 2 versus drainage index.

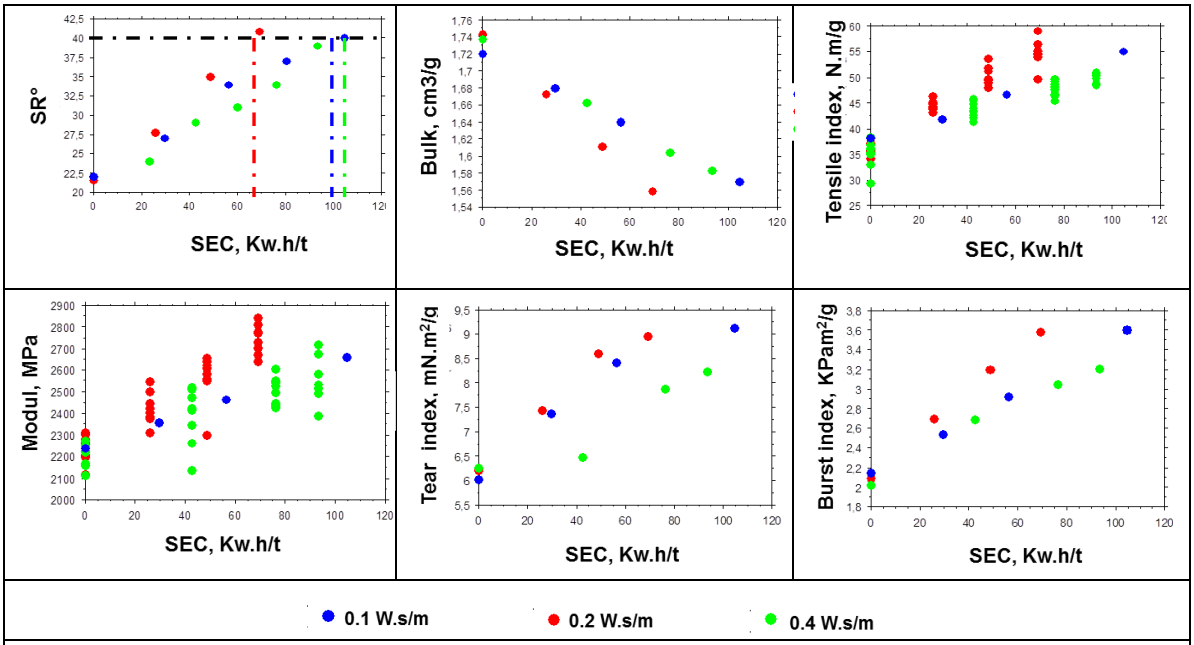


Figure 117 : Mechanical properties of b bleached Kraft pulp refined with different refining intensities.

Similar results were obtained for the Kraft pulp. The best mechanical properties were obtained with 0.2 Ws/m refining intensity.

The refining of Soda/Anthraquine was compared to the refining of Kraft pulp (Figure 49) in the best refining conditions (0.2 Ws/m refining intensity). Properties were also examined in annex 3 versus drainage index.

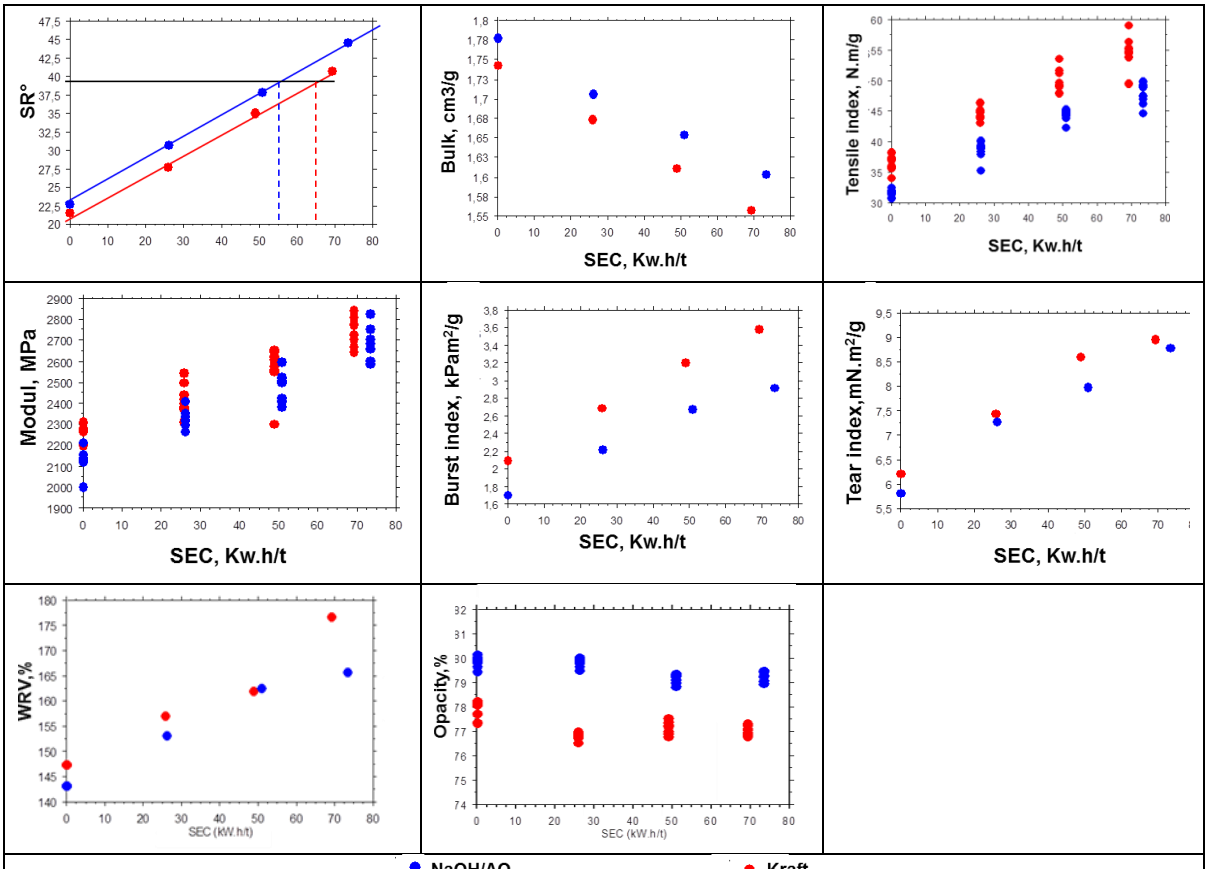


Figure118. Comparison between refining of Soda/AQ and Kraft pulp in the best refining conditions (0.2 Ws/m refining intensity)

Soda/AQ pulp consumed less energy than Kraft pulp to reach a given drainage index. Water retention value of Soda/Antraquinone pulp is lower and opacity is higher than Kraft pulp. However, mechanical properties of Soda-AQ pulp were lower than these of the Kraft pulp (Figure 118). Nevertheless, according to UFV results, the addition of xylans in Soda-AQ pulp allowed to improve mechanical properties and to compensate this difference.

Consequently, before refining, xylans from elephant grass pulp (EG1) were added to Soda-AQ pulp (3%). Refining was carried out on this pulp in the best refining conditions already determined (0.2 Ws/m refining intensity). Figure 50 presents mechanical properties of refined bleached Soda-AQ pulp with or without addition of xylans before refining.

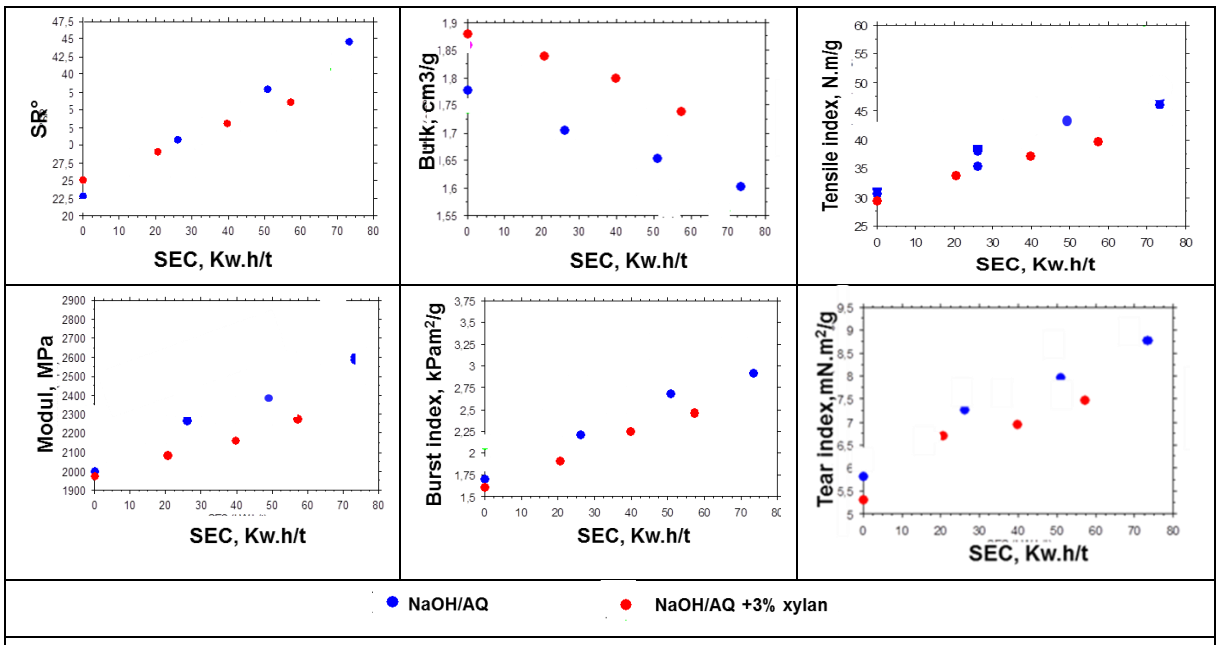


Figure 119. Comparison between refining of Soda/AQ pulp with and without addition of xylans (3%/ pulp)

According to Figure 50, the addition of xylans reduced the mechanical properties. This strange result was due to the bad solubilization of xylans in the pulp (Figure 51).

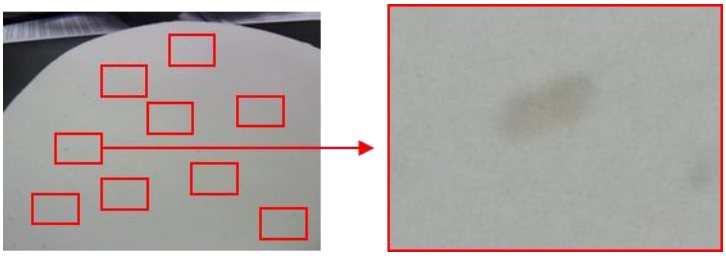


Figure 120 : Presence of xylan unsolibilized in the handsheet obtained after refining

Different techniques were tested to solubilize xylans before its addition in pulp:

- Mixing xylans with water at high speed
- Mixing xylans with water at high speed and at high temperature > 90°C
- Mixing xylans with water and ethanol
- Mixing xylans and sodium hydroxide with different concentrations. However the increase in sodium hydroxide was limited because at high pH, refining was very difficult to manage.

Consequently, other solution was tested to solubilize xylans in the pulp before refining. Bleaching stage (extraction stage) was performed on bleached Soda-AQ pulp and with addition of xylans.

The extraction stage was performed in the same conditions as O stage carried out during bleaching pilot trial (Table 8) without oxygen addition. However, after extraction stage, xylans were again present at the surface of the handsheet produced with this pulp.

Consequently another test with higher pH and retention time was performed (pH 12.5 and retention time of 90 min).
Mechanical properties of bleached Soda-AQ pulp after extraction stage carried out with or without addition of xylans were measured (Figure 52).
The addition of xylans in Soda/Antraquinone pulp during extraction stage did not improve mechanical properties. Insolubilized xylans were again present at the surface of the handsheet.

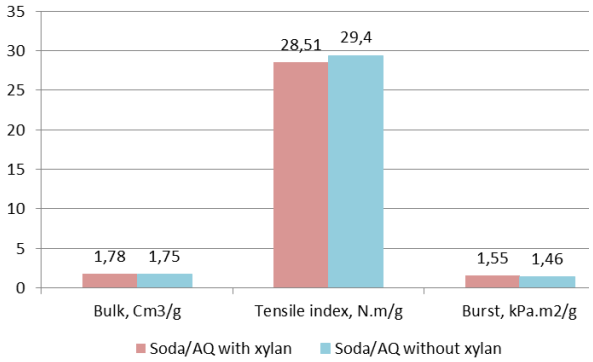


Figure 121. Mechanical properties of bleached Soda/AQ pulp after extraction stage performed with or without addition of xylans (9% /pulp o.d)

Consequently another trial was performed in order to solubilize xylans before the extraction stage. Xylans were added with sodium hydroxide at pH 12.5 in similar conditions as previously used without pulp addition. Then, solution was heated at 98°C during 90 min. This solution contained also insolubilized xylans which were filtered.
Then, This solution was used to perform extraction stage with Soda-AQ pulp. Extraction stage was performed with hydrogen peroxide in order to avoid the formation of chromophoric groups during this stage. After extraction stage, mechanical properties of this pulp were compared to those of Kraft pulp treated in similar conditions without addition of xylans. However, mechanical properties of Soda-AQ pulp were lower than Kraft pulp.

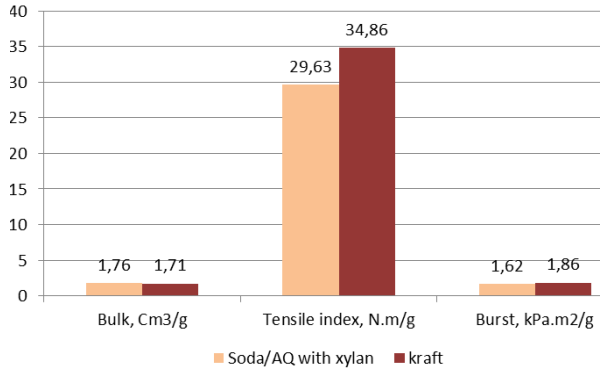


Figure 122. Mechanical properties of bleached Soda/AQ pulp with xylans (9%/ pulp o.d) after extraction stage

During laboratory trial performed by UFV, xylans were well solubilized in the pulp. Xylans used in UFV laboratory were not dried. On the other hand, xylans used at CTP were dried in UFV laboratory. These bad results with xylans could be attributed to their drying. Consequently, it was not interesting to perform pilot refining trial with these dried xylans.

Conclusions of WP5

The LGF organosolv cooking was successfully demonstrated also at pilot scale, producing well hydrolysable biomass with high ethanol yield. Nearly 90% of the theoretical ethanol yield was reached after the alkaline extraction of LGF pulp. This was higher compared to the alkaline oxidation, which also produced well hydrolysable pulp for bioethanol production. As reported in WP4, the bioethanol yield after enzymatic treatments at pilot scale was very limited.
Alkaline demonstration trial at pilot scale validated results obtained at laboratory. Soda/ Anthraquinone pulp could replace Kraft pulp. It is a sulfur-free process which is interesting for limiting air pollution. However, Soda/ Anhraquinone pulp had lower mechanical properties than Kraft pulp. However, according to UFV laboratory trial, addition of xylans from elephant grass on Soda/Anthraquine pulp can improve mechanical properties.

The enzymatic deconstructions using oxido-reductases or hydrolases performed in laboratory were also validated at pilot scale. Unfortunately, the corresponding production of bioethanol was not conclusive.

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3.2. Deviations from the Work Plan

No deviations were produced from the original Work Plan

4. PLANS FOR THE DISSEMINATION AFTER THE 36th months

1. UFV

During the course of the project we have already published several papers regarding the detailed composition of elephant grass and the selected Brazilian eucalypt hybrids. We are now preparing one more paper which is being submitted in the *Bioresources Journal* for publication, and it is undergoing revision following the demand of editors of the journal.

On the other hand, part of the results obtained during the course of the project will be presented in several scientific congresses, including "*6th International Colloquium on Eucalyptus Pulp*", that will be held in Colonia del Sacramento, Uruguay, during November 24-27, 2013; the "13th European Workshop on Lignocellulosic and Pulp", that will be held in Seville, Spain, during June 24-27, 2014; the 2013 International Symposium on Wood Fiber and Pulp Chemistry, in June 2013, Vancouver, Canada.

Also, one PhD Thesis will be presented at the Federal University of Viçosa with the main results obtained during the course of the project: "The biorefinery concept applied to pulp and paper industry" by Fernando José Borges Gomes.

2. IRNAS / CIB

During the course of the project we have already published several papers regarding the detailed composition of elephant grass and the selected Brazilian eucalypt hybrids. In addition, we also published a paper regarding the enzymatic pretreatment of elephant grass and eucalypt wood with the high redox potential laccase. We are now preparing three more papers that we intend to submit for publication during the next couple of months. Among these, one of the papers will be dealing with the very promising results obtained regarding the enzymatic pretreatment of eucalypt wood with the commercial low-redox potential laccase from *Myceliophthora thermophila*, and using methyl syringate as mediator. The second paper will be devoted to the structural characterization of the residual lignins and black liquors produced from elephant grass and eucalypt G1xUGL by different chemical alkaline deconstruction processes (kraft, soda-AQ and soda-O_2 processes) at different kappa numbers. Finally, a third manuscript is under preparation including the results on the enzymatic degradation of elephant grass stems by hydrolases and the influence of the pith and bark in the total hydrolysis.

On the other hand, part of the results obtained during the course of the project will be presented in several scientific congresses, including "*The 17th International Symposium on Wood, Fibre and Pulping Chemistry*", that will be held in Vancouver, Canada, during June 12-14, 2013; the "*6th International Colloquium on Eucalyptus Pulp*", that will be held in Colonia del Sacramento, Uruguay, during November 24-27, 2013; the "13th European Workshop on Lignocellulosics and Pulp", that will be held in Seville, Spain, during June 24-27, 2014.

Also, two PhD Thesis will be presented at the University of Seville with the main results obtained during the course of the project: "Study of the chemical composition of fast growing lignocellulosic crops and modification of their lignins during alkaline deconstruction" by Pepijn Prinsen, and "Optimization of laccase-based pre-treatments for the enzymatic deconstruction of woody and nonwoody lignocellulosic feedstocks" by Alejandro Rico.

3. VTT

The results of LGF pulping as a potential pretreatment method for bioethanol production will be presented in the 17th International Symposium on Wood, Fibre and Pulping Chemistry, Vancouver, Canada, June 2013.

- Tamminen, T., Barth, D., Colodette, J., Liitiä, T. Organosolv pulping as pretreatment for bioethanol production from Eucalyptus and Elephant grass
- Two joint papers together with IRNAS are under preparation on characterisation of residual and spent liquor lignins of alkaline and LGF cooking.

5. DELIVERABLES AND MILESTONES

List the deliverables and milestones you are responsible due during the period covered by the report indicating whether they have been achieved.

DL2.7 Pre-treatment conditions using selected enzymes (**CIB**, CTP, Novozymes; 24th month) has been achieved in due time.

DL2.8 Pre-treatment materials for characterisation and evaluation – 2 (UFV, **CIB**, IRNAS, VTT, Novozymes, Suzano, CTP; 24th month) has been achieved in due time.

DL2.9 Conditions for scaling up the enzymatic deconstruction (**CIB**, IRNAS, Novozymes; 28th month) has been achieved in due time.

DL3.4 Characterisation of pretreated non-woody materials using advanced analytical tools (**UFV**, IRNAS, VTT; 18th month) has been achieved in due time.

DL3.5 Characterisation of pre-treated woody materials using advanced analytical tools – 1 (UFV, IRNAS, **VTT,** CTP; 24th month) has been achieved in due time.

DL3.6: Characterisation of black liquors and other side streams (**UFV**; 24th month) has been achieved in due time.

DL3.7 Characterisation of pre-treated nonwoody materials using advanced analytical tools – 2 (UFV, **CIB**, IRNAS, VTT, CTP; 32th month) has been achieved in due time and submitted to UFV.

DL3.8 Characterisation of pre-treated woody materials using advanced analytical tools – 2 (UFV, **CIB**, IRNAS, VTT, CTP; 32th month) has been achieved in due time and submitted to UFV.

DL4.1 Pulp and papermaking evaluation after optimised pre-treatment (**UFV**, Novozymes; 32th month) has been achieved in due time.

DL4.2 Evaluation of pretreated materials and residues for bioethanol production (**VTT**; 36th month) has been achieved in due time.

DL4.3 Setup of application method for anaerobic digestion and evaluation of pre-treated materials for biogas production (**Novozymes**; 36th month) has been achieved in due time.

DL4.4 Procedure for improving eucalypt pulp with grass xylan additive (**UFV**; 36th month) has been achieved in due time.

DL5.1 Pilot scale solvent deconstruction trials (**VTT**; 14th month) has been achieved in due time.

DL5.2 Pilot-Scale enzymatic deconstruction trials and ECF pulp bleaching trials (**CTP**; 36th month) has been achieved in due time.

DL5.3 Pilot-scale pulp beatability trials (**Suzano**; 36th month) has been achieved in due time.

DL6.1 FUNARBE financial report (**UFV**; 36th month) has been achieved in due time.

6. NEW CONTACT PERSONS

In case that any of the responsible persons of any of the beneficiaries is replaced for a particular reason, please explain and indicate the name and contact details of the new contact person.

Not applicable

APPENDIX A

UFV

Grant agreement no: KBBE-2009-3-244362
Optimized pre-treatment of fast growing woody and nonwoody Brazilian crops by detailed characterization of chemical changes produced in the lignin-carbohydrate matrix

Second Periodic Report

1-Jul-2011 to 31-Dec-2012
Partner P1 (UFV)

Grant agreement no: **KBBE-2009-3-244362**

Project acronym: LIGNODECO

Project title: Optimized pre-treatment of fast growing woody and nonwoody Brazilian crops by detailed characterization of chemical changes produced in the lignin-carbohydrate matrix

Funding Scheme:	Collaborative project (small or medium-scale focused research project)		
Thematic Priority:	KBBE-2009-3		
Period covered:	From 1 July 2011 to 31 December 2013		
Date of preparation:	15 January 2013		
Start date of project:	1 January 2010	Duration:	36 months

Partner name:	**UFV**
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Author 4	*Rubens Chaves de Oliveira*
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Author 6	*Danila Morais de Carvalho*
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Author 22	*Marco Maurici Araújo Alves*
Author 23	*Marina Ulisses da Silva*
Author 24	*Nara Brandão Costa Santos*
Author 25	*Phillipe de Souza Ávila*
Author 26	*Vitor Rodrigues Matos*

*Use the font **Times New Roman**. Font size in text is **12 points**. Page numbering is header - right, no numbering on the front page. Line spacing single, 0 point before and after paragraph.*

For those who have been active in research, the appropriate length would be 5–10 pages. For the institutes/companies that have not worked, please report only the appropriate items e.g. plans.

1. SUMMARY OF THE WORK

The UFV report for these last eighteen months presents developments on task *T1.2- General characterisation of the lignocellulosic feedstocks* of WP1, *Task 3.4 - Analysis of black liquors and other side stream* of WP3 and ***Task 4.3- Upgrading xylans as paper grade pulp additives*** of WP4**. On Task 1.2, the** new *Eucalyptus globulus* wood batch was characterized and it was confirmed that its characteristics were similar to the previous sample and thus very suitable for the demonstrations on the production of pulp and bioproducts. The main traits of this new sample included: (1) low lignin content; (2) low metal content; (3) high basic density and; (4) high carbohydrate content. Therefore, this new wood batch used for the demonstration tasks without restrictions. Regarding **Task 3.4** the main findings on black liquor characterization were: (1) the black liquor sulfur content is lower for the pulp produced at kappa 20 than at kappa 15; (2) the black liquor gross heating value is superior for pulps produced at kappa 20 than at kappa 15; (3) the gross heating value of black liquor derived from Soda-AQ process is higher than that derived from kraft process; (4) the black liquor derived from soda-AQ pulping of elephant grass has lower heating value than that from wood at a given kappa number; (5) black liquor derived from pulping of elephant grass has lower carbon content and unusually high nitrogen content when compared to wood black liquor; (6) when cooking eucalyptus wood to kappa 20, a small but significant amount of xylans of reasonable MW remains in the black liquor; (7) recovery of such xylans (~1.5% on pulp weight) is possible by adding black liquor to the O-stage (alternatively through poor washing); (8) the addition of black liquor impairs the O-stage only slightly but impairs bleaching significantly (+2 kg ClO_2/odt); (9) yield and pulp quality gains must be weighed against increased ClO_2 demand. The **Task 4.3** focused on the xylan management in a pulp mill, either extracting xylans from a given pulp and evaluating its properties, or depositing such xylans into another pulp furnish and evaluating its properties as well. The main findings on the deposition of grass xylans onto wood pulp were: (1) O-stage grass xylans deposition on wood pulp was successful, increasing the wood pulp xylans content from 14.4 to 17.3%; (2) the deposited xylans were proven stable across pulp bleaching and pulp beating; (3) bleached yield gains of about 3% were achieved due to xylans deposition; (4) the deposited xylans caused improved wood pulp beatability and strength properties, including tensile and burst indexes, modulus of elasticity, tensile energy absorption and stretch. The main findings on the elephant grass xylan depletion (by alkali extraction) were: (5) the removal of xylans increased bulk, opacity and light scattering coefficient while decreased the tensile index; (6) the tensile index of the pulp was affected by the removal of xylans, however, after refining the pulps with low levels of xylans achieved the desired values of tensile index was for the production of tissue paper; (7) pulps with lower levels of xylans, within the range studied, had excellent characteristics

for production of tissue paper, including a very high bulk, high water absorption capacity by capillarity as measured by g/water per gram of pulp and high capillarity Klemm.

2. PROJECT OBJECTIVES FOR THE PERIOD

The activities developed on WP1, WP3 and WP4 in the last six months fall within the following LIGNODECO objectives, respectively: (1) evaluation of the feedstock for the pilot test; (2) characterization of the black liquor and other solid streams and their evaluation for energy production; (3) evaluation of xylans as a paper grade pulp additive; (4) evaluation of xylan depleted pulps for tissue grade applications**.**

3. WORK PROGRESS AND ACHIEVEMENTS DURING THE PERIOD

3.1 Work Progress

Provide a concise overview of the progress of your work (results and discussion). Report according to WPs and Tasks

- *Highlight clearly significant results.*
- *Explain the reasons for possible deviations from Annex1 and their impact*
- *as well as corrective actions taken / suggested*

Task 1.2 of WP1: General characterisation of the lignocellulosic feedstocks

A general characterization of the new *Eucalyptus globulus* sample batch intended to the demonstration work was accomplished. This characterization was necessary since the original sample provided by ENCE-Spain was totally consumed in the optimization part of the work and a new sample (large volume) was required for the demonstration work. The large batch of the new sample was collected and evaluated for the same traits as the previous samples. The goal here was proving that the sample to be used for the demonstration studies had similar characteristics when compared to the one used for the optimization study.

Biomass density: Density is a very important factor affecting biomass utilization since it influences harvesting, transportation and processing costs. In this regard, the new *E. globulus* batch showed a slightly higher density (591 kg/m^3) than the European *E. globulus* (532 kg/m^3). A high density means more weight of biomass charge per unit volume of the equipment used for biomass deconstruction, which improves mill productivity. However, the differences between the two materials were not so significant to the point of causing large impact in the demonstration studies.

Biomass Inorganic Composition: The most significant inorganic chemical traits of the various biomass types are presented in Table 1. The total inorganics measured on the new sample by complete biomass combustion (ash content) was 0.25%; this value was slightly lower than that of the previous sample used in the optimization study (0.31%). The low ash content is advantageous since minerals are very detrimental for most industrial utilization of biomass because of corrosion and deposits in equipment, reduced biomass heating value and decreased mill throughput. In general the amount of inorganics present in both eucalypt wood samples were very low and quite acceptable for most industrial applications.

Table 1: Inorganic composition of *Eucalyptus globulus* samples used in the optimization and demonstration studies

Composition		*E. globulus (optimization studies)*	*E. globulus (demonstration studies)*
Ash	Ash, %	0.31	0.25
Metals	Cu, mg/kg	2.5	1.5
	Fe, mg/kg	13.3	6.6
	Ca, mg/kg	668.5	307
	Mn, mg/kg	43.4	28.9
	Mg, mg/kg	227.1	175

Biomass Organic Composition: The basic organic composition of the *E. globulus* samples used in the optimization and demonstration studies is presented in the Table 2. Extractives are quite troublesome since they cause many difficulties in operating the industrial facilities, causing unexpected lost time in the operation for cleaning of equipment and instruments due to their stickiness and tackiness. There were no significant differences between the extractive contents of the *E. globulus* samples used for the optimization and demonstration studies, regardless of the extraction methods, i.e., acetone extraction or total extraction (ethanol/toluene (1:2) → ethanol → hot water solvent system).

In order to measure the biomass cell wall components, it is relevant to remove all extractives present in the material. The standard procedure for removing all extractives from biomass is through extraction using ethanol/toluene (1:2) → ethanol → hot water solvent system to prepare the so-called extractive free wood. Table 2 shows the chemical composition of the extractive free *E. globulus* wood samples. The main biomass components that find application for production of biofuel and bio-materials are the cellulose, hemicelluloses and lignin fractions. The new *E. globulus* wood batch showed slightly lower lignin content than the one used for the optimization studies. On the other hand, the new batch presented lignin of lower S/G ratio in relation to the previous sample, but still quite high. As a consequence of its lower lignin content, the new sample presented higher glucan content than the previous sample. In general, the two *E. globulus* samples presented similar hemicellulose sugars (xylans, mannans, galactans and arabinans), uronic acids and acetyl groups content. An overall assessment indicate that that the *E. globulus* samples used for optimization and demonstration studies are similar and shall not disturb the data comparison between the two project phases.

Table 2: Chemical characteristics of *Eucalyptus globulus* samples used in the optimization and demonstration studies

Sample	Acetone Extractive	Total Extractive	Sugar Composition, %					Acid Soluble Lignin, %	Klason Lignin, %	Total Lignin, %	Lignin S/G ratio	Acetyl groups, %	Uronic Acids, %
			Glu-cans	Xylans	Man-nans	Arabi-nans	Galac-tans						
Optimization studies	1.2	2.5	46.6	13.6	1.4	0.2	0.2	4.8	23.8	28.6	4.0	2.6	3.0
Demosntration studies	2.3	3.0	53.0	13.7	0.9	0.3	0.3	2.5	22.7	25.2	3.4	2.0	2.8

Conclusions of Task 1.2

The new *Eucalyptus globulus* sample batch aimed at the demonstration studies showed desirable characteristics for pulp and bioproducts production, namely: low lignin content, acceptable S/G ratio, low metal content, high density and high carbohydrate content. There were no large differences between the main wood traits when comparing this sample with the one used in the optimization studies.

Task 3.4 of WP3: Analysis of black liquors and other side stream

The composition of black liquors and other side streams derived from alkali deconstruction processes (Kraft and Soda-AQ) was characterized in *Task 3.4*. The following characteristics were determined: solid contents, including organic and inorganic, residual effective alkali, heating values and elemental analysis including Na, S, K, Cl, SiO_2, C, H, O and N. The analyses were carried out using elemental analyzer, atomic absorption spectrometer and ion chromatograph. Also studied was the potential of using the black liquor as a source of xylans, since it is known that the Kraft black liquor contains reasonable amounts of high molecular weight xylan.

Black liquor characterization: The material dissolved in the black liquor consist mainly of lignin and degraded carbohydrates (hemicelluloses and cellulose) while the minor part are extractives, proteins and inorganic constituents. Table 3 shows black liquor heating value, total solids, inorganic solids, organic solids and residual alkali. Results of inorganic /organic solids indicate that the eucalypt as well as the elephant grass black liquors obtained by kraft and Soda-AQ processes possess an average of 46% (44.1-52.8%) inorganic matter and 54% (50.2-56.1%) organic matter. Cooking terminated at kappa number twenty resulted black liquors of higher heating value and lower solids content in relation to cooking terminated at kappa 15, indicating less carbohydrate loss in the former case. The Soda-AQ process would be an excellent solution compared to kraft process, since the black liquor from this process presents a higher heating value than the kraft black liquor and is sulphur free. The absence of sulfur compounds in the Soda-AQ black liquor enormously facilitates its further fractionation into valuable components.

Chemically, black liquor is a mixture of several basic elements. The results of Na, S, K, Cl, SiO_2, C, H, O and N are presented in the Table 4 and are expressed in terms of the percentage of the element mass to the total mass of dry solids existing in the liquor. Potassium and chloride are particularly dangerous for their ability to decrease the ash melting point during combustion, thus causing sticky ash problems in recovery boiler systems. In addition, chlorides are highly corrosive and troublesome for most equipment regardless of metallurgy. The amounts of Cl, SiO_2, C, N, H and O contents were very close among the black liquors derived from woody materials. Kraft cooking terminated at kappa 20 resulted black liquor of lower sulfur content than that of cooking terminated at kappa 15.

Black liquors from elephant grass presented very high contents of potassium and SiO_2, which is very undesirable in most industrial processes for their ability to cause deposits in equipment during evaporation of liquid streams and combustion of solid streams. In addition, these liquors showed high nitrogen content, probably due to protein presence in the juvenile grasses with intense metabolic activities. The high nitrogen content is not favorable in combustion processes since nitrogen enhances NOx emissions. Another interesting observation is that the black liquors from elephant grass present low carbon content and, consequently, low gross heating values.

Table 3: Heating value, total solids, inorganic solids, organic solids and residual alkali of black liquors derived from Kraft and Soda-AQ cooking of eucalypt and elephant grass, with cooking ending in kappa numbers 15 and 20.

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		Kappa 15					**Kappa 20**				
		Heating value, cal/g	Total Solids,%	Inorganic Solids,%	Organic Solids, %	Residual AA, g/L NaOH	Heating value, cal/g	Total Solids,%	Inorganic Solids,%	Organic Solids, %	Residual AA, g/L NaOH
Kraft	U1 x U2	3963.1	13.0	46.5	53.5	10.2	3949.6	10.9	45.4	54.6	3.1
	G1 x UGL	3591.7	13.4	44.1	55.9	9.6	3970.6	12.8	49.4	50.6	2.7
	DG x U2	3669.0	12.7	46.3	53.7	9.0	4028.8	11.2	41.4	53.7	3.8
	IP	3657.0	12.5	46.8	53.2	9.4	3920.3	11.0	49.4	50.6	2.6
	IB	3818.5	11.3	48.5	51.5	5.7	3917.6	10.2	43.3	56.7	2.3
	E. Grass	3395.0	12.9	46.0	54.0	5.1	3554.0	10.4	47.5	52.5	4.2
Soda-AQ	U1 x U2	3442.5	13.4	45.4	54.6	22.4	4000.2	13.0	45.2	54.8	12.2
	G1 x UGL	3705.1	13.5	49.7	50.3	20.2	4049.1	11.2	47.6	52.4	10
	DG x U2	3691.3	15.7	46.9	53.1	22.7	4071.3	12.4	43.7	56.3	8.6
	IP	3602.3	13.0	49.8	50.2	18.9	4072.6	11.3	46.1	53.9	7.2
	IB	3748.7	11.7	52.8	47.3	11.8	3851.3	10.3	43.1	56.9	5.3
	E. Grass	3510.5	11.0	46.6	53.4	2.7	3696.0	10.4	43.9	56.1	1.2

Table 4: Elemental analyses of black liquors derived from Kraft and Soda-AQ cooking of eucalypt and elephant grass, with cooking ending in kappa numbers 15 and 20.

		Kappa 15									**Kappa 20**								
Element, %		**Na**	**SiO_2**	**Cl**	**K**	**C**	**H**	**N**	**S**	**O**	**Na**	**SiO_2**	**Cl**	**K**	**C**	**H**	**N**	**S**	**O**
Kraft	U1 x U2	18.1	1.6	0.08	0.08	41.1	3.7	0.1	3.5	33.7	20.7	1.3	0.07	0.08	43.0	3.9	0.1	2.8	34.1
	G1 x UGL	17.8	1.1	0.10	0.07	39.2	3.7	ND	4.5	34.7	17.4	1.4	0.10	0.08	42.0	3.8	0.1	3.6	34.7
	DG x U2	22.3	1.1	0.06	0.09	41.2	3.8	0.1	3.9	34.3	14.6	1.2	0.06	0.07	43.0	3.9	ND	3.3	34.4
	IP	20.5	1.4	0.05	0.06	40.6	3.8	0.1	3.4	34.8	17.9	1.4	0.12	0.07	44.7	4.0	0.1	2.9	34.0
	IB	14.7	1.0	0.06	0.06	41.3	3.9	ND	4.0	35.5	13.8	1.3	0.06	0.07	41.7	3.9	ND	3.1	35.8
	E. Grass	14.9	4.0	1.32	0.33	35.8	3.6	0.5	3.4	34.2	15.4	3.3	1.98	0.50	36.0	3.8	0.6	3.0	34.2
Soda-AQ	U1 x U2	18.3	1.4	0.06	0.06	40.2	3.9	ND	ND	35.2	14.9	1.9	0.07	0.09	44.4	4.0	ND	ND	34.3
	G1 x UGL	17.8	1.0	0.09	0.10	40.5	3.8	ND	ND	35.0	15.3	0.7	0.08	0.07	44.1	4.0	ND	ND	34.9
	DG x U2	17.8	1.4	0.07	0.06	41.9	3.8	ND	ND	35.4	15.6	1.7	0.07	0.06	44.4	3.9	ND	ND	34.5
	IP	17.9	1.2	0.08	0.09	40.4	3.9	0.1	ND	34.7	14.7	1.5	0.76	0.08	44.4	4.0	0.1	ND	34.7
	IB	16.2	1.5	0.07	0.05	43.7	3.8	ND	ND	34.9	16.3	1.6	0.13	0.05	45.6	3.9	ND	ND	35.1
	E. Grass	14.9	3.1	2.60	0.44	38.6	3.9	0.4	ND	35.3	15.4	3.4	2.21	0.56	39.3	4.0	0.4	ND	34.8

Black liquor application studies

Black liquor contains a variety of low molecular weight materials, mainly organic acids, but also contain sizeable amounts of lignin and a small but significant amount of polymeric xylans. Many efforts have been made in the isolation and utilization of the lignin, but not so much has been done on the utilization of the xylans. The aim of this study was developing a novel and practical way of reclaiming the polymeric xylans existing in black liquor streams. The technique involved the addition of black liquor in the oxygen delignification stage under proper conditions for the xylans contained therein to precipitate. The proposed technique is easy to implement on an industrial scale, since most of the infrastructure required for its implementation already exists in most Kraft pulp mills. The technique has the potential of increasing the content of xylans in the pulp by 2%, thus improving yield and pulp quality. The xylans would otherwise be burnt along with the black liquor in the recovery system, but producing very little heat since they are highly oxygenated.

Generation and characterization of pulp and black liquors: Kraft black liquors from *Eucalyptus urograndis* (IP) and *Eucalyptus globulus* were used. The two woods were cooked at 4L/1 kg liquor to wood ratio, 165^{o}C, 90 min to maximum temperature, 60 min at maximum temperature and 35% sulfidity. The kraft pulping results are presented in Table 5, which shows a slightly better performance for the European *Eucalyptus globulus* attributable to its high S/G ratio of 4/1 (Table 2) in comparison with the *IP* clone (2.7/1). The fate of the xylans of the two different woods was determined. The xylans in the pulps were measured according to Wallis et 1996 and the xylans in the black liquors derived from the two woods were extracted according to the procedure described by Teleman et al. (1995). The amount of xylans distributed in the pulps and black liquors are presented in Table 6.

Table 5. Cooking results of the eucalypt clones evaluated.

Wood species	AA, % NaOH	Screen Yield, %	Rejects, %	Viscosity, dm^3/kg	HexA, mmol/kg	Xylans, %	Balck liquor solids, %
European Eucalyptus globulus	17.2	54.5	0.7	1350	63.3	17.4	15.1
Eucalyptus urograndis (IP)	18.9	53.3	0.0	1290	57.4	15.8	15.8

*Kraft conditions: 4L/1 kg liquor to wood, 165^{o}C, 90 minutes to maximum temperature, 60 minutes at maximum temperature and 35% sulfidity.

About 54-56%% of the xylans from the original wood ended up in the pulp and the remaining dissolved in the black liquor either in the polymeric form (9-10%) or degraded (34-38%). No significant differences were observed between the xylan fate in the kraft process for *E. urograndis and E. globulus*. Although only 9-10% of the xylans remained in the polymeric form and were recovered, it is a significant a number if they can be adsorbed back onto the fibers by some ingenious technique. Table 7 shows the molecular weight of the wood, pulp and black liquor xylans. It is noticeable that that the polymeric xylans in the black liquor are smaller than the ones in the original chips and in the kraft pulp, but still valuable if they could be adsorbed back onto the pulp. Since these xylans still contain significant amounts of uronic acids (Table 8), i.e., approximately 1 molar weight unit of uronic acids (4-O-methyl-D-glucuronic acid and hexenuronic acids) per ten xylose units, they present good potential for improving pulp properties in addition to their role in improving process yield.

Table 6. Distribution of Eucalyptus wood xylans in pulp and black liquor after kraft pulping to Kappa 20.

Wood species	Xylans, % of original wood		
	Pulp	Black Liquor	
		Polymeric	Degraded
Eucalyptus globulus	55.7	9.9	34.4
Eucalyptus urograndis (IP)	53.8	8.6	37.6

Table 7. Molecular weight (g.mol-1) of xylans in the wood, kraft pulps and corresponding black liquor after cooking at Kappa 20.

Wood species	**Wood**	**Kraft Pulp**	**Black Liquor**
New *Eucalyptus globulus*	27,160	20,567	15,257
Eucalyptus urograndis (IP)	27,512	20,314	15,322

Table 8. Molar ratios of MeGlcA and HexA/10 xyloses in kappa 20 pulp and corresponding black liquor as measured by 1H NMR.

Wood Species	**Kraft Pulp at Kappa 20**			**Black Liquor at Kappa 20**		
	MeGlcA	HexA	**Total**	MeGlcA	HexA	**Total**
New *Eucalyptus globulus*	0.60	0.47	**1.07**	0.40	0.55	**0.95**
Eucalyptus urograndis (IP)	0.54	0.34	**0.88**	0.28	0.70	**0.98**

MeGlcA: 4-O-methyl-D-glucuronic acid.
HexA: hexenuronic acids.

Reclaiming Xylans from Black liquor in the Oxygen Delignification Stage: The recovery of polymeric xylans from Kraft black liquor has always been a challenge, since it is mixed with a large variety of organic and inorganic materials, including sulfur based ones. Instead of separating the xylans from the black liquor, an approach of redepositing them directly from the black liquor onto the pulp in the oxygen delignification stage was evaluated in this study. Black liquor from *Eucalyptus urograndis* (IP) produced according to Table 5 was added to kraft pulp also produced according to Table 5. The xylan contents of the pulps produced under reference and black liquor assisted processes were 15.2% and 16.4%, respectively. Therefore, about 1.4% of xylans were reclaimed from the black liquor across the oxygen delignification stage, and that reflected in a process yield increase of the same proportion. However, the oxygen delignification performance was negatively affected by the addition of black liquor, mainly the brightness gain and the kappa drop across the stage. This loss of performance was anticipated since black liquor contains lignin, which consumes alkali an oxygen, thus decreasing overall O-stage performance.

Table 9 Results of the black liquor* assisted oxygen delignification** of *Eucalyptus urograndis* kraft pulp***.

Parameter	**Reference**	**Black Liquor Assisted**
Kappa number	12.7	13.1
Viscosity, dm^3/kg	1052	1073
Brightness, % ISO	56.1	52.3
Kappa Drop, %	35.5	33.5
Viscosity Drop, dm^3/kg	238	217
Brightness gain, % ISO	19.3	15.5
Xylans, %	**15.2**	**16.4**

Yield, %	**98.1**	**99.5**

*15.8% solids, 45.7% organics, 54.3% inorganics, 8.6% xylans on wood weight.
**10% consistency, 600 kPa, 105°C, 60 min, 20 kg NaOH/adt, 30 kg O_2/adt, 200 kg COD/adt added to the O-stage in the process with black liquor.
***kappa number 9.7, viscosity 1290 dm^3/kg, brightness 36,8% ISO, xylans 15.8%, 5.5 kg COD/odt pulp.

Impact of black liquor application on oxygen delignification stage on bleaching performance: The pulps produced according to Table 9 were bleached with the D-P-D sequence in order to determine the impact of black liquor addition in the O-stage on the chemical consumption during bleaching (Table 10). As anticipated, the pulp produced by the black liquor assisted O-stage required a higher quantity of chlorine dioxide (1.8 kg ClO_2/odt pulp) to reach full brightness in relation to the reference. This is explained by the higher kappa number (0.4 units) and lower brightness (3.8% ISO) of the black liquor treated pulp. The cost related to the additional chlorine dioxide demand must be weighed against the yield gain caused by the xylan deposition. The xylan deposition also resulted in decreased bleached pulp viscosity, which can be explained by the low molecular weight of the xylans in relation to cellulose. The deposited xylans were stable across the bleaching process. The xylans losses across bleaching for the reference and xylans enriched pulps were similar. After bleaching, the xylans content were 14.9% and 16.2% for the pulps produced with conventional and black liquor assisted oxygen delignification stages, respectively.

Table 10. Bleaching results with the sequence D-P-D*, for the pulps produced with conventional and black liquor assisted oxygen delignification stages

Parameter	Reference	Black Liquor Assisted
ClO_2, kg/odt	10.8	12.6
H_2O_2, kg/odt	5	5
NaOH, kg/odt	10.0	11.0
H_2SO_4, kg/odt	3	5
Brightness, % ISO	90.2	90.3
Viscosity, dm^3/kg	798	769
Xylans, %	14.9	16.2
Yield, %	97.9	97.8

*D: 10% consistency, 90°C, 120 min, pH 3, 8 kg ClO_2/odt pulp; P: 10% consistency, 80°C, 90 min, pH 11, 10-11 kg NaOH/odt pulp, 5 kg H_2O_2/odt pulp; 10% consistency, 80°C, 120 min, pH 5.5, 2.8 and 4.6 kg ClO_2/odt pulp for reference and black liquor assisted, respectively,

Conclusions Task 3.4

Different pulping processes and raw materials result in black liquors with distinct chemical compositions that must be considered upon their utilization. The main findings on black liquor characterization were: (1) the black liquor sulfur content is lower for the pulp produced at kappa 20 than at kappa 15; (2) the black liquor gross heating value is superior for pulps produced at kappa 20 than at kappa 15; (3) the gross heating value of black liquor derived from Soda-AQ process is higher than that derived from kraft process; (4) the black liquor derived from soda-AQ pulping of elephant grass has lower heating value than that from wood at a given kappa number; (5) Black liquor derived from pulping of elephant grass has lower carbon content and unusually high nitrogen content when compared to wood black liquor; (6) when cooking eucalyptus wood to kappa 20, a small but significant amount (8-10%) of xylans of reasonable MW remains in the black liquor; (7) recovery of such xylans (~1.5% on pulp weight) is possible by adding black liquor to the oxygen delignification stage (O-stage) resulting in yield gain of the same proportion; (8) Such practice impairs the O-stage performance only slightly but impairs

bleaching significantly (+2 kg ClO_2/odt); (9) yield and pulp quality gains must be weighed against increased ClO_2 demand for pulp bleaching.

Task 4.1 of WP4: Bleaching of the pretreated materials obtained from alkaline deconstruction

The elephant grass and eucalypt pulps derived from the alkaline deconstruction at kappa number 15 and 20 were bleached by the elemental chlorine free sequence (ECF) O-D-P-D to 90% ISO brightness. Table 11 presents the characterization of materials after the alkaline deconstruction. The oxygen delignification (O-stage) was run at 10% consistency, 100°C, 60 min, 700 kPa pressure, 20 kg NaOH/odt pulp and 20 kg O_2/odt pulp. The first chlorine dioxide stages (D) were carried out at 10% consistency, end pH 3.5, 85ºC, 120 min, with kappa factor of 0.20 for pulps with kappa number 15 and 0.24 for pulps with kappa number 20. The P stages were run at 10% consistency, end pH 10.5, 85ºC, 120 min, with hydrogen peroxide doses of 0.5% on pulp weight. The second chlorine dioxide stages (D) were carried out at 10% consistency, end pH 5.5, 70ºC and 120 min, with variable chlorine dioxide charges to achieve the desired brightness. The pulp bleachabilities were determined on the basis of the total active chlorine consumption per kappa unit removed across bleaching to a target brightness of 90% ISO. The bleached pulps were evaluated for their brightness, brightness stability and viscosity. All bleaching stages were controlled on the basis of end pH and chemical consumption across the stage so that a minimum oxidant residual was maintained but no excess residuals were accepted.

Table 11. Characteristics of the elephant grass and eucalypt pulps obtained by the Kraft and Soda-AQ processes at kappa 15 and 20, according to task T2.1

		Kappa 15						Kappa 20					
		Bright-ness, % ISO	Visco-sity, dm^3/kg	Glucans	Xylans	Uronic Acids, %	HexA, mmol/kg	Bright-ness, % ISO	Visco-sity, dm^3/kg	Glucans	Xylans	Uronic Acids, %	HexA, mmol/kg
Kraft	U1xU2	33.6	1032	82	13.9	0.6	39.8	31.7	1073	81.2	14.2	1.3	48.8
	G1xUGL	33	1144	81.6	15.4	0.7	41.8	32.2	1193	80.8	15.8	1.2	48.6
	DGxU2	34.2	892	82.2	13.4	0.7	39.1	31.3	1054	81.2	14	1.3	48.9
	IP	34.1	939	81.9	14	0.6	39.5	31.0	1100	80.6	14.9	1.3	46.4
	IB	34.6	990	79.5	17.4	0.8	39.1	31.6	1064	79.3	17	1.1	37.3
	E. Grass	32.9	1100	77.6	18.9	0.7	13.2	31.1	1359	77.7	18.5	0.8	11.1
Soda-AQ	U1xU2	33.3	875	88.1	8.5	0.2	15.8	30.8	919	84.9	11.3	0.5	40.3
	G1xUGL	34	748	86.2	10.4	0.3	21.2	32.1	917	83.9	12.2	0.7	48.2
	DGxU2	34	951	88	8.8	0.3	15.9	30.8	1020	84.2	11.8	0.6	43.4
	IP	33.9	972	87.1	9.6	0.3	218	29.6	1028	84.5	11.2	0.6	45.7
	IB	35.5	990	83.9	12.9	0.7	29.4	34.1	1064	82.1	14.1	1.3	41.6
	E. Grass	31.6	875	80.3	16.5	0.4	15.15	28.5	883	78.1	17.6	0.6	16.3

Oxygen Delignification Results*:* The pulps produced by two processes (kraft and Soda-AQ) and two kappa numbers (15 and 20) were submitted to oxygen delignification. Table 12 shows the oxygen delignification (O-stage) results. The overall oxygen delignification stage performance was measured by the kappa drop and brightness gain across the O-stage. It was observed that the kappa drop decreased with increasing kappa number, a result likely explained by the decreased content of lignin containing free phenolic hydroxyl groups and increased HexA content with increasing kappa (COLODETTE et al., 2007). Oxygen delignification performance is hampered by the lack of free phenolic hydroxyl groups in the pulp since such groups are the main sites of oxygen reactions during O-stage. On the other hand, HexA groups are resistant to oxygen reactions and little of it is actually removed in the O-stage. Hence, pulps containing large HexA amounts comprising its kappa number will respond poorly to the O-stage

(VENTORIM et al, 2006; EIRAS, 2003). This fact explains why the elephant grass pulps showed better performance in the O-stage than the eucalypt ones, as measured by the kappa drop across the stage. The same rationale can be applied to explain why the Soda-AQ pulps performed better in the O-stage than the Kraft ones (Table 12). Another relevant point that helps to explain the higher efficiency of the pulp obtained by the Soda-AQ process is a possible higher content of free phenolic hydroxyl groups (ZONG LAI (1999), which are the main sites for oxygen reactions (COLODETTE et al., 2007). It is known that in Soda-AQ pulping, AQ oxidizes the reducing end groups of carbohydrates, thus stabilizing them towards peeling reactions in alkaline media. The reduced form, AHQ, cleaves part of the β-aryl ether linkages in lignin. Thus, the molecular mass of the residual lignin is reduced and new phenolic hydroxyl groups are formed. Both effects render the lignin more soluble (KLEEN et al., 2002). Since phenolic hydroxyl groups are essential to lignin dissolution in alkali, a higher content of this functional group in the residual lignin may be partly related to the residual lignin being more condensed. For woody samples, in his work ZONG LAI (1999) showed that the tendency of alkaline lignin condensation reactions would increase in the order of: high sulfidity < kraft < Soda-AQ < soda cooks.

Table 12. Oxygen delignification performance for eucalypt and elephant grass Kraft and Soda-AQ pulps of kappa 15 and 20

		Kappa 15		Kappa 20	
		Kappa drop, %	Brightness gain, % ISO	Kappa drop, %	Brightness gain, % ISO
Kraft	U1xU2	48.4	19.6	46.1	17.7
	G1xUGL	46.2	18.1	46.1	16.5
	DGxU2	46.6	18.4	46.2	17.7
	IP	47.4	20.1	43.8	16.2
	IB	44	19.3	46.3	17.9
	E. Grass	61.9	12	50.9	7.5
Soda-AQ	U1xU2	57.5	18.1	50.5	17.1
	G1xUGL	57	19.8	45.5	15.8
	DGxU2	57.7	18.2	47.8	15.1
	IP	54	18.3	47.3	16.6
	IB	53.3	18	47.4	16.7
	E. Grass	73.7	14.5	69.4	14.7

***Bleachability Results**:* The pulps were bleached aiming brightness of 90% ISO as described in the methodology. For a fair analysis of all pulps, the parameter bleachability was used to compare the behavior of the various pulps across bleaching. In this work bleachability has been defined as the ratio between kappa drop across the bleaching sequence and total active chlorine (TAC) required for attaining the target brightness of 90% ISO. Total active chlorine was defined by the following equation:

$$\mathbf{TAC=[(ClO_2\times 2.63)+(H_2O_2\times 2.09)]}$$

In the equation above, the factors 2.63 and 2.09 are simple conversions of ClO_2 and H_2O_2 into active Cl_2 based on their oxidation equivalents. The parameters TAC, ClO_2 and H_2O_2 are expressed in kg/odt pulp. For the eucalypt pulps (Fig.1), the highest bleachabilities were achieved with the Kraft pulps of kappa 20 whereas the worst ones were seen for the Soda-AQ pulps of kappa 15. The same trend was observed for the elephant grass pulps. In general, the elephant grass pulps showed lower bleachabilities in relation to

the eucalypt ones, a result probably explained by the more condensed nature of the grass residual lignin and their higher transition metal contents in relation to the eucalypt ones.

Among the eucalypt pulps, the highest bleachabilities of the kraft pulps at kappa 20 (0.223 kappa unit/ kg TAC) and 15 (0.193 kappa unit/ kg TAC) were achieved with the G1xUGL clone. On the other hand, for the Soda-AQ pulps the highest bleachabilities at kappa 20 (0.172 kappa unit/ kg TAC) and 15 (0.136 kappa unit/ kg TAC) were attained with the clones IP and G1xUGL, respectively (Figure 2). For the elephant grass, the bleachabilities of the kraft pulps at kappa number 20 and 15, were 0.154 and 0.112 kappa unit/ kg TAC, respectively. On the other hand, bleachabilities values of 0.119 and 0.107 kappa unit/ kg TAC, respectively, were obtained for the Soda-AQ pulps at kappa 20 and 15 (Fig. 2). It is worth noting that the elephant grass used in this was not genetically improved as was the case for the eucalypts. Potential genetical improvements could be achieved aiming at the pulp production, so that the TAC values could be reduced. For example, the transition metal contents of the elephant grass pulps were higher than desired.

It is worth noting that Soda-AQ pulps of kappa 20 produced showed bleachabilities somewhat similar to Kraft pulps of kappa 15. Thus, cooking at kappa 20 could be a practical alternative to implement the Soda-AQ process give its many advantages compared to the Kraft counterpart, particularly in the light of the biorefinery prospects. The Soda-AQ process is highly desirable when the use of black liquor for biorefinery purposes is at stake. The absence of sulfur compounds in the Soda-AQ black liquor enormously facilitates its further fractionation into valuable components.

The total active chlorine (TAC) demands to bleach the pulps to 90% ISO are shown in Figures 3. Although the bleachability of the eucalypt and elephant grass kappa 20 kraft pulps were the highest, their TAC were not the lowest due to the effect of the kappa number value. Actually the lowest TAC was achieved for the eucalypt kraft pulp at kraft kappa 15. In general the highest values of TAC were seen for the Soda-AQ pulps of kappa 20.

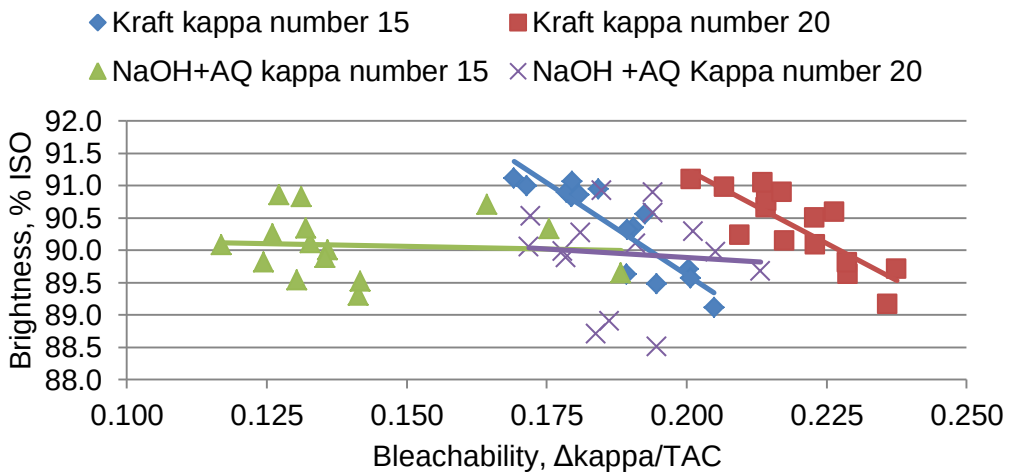


Figure 1. Bleachability (kappa unit/ kg TAC) of eucalypt kraft and Soda-AQ pulps obtained at kappa 15 and 20.

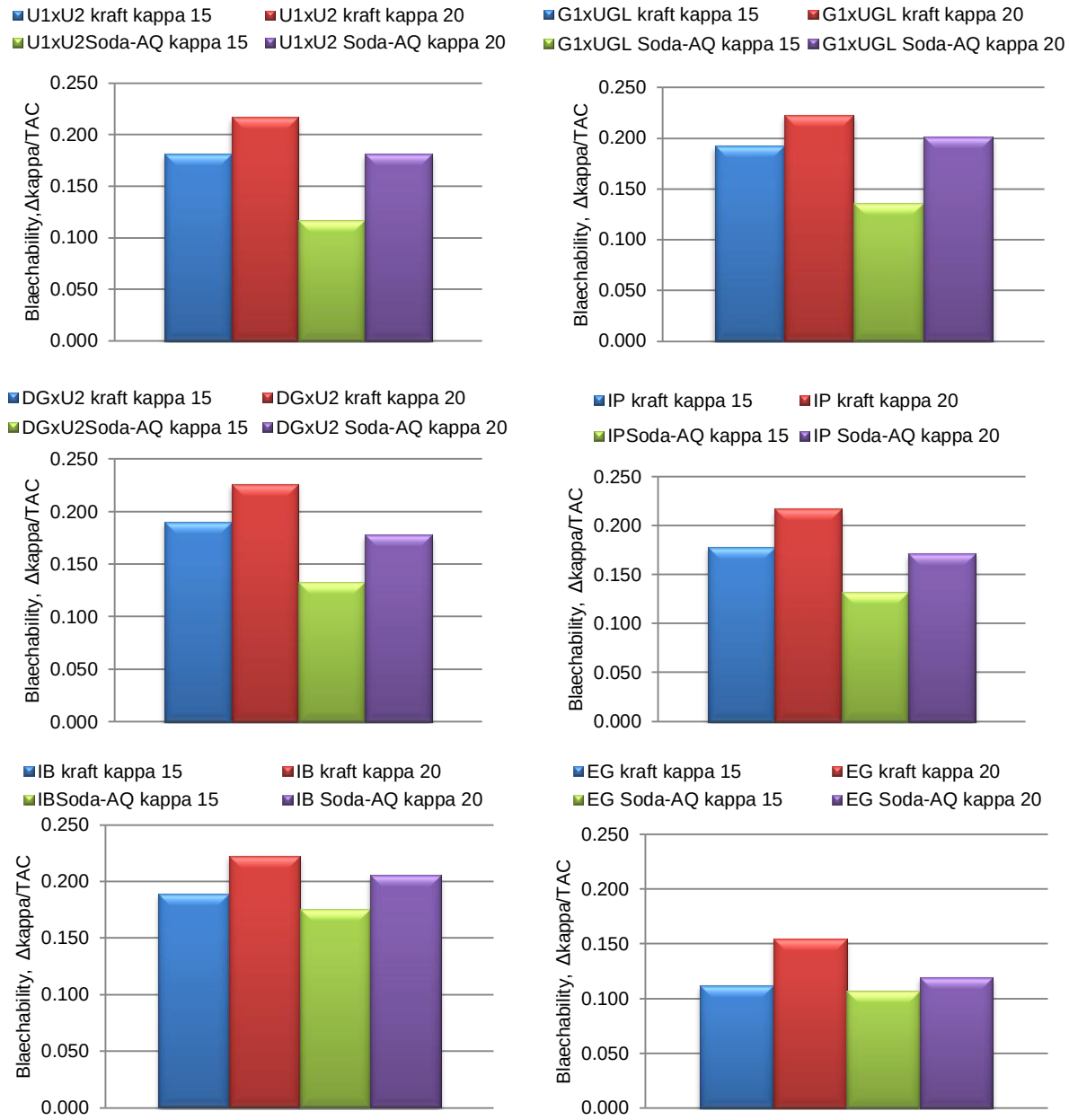
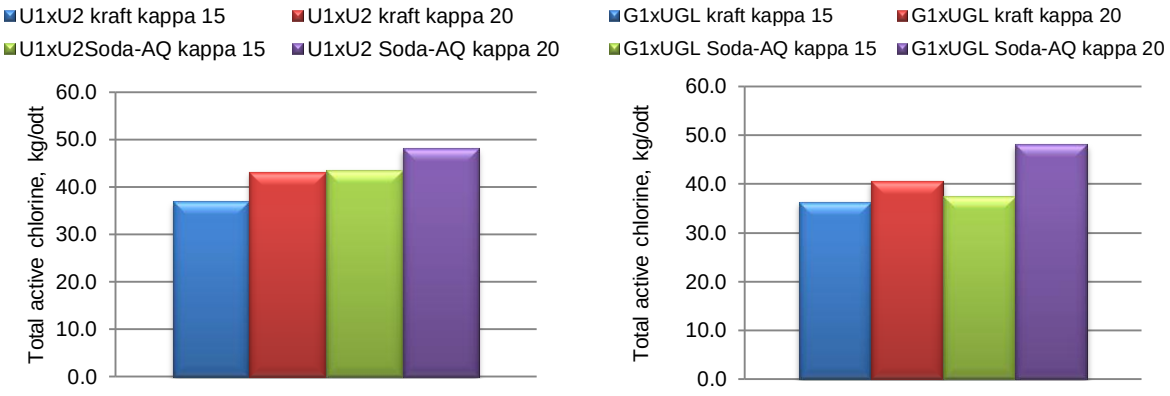


Figure 2. Bleachability (kappa unit/ kg TAC) of bleached pulps from eucalypts and elephant grass obtained by the Kraft and Soda-AQ processes at kappa 20 and 15.



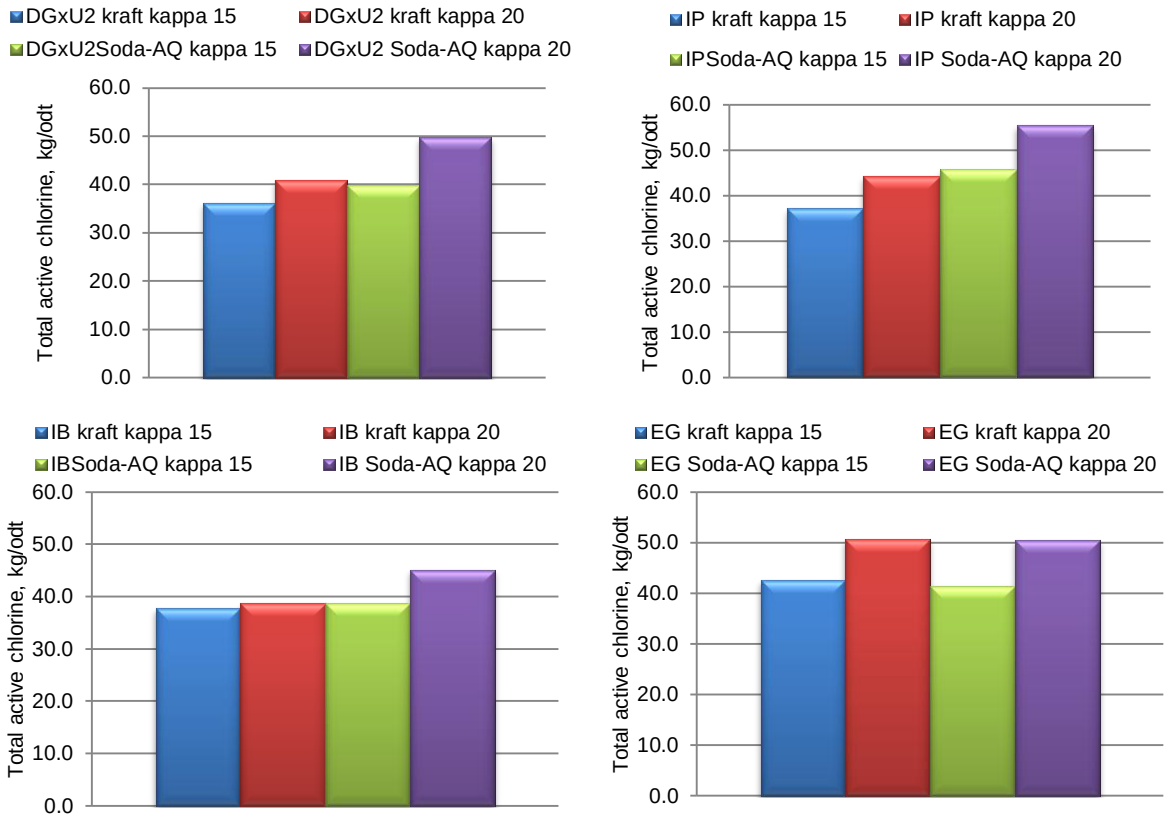


Figure 3. Total active chlorine used in the bleaching of eucalypt and elephant grass kraft and Soda-AQ pulps obtained at kappa 15 and 20.

***Bleached Pulp Viscosity and Brightness Stability*:** Figure 4 shows viscosity results for eucalypt and elephant grass bleached pulps. Among the eucalypt pulps, the kraft kappa 20 IP bleached pulp showed he highest value (760 dm^3/kg). The lowest viscosity value was obtained for the IP Soda-AQ at kappa number 15 showed the lowest viscosity value (427 dm^3/kg), but still sufficient high for applications in most paper grade pulp products. In regard to viscosity among all samples studied, the elephant grass kraft Kappa 20 bleached pulp showed the highest value (886 dm^3/kg), a result explained by the low xylan content of the elephant grass pulp. In spite of the kraft process to present the higher values to viscosity, the Soda-AQ process resulted in quite acceptable viscosity for IB (European E. globulus). As stated previously, the Soda-AQ process is the most indicated for a more efficient use of biomass in biorefinery applications, so among the eucalypt clones the IB seem to be the most desired raw material for this project. On the other hand the elephant grass also presented a quite acceptable viscosity by Soda-AQ process at kappa 20.

Brightness stability of the bleached pulps was expressed as post color number for both elephant grass and eucalypt pulps. The brightness stability increased with increasing kappa number (increasing TAC) as tendency previously reported (EIRAS, 2001), this result is shown in Figure 5. However, no significant and logical effects of pulping process and biomass raw materials were observed on brightness stability.

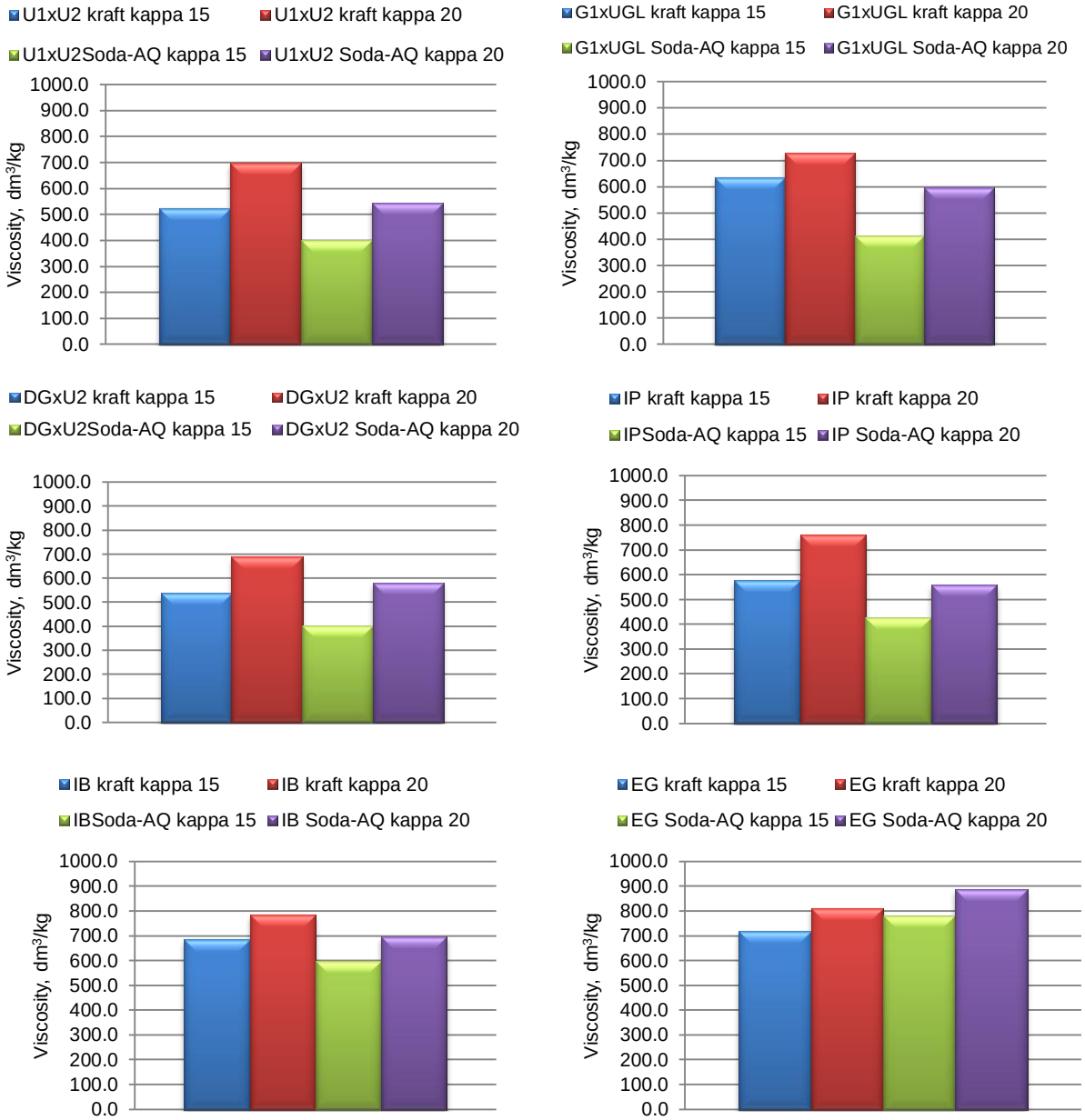
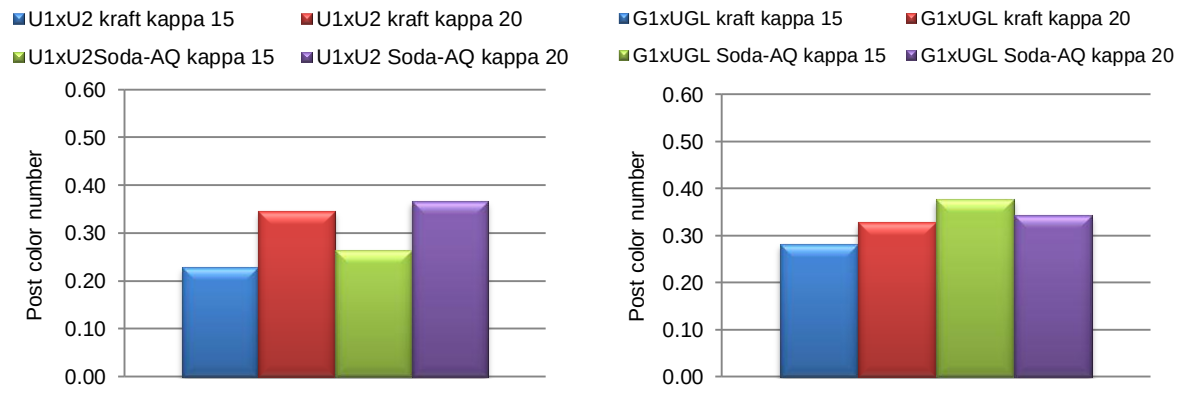


Figure 4. Viscosity of bleached pulps from eucalypt and elephant grass obtained by the Kraft and Soda-AQ processes at kappa 20 and 15.



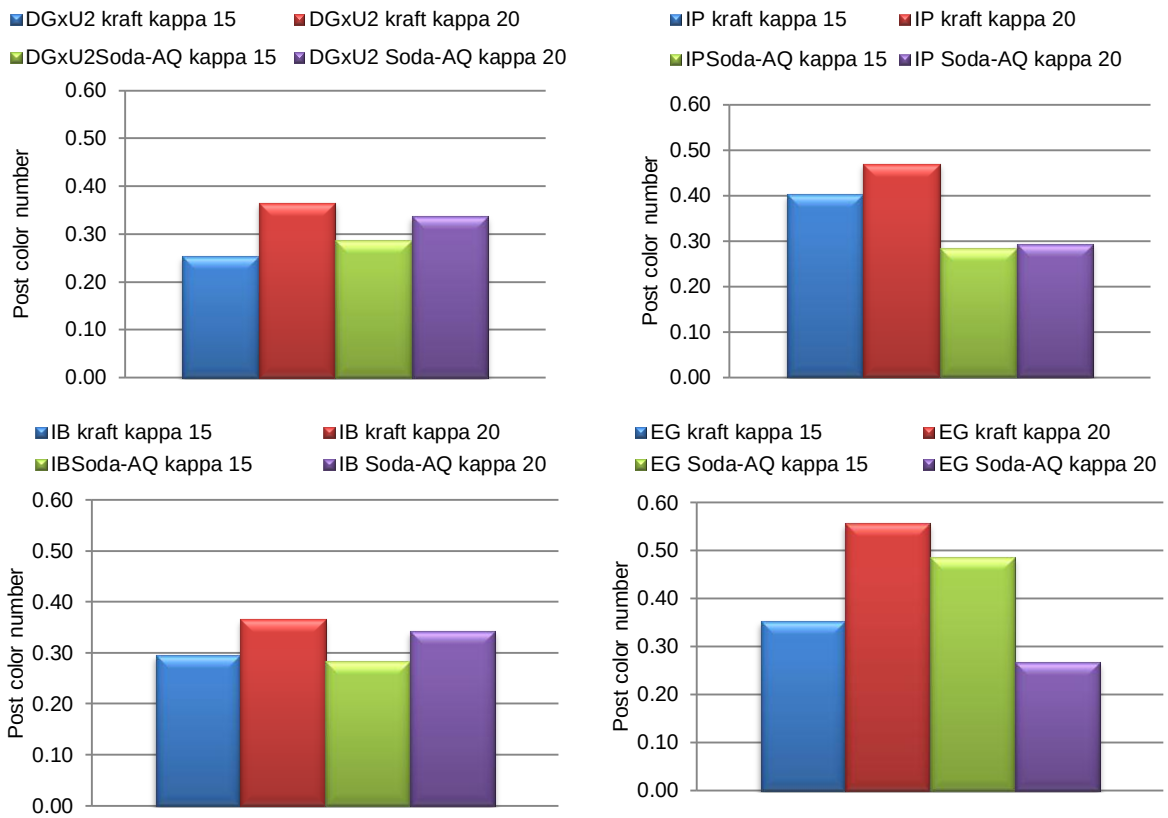


Figure 5. Post color number of bleached pulps from eucalypts and elephant grass obtained by the Kraft and Soda-AQ processes at kappa 20 and 15

Conclusions of Task 4.1

Among the pulps obtained from the Kraft and Soda-AQ processes, the kraft process at kappa number 20 presented a best bleachability and the Soda-AQ process at kappa number 15 presented the worst. The kraft process presented the higher values to viscosity, but the Soda-AQ process resulted in quite acceptable viscosity for IB (European E. globulus) and Elephant grass. The Elephant grass pulp of kappa 15 showed the lowest bleachability. Among the eucalypt the IB and hybrid DGxUGL showed the best bleachability.

Task 4.3 of WP4: Upgrading xylans as paper grade pulp additives

This task is divided into two main parts, namely: (I) production of special printing and writing paper grade pulps by using xylan as additive; (II) Production of special tissue grade pulps by partial xylan removal.

Part I: production of special printing and writing paper grade pulps by using xylan as additive

The use of non-woody xylan (obtained by extraction pre-treatment in WP2) as additive for eucalypt paper grade pulps were investigated. In this study three novelties were introduced: (1) the ideal conditions for xylans extraction, (2) the resorption of xylans during the oxygen delignification phase of the pulp bleaching process, (3) the production of special tissue grade pulp by partial removal of its xylans. The advantage of resorbing xylans during the oxygen delignification phase of the process is that the extracted xylan color coming from lignin and extractive impurities becomes irrelevant since they will go through subsequent bleaching. Therefore, much less purification of the extracted xylan is required. Eucalyptus fibers are largely used for printing & writing (P&W) paper grades. Pulps for P&W papers require significant refining and can benefit from a high content of xylans, which purportedly facilitate

this operation. High quality printing and writing paper grades require pulps of high tensile strength to withstand the forces the paper undergoes during manufacturing and use in high speed printing machines. In this way two different pulps were compared regarding their behavior during papermaking: (1) reference pulp without xylan addition; (2) xylan treated pulp, in which xylans extracted from elephant grass were redeposited onto the *E. globulus* pulp during the oxygen delignification stage. On the other hand it was investigated an application for the pulps which the xylans were extracted (xylan depleted pulps) aiming at special tissue grade pulp production.

The ideal conditions for xylans extraction: For the xylans extraction it was used the Cold Caustic Extraction technology (CCE) under well optimized conditions of alkali charge, time and reaction consistency at room temperature. The studies were carried using a kappa 20 elephant grass brown pulp produced by the Soda-AQ processes. Figure 6 shows the effect of the alkali charge on xylan removal for experiments carried out at 10% consistency and 60 minutes reaction time at room temperature (Figure 6). The dosage of 550kg NaOH/odt pulp was considered the optimal value to run the CCE stage (10% consistency, 60 min, room temperature) since charges above this value had only small effects on xylans removal. On the other hand, charges lower than this would not remove the targeted 7% of xylans established as a minimum in our previous studies. The value of 7% is the number that can actually be redeposited onto fibers.

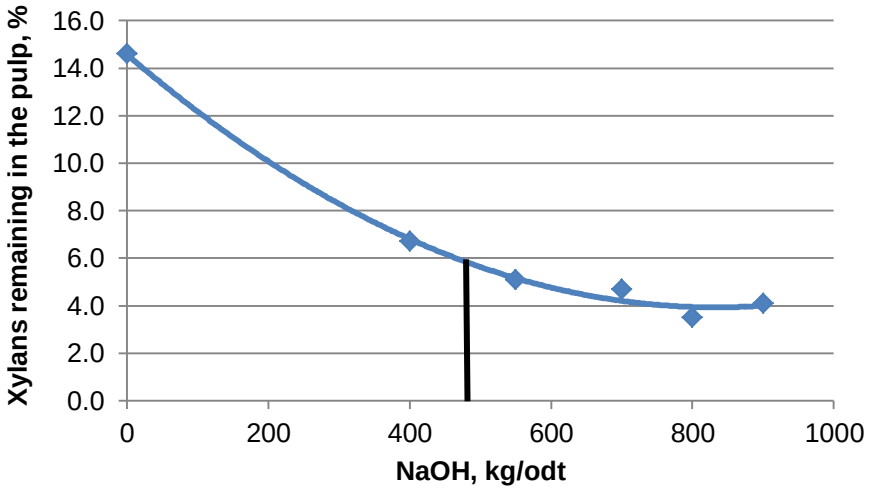


Figure 6. Effect of NaOH charge on xylan removal during the CCE treatment run at room temperature, 10% consistency and 60 minutes.

The effects of reaction time and consistency on CCE performance were evaluated at two alkali charges, namely 400 and 550 kg NaOH/odt pulp. The consistency affects the CCE performance because it influences de alkali concentration in the system and the alkali concentration is the main variable affecting xylan removal in the CCE stage. Figure 7 shows experiments run at room temperature and 15 min reaction where a more efficient xylan removal is visible at the higher consistency values, in the range of 10-20%, for a given alkali charge; the xylans extracted with 400 kg NaOH/odt at 15% consistency is actually higher than that extracted with 550 kg NaOH/odt at 10% consistency. The effect is very significant when the consistency is increased from 10 to 15% and not so significant when it is increased from 15 to 20%. Therefore, the 15% consistency is likely the most desirable value to run the CCE stage. On the other hand, one needs to pay attention to the consistency since too high a consistency may cause conversion of cellulose I into cellulose II, which makes the pulp useless for paper applications. Regarding reaction time, the results in Figure 8 show that 15 min suffices for efficient CCE stage. Increasing reaction time to 60 min resulted only slight xylan removal improvement.

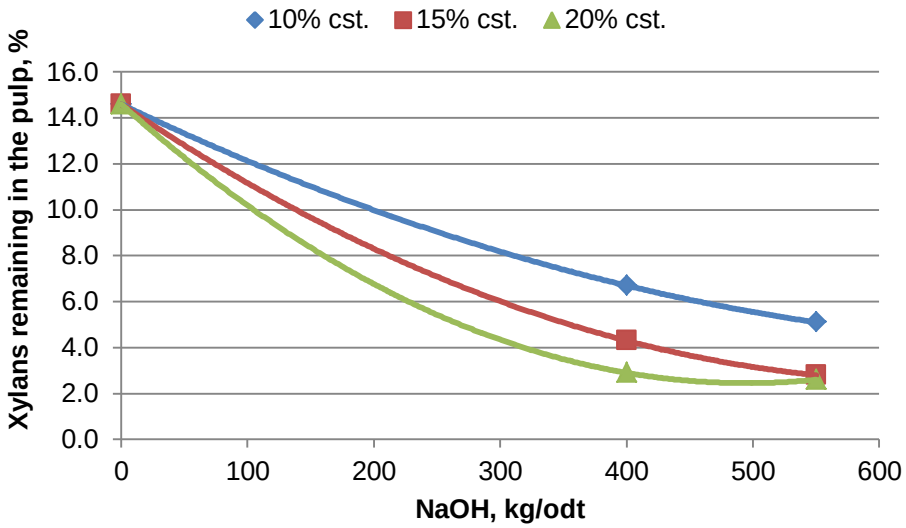


Figure 7. Effect of reaction consistency on xylan removal in the CCE stage run at room temperature and 15 minutes with different alkali charges.

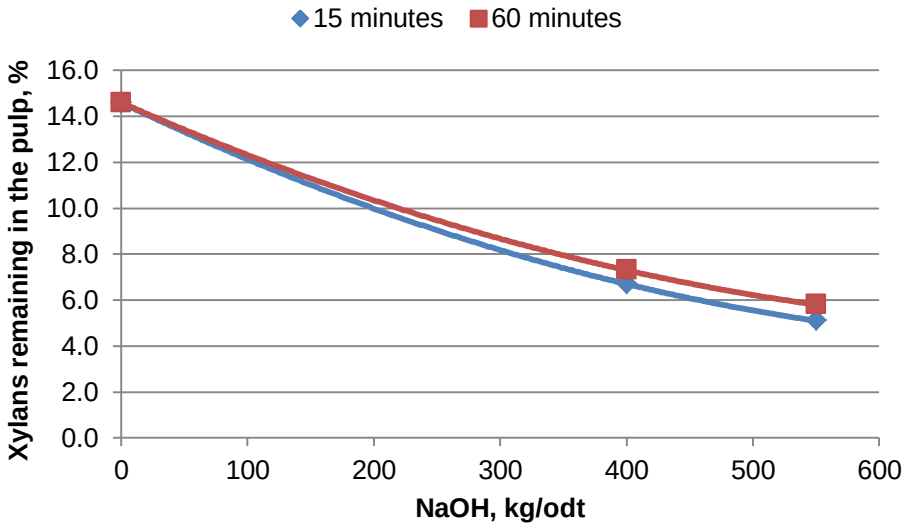


Figure 8. Effect of reaction time on xylan removal in the CCE stage run at room temperature and 15% consistency with different alkali charges.

Impact of xylans deposition on oxygen delignification performance: Xylans were extracted from elephant grass brown pulp by cold caustic extraction (CCE) and were added to *E. globulus* kappa 20 brown pulp (produced by the Soda-AQ pulping processes) during the oxygen delignification stage. Table 13 summarizes the results of the xylan assisted oxygen delignification. The xylan contents of the reference and xylan assisted processes after the oxygen delignification stage were 14.4% and 17.3%, respectively. The xylan addition onto pulp caused an increase of 2.9% of gravimetric yield. It was observed a slight effect of the xylan deposition on the oxygen stage end pH, which was lower than the reference, indicating the need for increased alkali addition in the O-stage, when xylan is added to this stage. The oxygen delignification efficiency was affected by the presence of lignin in the xylan extract, since the pulp rich in xylan showed higher kappa number and lower brightness than that of the reference treatment. This additional lignin consumed part of the alkali and oxygen required for the reactions during the oxygen delignification. The xylan deposition resulted in decreased pulp viscosity, which can be explained due to the low molecular weight of these hemicelluloses in relation to cellulose.

Table 13. Results of oxygen delignification carried out under reference conditions and assisted with xylans addition.

Conditions and Results	**Reference Process**	**Process assisted with xylans***
Consistency, %	10	10
Temperature, ^{o}C	100	100
Time, min.	60	60
Pressure, kPa	700	700
O_2, kg/odt	18	18
NaOH, kg/odt	20	20
Final pH	12.1	11.5
Brightness, %ISO	49.2	48.1
Viscosity, dm^3/kg	1011	951
Kappa number	12.7	13.2
Hexenuronic Acid, mmol/kg	64.4	68.3
Xylans, %	**14.4**	**17.3**
Yield, %	**98.1**	**101.1**

CCE stage was done in a non-woody pulp after O_2 delignification. The xylans were precipitated from the CCE liquor by ethanol addition followed by centrifugation. Xylans, were dissolved in water to a known concentration (70 g L^{-1}) and added to the brown pulp during the oxygen delignification at 7.0% of dry basis weight of pulp.

Impact of O-stage xylans deposition on bleaching performance: The reference pulp and xylan enriched pulp during the oxygen delignification were subsequently bleached with the D(EP)D sequence under conditions shown in Table 14. As expected the pulp rich in xylan showed a lower brightness than the reference, which can be explained by the sizable amount of lignin existing in the xylan extract, which contaminated the pulp during the oxygen delignification stage to a point that the post-oxygen kappa number was 0.5 unit higher for this sample than for the reference. The additional lignin and hexenuronic acid in the pulp rich in xylans consumed part of the bleaching reagents and decreased pulp brightness and pulp brightness stability. Note that the issues of lower brightness and brightness stability are easily solvable by adding additional amounts of chlorine dioxide during bleaching. The xylan deposition resulted in decreased bleached pulp viscosity, which can be explained by the low molecular weight of these hemicelluloses in relation to cellulose.

The deposited xylans were stable across the bleaching process. The xylans losses across bleaching for the reference and xylans enriched pulps were similar. After bleaching, the xylans content were 13.7% and 16.8% for the reference and xylans enriched pulps, respectively. The xylans deposition produced a total yield gain of 3.1% across the whole process, including oxygen delignification and bleaching.

Table 14: Conditions and results of the bleaching process for reference and xylans enriched pups.

Pulp Bleaching Conditions			
Conditions	**D**	**P**	**D**
Consistency, %	10	10	10
Temperature, ^{o}C	85	85	70
Time, min.	120	120	120
ClO_2 , kg/odt	8	-	6.5
H_2O_2, kg/odt	-	6.5	-
NaOH, kg/odt	-	8.0	-
Final pH	3.5	10.5	5.5
Pulp characteristics after DPD bleaching			

Samples	Reference Pulp	Xylans Enriched Pulp
Brightness, %ISO	89.6	88.7
Post Color Number	0.17	0.44
Viscosity, dm^3/kg	792	704
Kappa number	0.4	0.9
Hexenuronic Acid, mmol/kg	4.9	10.5
Xylans, %	13.7	16.8
Δ xylans across bleaching	**-0.7**	**-0.5**
Yield across bleaching, %	**96.4**	**99.5**

Impact of xylans deposition on pulp properties: The stability of the deposited xylans and their effect on pulp beatability and properties was evaluated after the bleaching and in papers sheets with a schopper riegler degree around 35, which is typical for printing and writing paper grades for eucalypt. The deposited xylans resisted well to the bleaching operation and to the mechanical forces during beating (Figure 9). So, xylans deposition during oxygen delignification showed to be a good alternative for increasing fiber line yield.

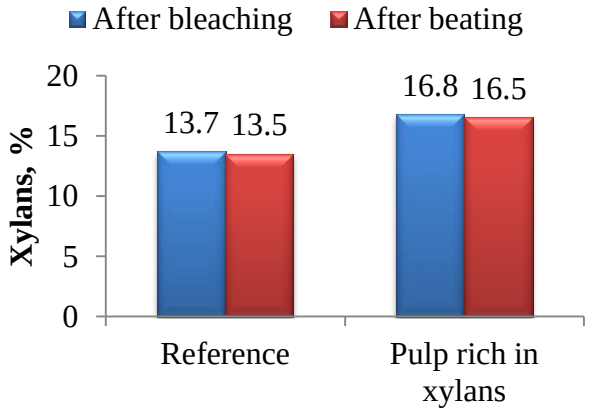


Figure 9: Xylans behavior after bleaching and beating for the reference and xylans assisted oxygen delignification process.

For the determination of pulp physical and mechanical properties, the samples were refined in the laboratory using PFI mill method at 10% of pulp consistency. Physical and mechanical tests were performed using laboratory hand sheets according to TAPPI test methods (Table 15) after pulp conditioning for 24 h in a room at 50 ± 2% of relative humidity and temperature of 23 ± 1°C. Tensile tests were done according to TAPPI T494 om-96 using INSTRON tester (model 4204 computer controlled) under the following conditions: Cross head speed=25 mm/min.; Load cell capacity=1.0 KN; specimen dimension=160x15 mm; Grip distance=100 mm.

Table 15: Test methods for pulp beating and strength property evaluations.

Laboratory beating of pulp	T248 sp-08
Forming Hand sheets for Physical Tests of Pulp	T205 sp-06
Physical Testing of Pulp Hand sheets	T220 sp-06
Internal Tearing Resistance of Paper (Elmendorf)	T414 om-04
Tensile Breaking Properties of Paper	T494 om-06

High quality printing and writing paper grades require pulps of high tensile strength to withstand the forces the paper undergoes during manufacturing and use in high speed machines. The pulps were evaluated in papers with a schopper riegler degree around 35, which is industrially used to printing and

writing paper grades. The results are shown in Figure 10. The xylans enriched pulp tended to form papers of higher tensile energy absorption, specific elastic modulus, tensile index, burst index and air resistance permeance, when compared to the reference pulp. However, the xylan enrichment resulted pulp of lower tear index and bulk compared to the reference. When the results of properties at a given beating energy consumption were analyzed, all properties evaluated showed higher values for the xylan treated pulp with much less energy beating energy demand. The only exception occurred for the tear index. This is explained by the lower amount of cellulose chains in the xylans enriched samples compared to the reference; the tear strength property benefits from cellulose rich pulps.

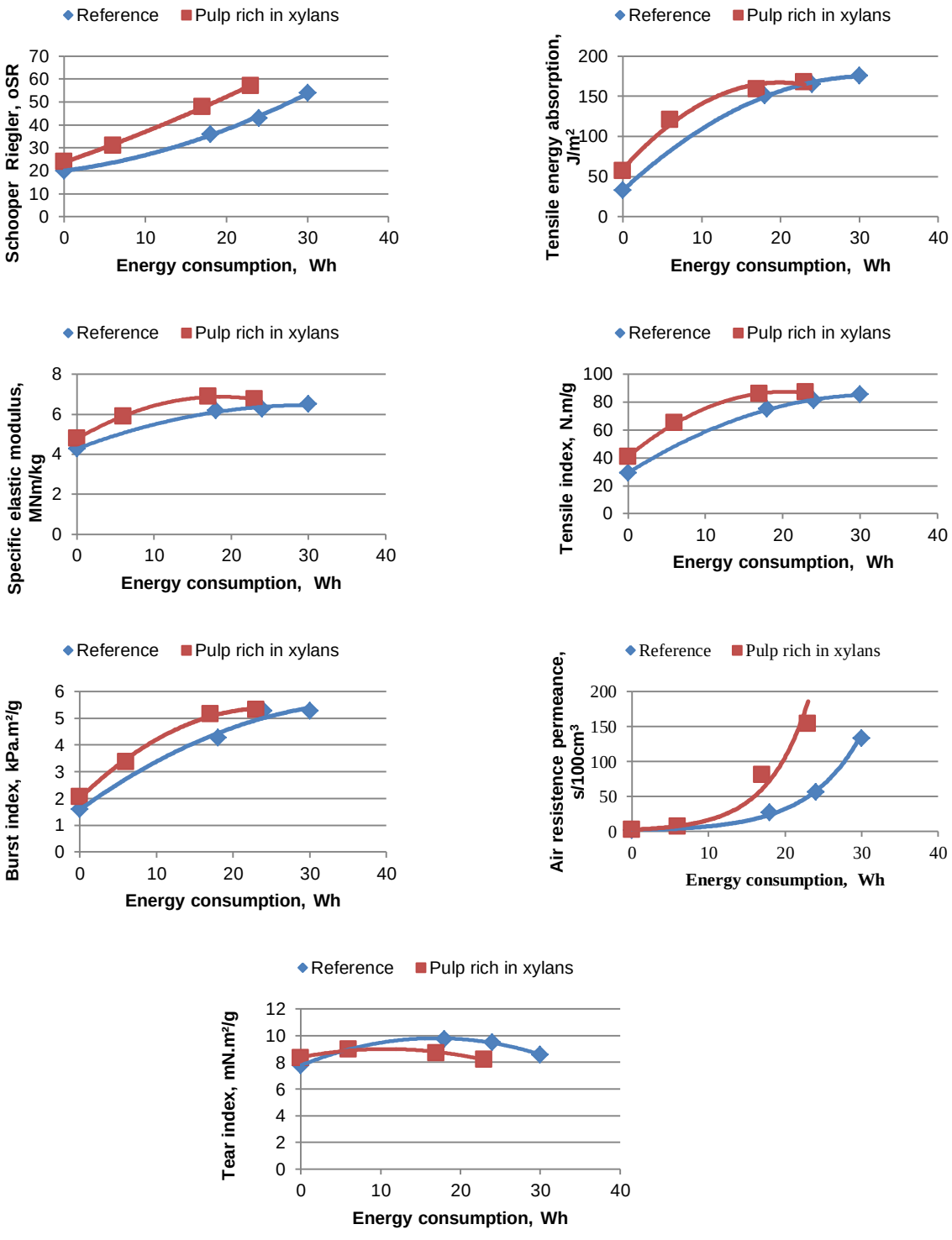


Figure 10: Energy consumption versus pulp properties for the reference and xylan enriched pulp samples.

Part II: Production of special Tissue Grade Pulps by Partial Xylan Removal

Tissue papers are responsible for a significant portion in the total paper manufacture (8-10%). Among the requirements of pulps quality for these papers, their level of xylan stands out, which has been a controversial matter. The aim of this study was evaluating the influence of eucalypt pulp xylan content on its tissue grade properties. A brown eucalyptus kraft pulp was treated with variable alkali charges in a CCE stage in order to obtain materials with different xylans content. The resulting samples were bleached by the ODPD sequence and evaluated for their main properties as applicable to tissue grade pulps. For this study, it was uses an industrial *Eucalyptus urograndis* kraft pulp was used: kappa number = 16.1, viscosity = 1226 dm^3/kg, brightness = 42.1%ISO, xylan content = 15.6% and HexA = 61.8 mmol/kg.

Four alkali charges (10, 30, 50 and 70g/L NaOH) were used to produce pulp of different xylan contents, maintaining constant other operating conditions (30 min, 30°C, 10% consistency). This alkaline treatment, also designated as Cold Caustic Extraction (CCE) was carried out with 100 grams of pulp in polyethylene bags. The pulp sample was thoroughly mixed with sodium hydroxide and water and let stand for 30 minutes at 30°C, after which a sample of liquor was squeezed out from the pulp for analyses. Following, the pulp was washed with distilled water in excess. The filtrates collected after extraction were analysed for COD **(Standard Methods 5220D). All experiments were performed in duplicate.** These four CCE treated pulp samples and the reference pulp were subjected to analysis of viscosity (SCAN cm-15: 99), gravimetric yield and xylan content (HPLC - after acid hydrolysis according to TAPPI T249).

Following, they were oxygen delignified in a reactor/mixer, model Mark V (Quantum Technologies Inc), and further bleached with D-P-D sequence. The bleaching stages were carried out n polyethylene bags under the conditions shown in Table 16. By the end of each stage, the pulp was washed with the equivalent of 9 m^3 of distilled water per ton of pulp. All stages were performed in duplicate. The bleached pulps were evaluated for their kappa number (TAPPI T236cm-85), brightness (TAPPI T525 om-92), brightness reversion (TAPPI UM200 - 4h, 105±3°C) and hexenuronic acid content (TAPPI T282pm-07).

Table 16. General Bleaching Conditions

Conditions	O	D	P	D
Consistency (%)	10	10	10	10
Time (minutes)	60	120	60	120
Temperature (°C)	100	75	82	76
Pressure O_2 (kPa)	500	-	-	-
ClO_2 as Cl_2 (kg/odt)	-	kappa factor = 0.16	-	5; 10; 15
H_2O_2 (kg/odt)	-	-	3.0	-
NaOH (kg/odt)	15	-		-
End pH	-	3.0	10.8	4.5

The bleached pulps were refined in a PFI mill, according to TAPPI 248 om-00. The beating intensity was expressed in Schöpper-Riegler degrees (°SR), according to TAPPI 200 sp-01. The number of PFI revolutions was variable, in order to obtain three levels of beating, allowing the development of refining curves in the range 15 to 50°SR. The hand sheets were formed using a TAPPI former, with a grammage of approximately 60 g/m^2, according to TAPPI 205 sp-02 and TAPPI T218 sp-97. The hand sheets were placed in acclimatized room at temperature of 23 ± 1°C and relative humidity of 50 ± 2% before testing.

The pup hygroscopic properties were evaluated for water absorption capacity test - the basket test (NBR 15,004), Water Retention Value - WRV (TAPPI UM 256) and Klemm capillary (ISO 8787:86). The pulp optical properties of light scattering coefficient (ISO 2471:98) were measured in a spectrophotometer Datacolor, model Elrepho 450X. Tests relating to tensile index were made in Instron

apparatus, with a distance between jaws 100 mm, test speed of 25 mm / min load cell 1000 N and computerized data acquisition and analysis (TAPPI T494 om- 01). Tests for tear strength (TAPPI T414 om-98) were performed on the Eldendorf device. The test for the specific apparent volume (bulk) followed TAPPI T220 sp-01 standard.
The results were statistically analysed using the Statistica software (version 7.0). The analysis of variance (ANOVA) was done at the 5% significance level. The hypotheses tested were: H0: all means are equal, i.e., there is no significant difference between treatments, and Ha: there is at least an average statistically distinct from the others. Since the ANOVA showed significant difference between treatments, the Tukey test was applied to find out which treatments were distinguished from each other.
In addition, it was used the Curve Expert software (version 1.4) to obtain the models from the drainability and beatability analysis of the pulp samples and their different levels of refining. The adjusted equations were compared by F test, using the identity test models at the 5% significance level, according to the methodology for linear (Regazzi, 1993) and for nonlinear models (Regazzi, 2004). The hypotheses tested were: H0: all equations are equal and can be represented by a reduced common equation, and Ha: the equations are statistically different and cannot be reduced to a common equation.

Xylan Extraction from Kraft Pulp by Alkali Treatment: The alkaline treatment aimed at producing brown pulp with different levels of xylan content. The resulting pulps were evaluated for bleachability and their properties for production of special tissue papers. The effect of the NaOH charge on the pulp characteristics are shown in Table 17.

Table 17. Effect of the NaOH concentration on brown pulp characteristics after the CCE treatment (30 min, 30°C, 10% consistency)

Results	**NaOH concentration, g/L**				
	0 (Ref.)	**10**	**30**	**50**	**70**
Xylans (%)	15.6[a]	14.5[a]	10.8[b]	8.1[c]	5.9[d]
HexA's (mmol/kg)	61.8[a]	61.4[a]	50.0[b]	34.7[c]	22.8[d]
Yield (%)	-	98.5[a]	96.7[b]	89.2[c]	85.7[d]
Kappa number	16.1[a]	15.2[b]	13.3[c]	10.5[d]	7.9[e]
ISO Brightness (%)	42.1[a]	40.1[b]	41.4[a]	43.2[c]	42.9[d]
Viscosity (dm^3/kg)	1226[a]	1204[a]	1245[a]	1278[a]	1308[b]
COD of the filtrate (kg/t)	-	6.3[a]	66.3[b]	196.4[c]	206.6[d]

As anticipated the amount of xylan remaining in the pulp decreased with increasing alkali concentration in the CCE stage, reaching 62% removal when treated with 70 g/L NaOH. This result ins in agreement with those of Wang, 2008. According some authors, xylans are soluble in alkali and water, and therefore they are easily removed in alkaline treatments (Al-Dajani, 2008; Sjostrom, 1999; Hashimoto, 1975; Scott, 1989; Cunninghan, 1986; Walton, 2010). The xylan removal had a direct effect on pulp HexA content and pulp yield. The HexA are directly linked to the xylan chain, and thus removed along with these polymers (Petit-Breuilh et al, 2004). The yield drop is caused by the xylan losses. Other materials such as degraded cellulose and lignin also contribute to yield drop.
The pulp kappa number drop across the CCE treatment indicates that lignin and HexA are partially removed. The HexA contribution to the kappa drop can be determined considering that each 10 mmol/kg of HexA lost represents one kappa unit.
The pulp brightness showed a small increase in the CCE treatment due to the partial removal of lignin chromophores present in the brown pulp. Although the viscosity is influenced by the low molecular weight carbohydrates such as hemicellulose and degraded cellulose, there was no statistical difference in the results thereof, except for the pulp treated with NaOH 70 g/L. The results for the COD in the filtrate correlated with the xylan and kappa losses across the CCE stage. An increase in the filtrate COD values was observed as the alkaline extraction became more severe.

Oxygen Delignification and D-P-D Bleaching of the Xylan Depleted Pulps: The results of the oxygen delignification are shown in Table 18. The reduction in kappa number for the alkali pre-treated pulp ranged from 31.7 to 33.6%, i.e. the decrease in xylans content had no significant impact on the efficiency of this stage. For the reference treatment the efficiency was 32.3%. It was expected that this efficiency would increase in the pulps treated with alkali, because they contained a lower content of HexA, which notoriously have a negative effect on the efficiency of the O-stage. However, this effect was masked by the fact that the alkali-treated pulps had different initial kappa numbers. It is not correct to compare the efficiency of the O-stage in pulps of different kappa numbers. As expected, the HexA removal in the range of 2.6 to 4.6% was very low in the O-stage, since they do not react with oxygen and do not contribute significantly to kappa number reduction (Vuorinen, 1996)

Table 18. Oxygen delignification results for pulps containing variable amounts of xylans

Results	Xylans content, %				
	15.6	14.5	10.8	8.1	5.9
Kappa number	10.9[a]	10.1[a]	9.0[b]	7.1[c]	5.4[d]
HexA (mmol/kg)	59.8[a]	59.8[a]	48.1[b]	33.1[c]	22.0[d]
Viscosity (dm^3/kg)	1101[a]	1071[b]	915[c]	-	904[c]
ISO Brightness (%)	49.2[a]	53.4[b]	54.0[c]	55.2[d]	57.8[e]
Kappa reduction (%)	32.3	33.6	32.3	32.4	31.7
HexA reduction (%)	3.1	2.6	3.7	4.6	3.5
Viscosity reduction (%)	10.2	11.0	26.5	-	30.9
Brightness gain (%)	7.1	13.3	12.6	12.0	14.9

The viscosity of the pulps were lower in the samples treated with higher concentrations of NaOH (904-1071 dm^3/kg) when compared to the reference (1101dm^3/kg). The pulps treated with alkali are more susceptible to degradation since they are devoid of hemicellulose in the fiber surface, which protect the cellulose chains against the attack of radical species common in the O-stage. The brightness gain in the oxygen delignification increased with increasing concentration of the NaOH liquor, which can be explained by the lower kappa numbers achieved.

The oxygen delignified pulps were bleached under similar conditions with the sequence D-P-D, keeping a constant kappa factor (0.16) in the first chlorine dioxide stage and varying the dosage of chlorine dioxide in the second stage. All the experiments aimed at achieving final brightness of 90% ISO. The bleaching results are presented in Table 19.

Table 19. Bleaching results with the D-P-D sequence for pulps containing variable amounts of xylans

Results	Xylans content, %				
	15.6	14.5	10.8	8.1	5.9
Total active chlorine [1/] (%)	3.87[a]	3.75[a]	3.07[b]	2.27[c]	1.99[d]
Bleachability (kappa ud / % active Cl)	2.8[a]	2.7[a]	2.9[a]	3.1[a]	2.7[a]
ISO Brightness (%)	89.8[a]	89.8[a]	89.9[a]	89.8[a]	90.1[a]
ISO Brightness reversion (%)	2.7[a]	2.6[a]	2.4[a]	2.5[a]	2.2[a]
Viscosity (dm^3/kg)	926[a]	911[a]	813[b]	-	767[c]
HexA's (mmol/kg)	6.6[a]	7.3[b]	7.3[b]	9.4[c]	5.5[d]

In general, the xylan removals in the CCE treatment decrease bleaching chemical demand. The lowest value of total active chlorine demand for bleaching was achieved with the pulp containing the least xylans, and this is explained by the lower kappa number of this pulp after the oxygen delignification stage (Table 18). The results also show that the bleachability between 2.7 to 3.1 did not vary greatly among the samples. There was no statistical difference among treatments for brightness reversion. The pulp brightness reversion ranged from 2.2 to 2.7 %, with the lower values observed for the pulps containing less xylan. On the other hand, the bleached pulp HexA content varied between 5.5 and 9.4 mmol/kg, with the highest value observed in the pulp with highest xylan content. The highest brightness reversion in the pulps with lower xylan content can be explained by its lower HexA content. These components have been pointed out as the greatest responsible for the pulp brightness reversion (Vuorinen, 1996). The pulp viscosities ranged from 767 to 926 dm^3/kg, with the highest value found for the reference sample and lowest for the samples treated with higher concentration of alkali solution. The results confirm the role of xylans in protecting the cellulose chains against degradation by oxidants during bleaching. Thus, by removing xylans, the cellulose chains become susceptible to degradation.

Properties of xylan depleted pulps: By decreasing the xylan content in the pulp, the energy consumed during beating is increased. It is well known that the xylan exerts positive influence on the swelling, hydration and fiber flexibility, which favors the refining process. Pulps that are used in the manufacture of tissue paper are slightly refined, to about 20°SR in order to produce a tensile tensile index in the range of 20Nm/g. Extensive beating is not desirable because it impairs the ability of the paper to absorbs and retain water through capillarity.

Figure 11A shows the impact of pulp xylan content on beating energy consumption to achieve the tensile index of 20 Nm/g. It is observed that by increasing the xylans content from 6 to 16%, the power consumption is decreased by 88%, which corresponds to approximately 9% energy savings per each 1% increase in xylans content. This trend has been observed by other workers (Vuorinen, 1999). Figure 11B shows the effect of xylan content on the tensile index for the energy consumed to achieve 20°SR (this value is considered a target in the most common mills). It can be concluded that pulps with lower contents of xylans have lower tensile index and consume more energy for the same Schöpper-Riegler. This implies that for a given energy consumption, pulps with lower levels of xylans will have lower tensile index.

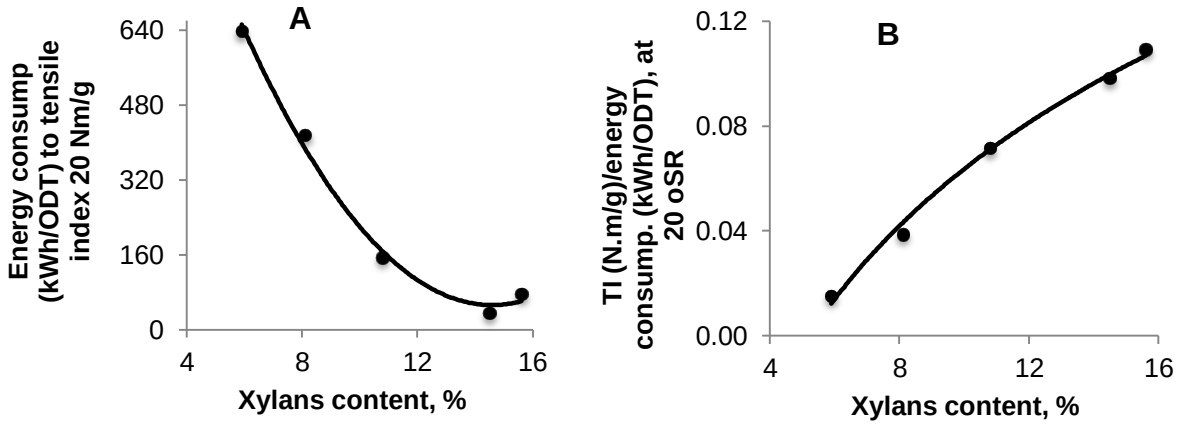


Figure 11. Effect of pulp xylan content on beating energy demand expressed two different ways: (A) at a fixed tensile of 20 N.m/g and (B) at fixed drainage resistance degree of 20°SR.

The drainability of the pulps affects the paper machine speed and its runnability. It is measured by the drainage resistance (^{o}SR) at a given energy consumption for refining. Figure 12A shows that pulps with lower xylans content have lower drainage resistance at a tensile strength of 20 ^{o}SR. This implies that lower the xylan content in the pulp, for each unit of energy (Wh or kWh), the lower the drainage resistance of the pulp. Figure 12B also shows that resistance to drainage is highly minimized when pulp xylan content is decreased. These results confirms the action of hemicellulose in the fluid retention during drainage of the cellulosic material. Therefore, pulps of low xylan contents are desirable for tissue paper production because of their enhanced drainability, which indirectly means improved runnability.

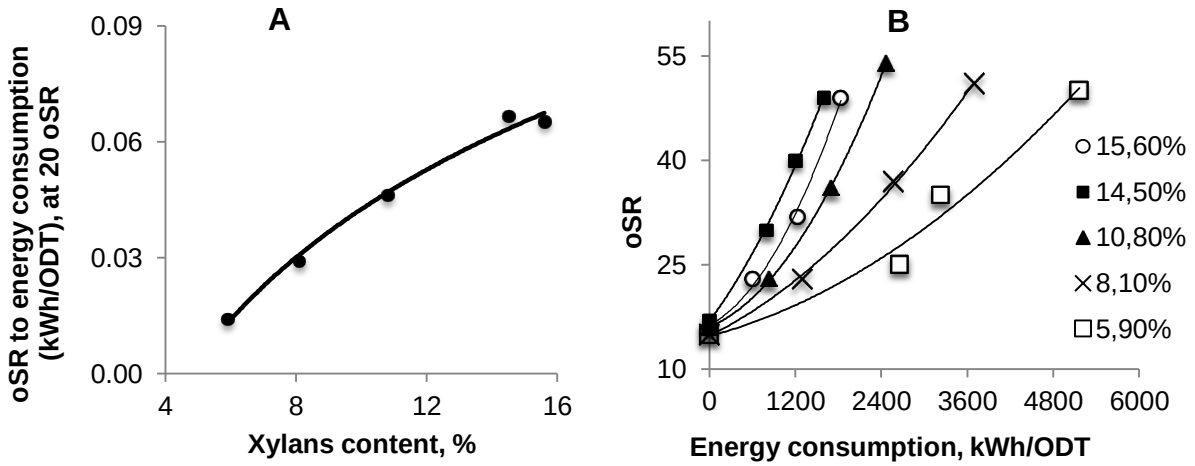


Figure 12. Effect of pulp xylan content on drainability expressed two different ways: (A) at fixed drainage resistance degree of 20^{o}SR, (B) at various drainage resistance degrees.

The Water Retention Value (WRV) data for the five bleached pulps samples containing different amounts of xylan are shown in Table 20. There was a clear trend of decreasing WRV with decreasing pulp xylan content, with the effect being more pronounced at the very low levels of xylans. These results were anticipated since xylans are well known to be highly hydrophilic and thus their removal from the pulp tend do decrease pulp water retention capacity. The WRV is a combined property involving chemical effects (hemicellulose content) and physical effects which include fiber population, total pore volume and distribution of the capillaries (Foelkel, 2007). The properties of swelling and hydration of the fibers are very much influenced by the cooking and bleaching operations, since they affect the amount of hemicelluloses content and the cell wall integrity. The high content of hemicelluloses, associated with large number of fibers and degraded fibers (low viscosity), leads to pulps with high water retention. Therefore, it makes the pulp very difficult to drain and flow into the paper machine. These pulps even when unrefined, have high Schöpper-Riegler (°SR) and this shows that the drainability is hampered even for unrefined pulps (Foelkel, 2007). Of course, lowering the water retention value of the pulp by decreasing their xylan contents can be advantageous for runnability and positive for certain tissue paper grades.

Table 20. **Effect of pulp xylan content on water retention value (unbeaten pulp).**

Xylans content, %	15.6	14.5	10.8	8.1	5.9
WRV, %	162[a]	161[a]	154[b]	141[c]	131[d]

Figure 13A shows the effect of xylan content on pulp water absorption capacity at 20ºSR. It is shown that water absorption capacity increases with decreasing pulp xylan content. Water absorption capacity is one of the most fundamental properties of tissue grade pulps. The presence of xylan in the pulp creates difficulty in the movement of water molecules in the pulp fibers, thus preventing a good absorption of

water therein. The pulp absorption capacities at different refining levels are presented in Figure 13B. The reference sample and the sample containing 14.5% of xylan were represented by a single curve, since they were statistically equal according to the identity test models; this similarity occurred because the xylan content of this pulp and that of the reference were somewhat similar as well. With the progress of pulp beating, the water absorption capacity of the pulps decreased. At the higher beating levels (> 30°SR) the water absorption capacity of the pulps were no longer affected by the pulp xylan content. Therefore, the pulp xylan content influences the water absorption capacity only at low beating levels, but low beating levels are typical of tissue grade pulps.

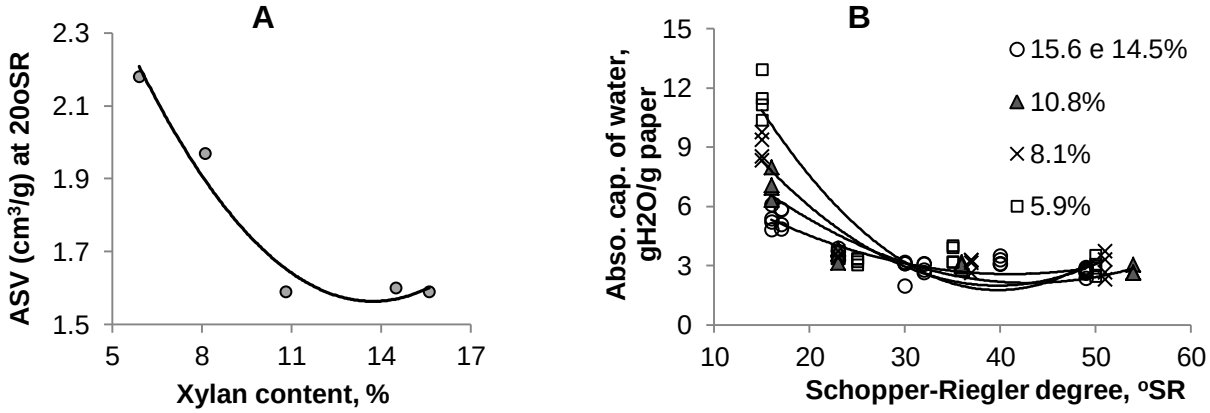


Figure 13. Effect of pulp xylan content on water retention capacity expressed two different ways: (A) at fixed drainage resistance degree of 20°SR, (B) at various drainage resistance degrees.

The Klemm capillarity is an assay that measures the rate of absorption of liquids, being a very important in test for tissue grade pulps. This property indicates the easiness by which the water is absorbed by the capillaries within the paper. Thus, pulps that present higher Klemm capillarity tend to have less bonding and greater amounts of empty spaces (Castanho, 2000). The beating aims to increase inter-fiber bonding *via* hydrogen bonding by the hydroxyl groups in the cellulose chains. The frequency of these bonds can be increased with a larger contact area between fibers. Therefore, the movement of water molecules in the fiber is restricted. As a result, the values of Klemm capillarity tends to decrease with increasing beating intensity.

It can be seen in Figure 14A that the decreases of xylan content in the pulp increased Klemm capillarity, i.e., increases the distance that the water flows into the paper structure. One explanation for this is that water that moves through the paper structure interacts greater with the hemicelluloses than with the cellulose chains. It is known that hemicelluloses are amorphous, thus hampering the displacement of water in the paper structure. Thus, papers made from pulps with lower xylan content are favourable for the production of tissue paper because they have good absorption of liquids, which is desirable during the tissue paper applications**.** Figure 14B shows the results of the Klemm capillarity according to the refining energy consumption for the papers produced from five samples of bleached pulps. One of the major effects of the refining process is the increase of pulp fibrillation which results in an increase in surface area for the beaten fibers. Besides the increase of interfiber bonding, the refining also produces fines. As a result, the movement of water molecules within the fiber network is more difficult. Then, the Klemm capillarity values tend to decrease by increasing the refining energy.

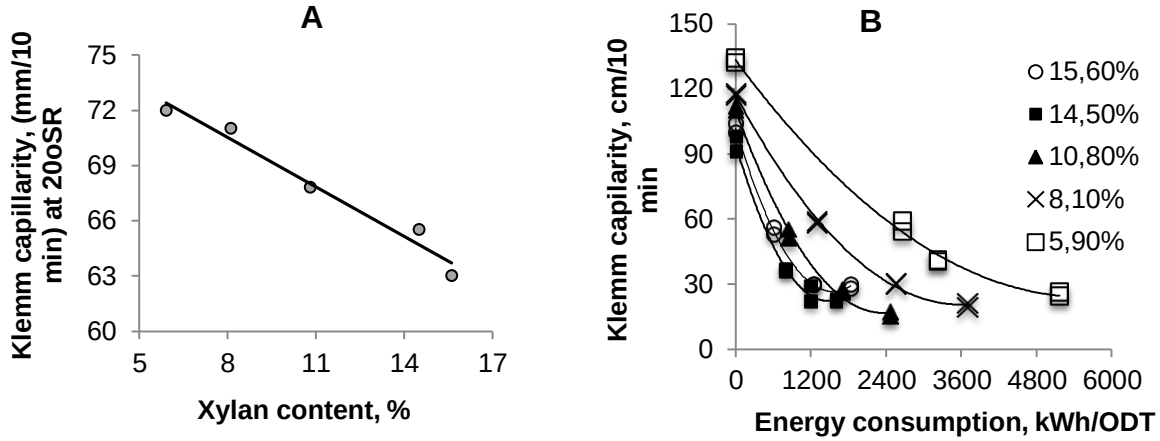


Figure 14. Effect of pulp xylan content on capillarity Klemm expressed two different ways: (A) at fixed drainage resistance degree of 20°SR, (B) at various beating energy consumptions.

It is desired that the tissue paper possess a minimum tensile strength enough to run the paper machine but not too high to a point where it becomes harmful for paper softness. It is observed in Figure 15A that xylan content negatively influences pulp tensile index. However, a pulp containing about 8% xylans possess a tensile index sufficiently high for tissue grade applications with the benefit of being softer that the reference pulp for example. This is advantageous for tissue grade pulp production. Figure 15B shows the tensile index curves for pulps produced at various refining energy consumptions. It can be seen that pulps with higher xylan contents require less energy to achieve a given tensile index.

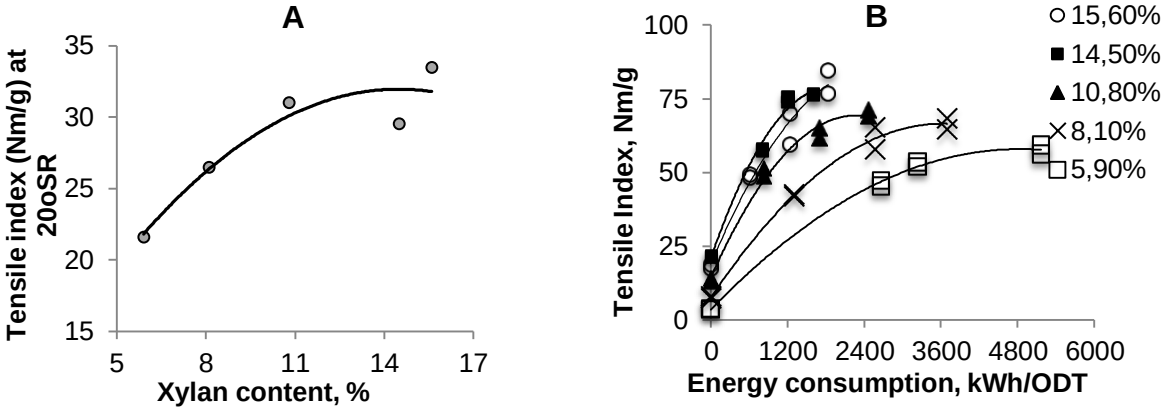


Figure 15. Effect of pulp xylan content on tensile index expressed two different ways: (A) at fixed drainage resistance degree of 20°SR, (B) at various beating energy consumptions.

Tear index is the average force required to tear a paper sheet divided by its weight. The tear resistance test is used to evaluate the resistance of papers such as bags, labels, printing and writing, tissues and other papers that requires tear strength during their use (‘DAlmeida, 1988). Figure 16A shows that that tear strength correlate positively with pulp xylan content at a 20 °SR. Tear index indirectly measures the strength of individual fibers. The results obtained for the tear index in this study is in accordance with published data (Rebuzzi, 2006), that report tear values of 3 and 9 mN.m2/g for sulphite pulp and kraft pulps containing 6% and 17% pentosans, respectively. Figure 16B shows the curve of tear index for pulps produced at various bating energy consumptions. It is observed that pulps with higher contents of xylan, at the same tear index, required less energy when compared to the pulps with lower contents of xylan. For all pulps, the tear index increased with increasing beating energy to a certain level but with the intensification of beating the tear index decreased. This beating causes damage to the fiber structure, causing reduction of fiber length. Pulps of low xylan content tend to have low tear index due their high refining intensity requirements and consequent larger fiber damage.

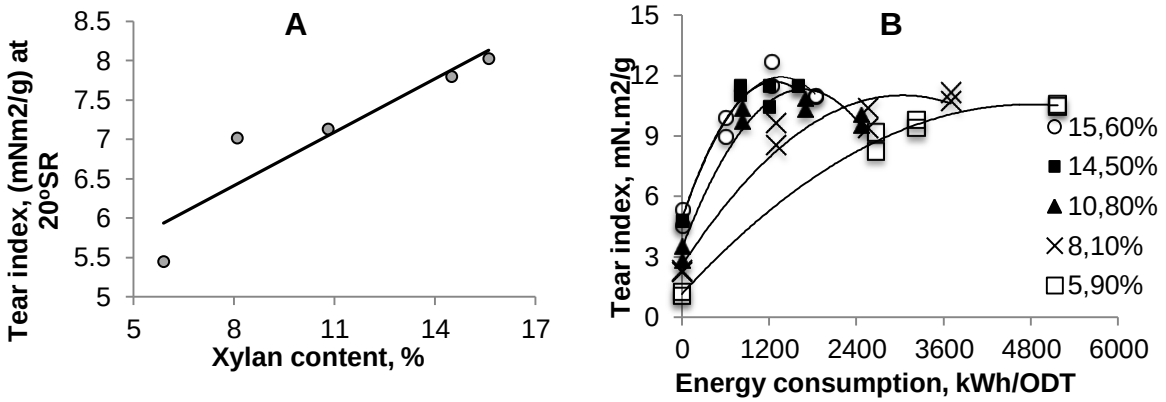


Figure 16. Effect of pulp xylan content on tear index expressed two different ways: (A) at fixed drainage resistance degree of 20°SR, (B) at various beating energy consumptions.

The apparent specific volume, also known as bulk, is calculated by dividing the thickness (µm) of the paper for its weight (g/m^2). The structural characteristics of the fibers also influence the determination of this property. Stiffer fibers produce bulkier papers due to their low ability to form a fiber mat (Howard, 1992). Figure 17A shows the effect of pulp xylan content on bulk calculated for 20°SR, and indicates that bulkier paper is obtained with pulps of lower xylan contents. Bulkier pulps are interesting for tissue grade paper manufacture because they present high internal softness. It can be observed that the reference sample and the one containing 14.5% xylans showed similar bulk, and are represented by a single curve in Figure 17B. The other samples had significant differences in their bulk values. With the increasing beating energy the bulk property tended to decrease for all samples regardless of their xylan contents, but the trend was maintained.

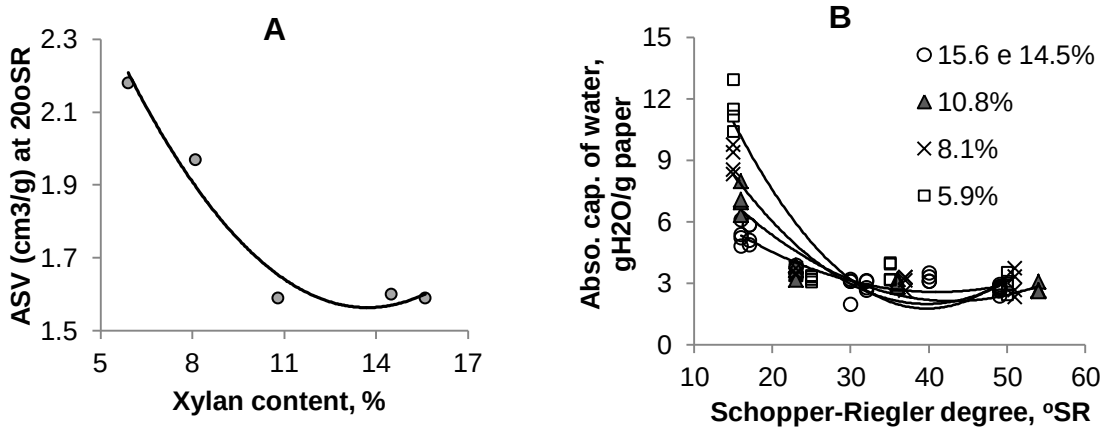


Figure 17. Effect of pulp xylan content on apparent specific volume (ASV) – bulk, expressed two different ways: (A) at fixed drainage resistance degree of 20°SR, (B) at various beating energy consumptions.

Paper optical properties are attributable to the amount of inter-fiber bonding, the number of optical surfaces and the refractive particles of light scattering potential (Carpim, 1987). Pulps with low xylans content have higher light scattering coefficients (LSC) and opacity, which can be explained by the influence of xylans in the inter-fiber bonding. The decrease in xylans reduces the interfaces fiber - air, thereby reducing the LSC of the pulp (Figure 18A**).** Figure 18B shows that the LSC of the pulps was influenced by the content of pulp xylans regardless of beating energy applied. Pulps with higher xylan

content demanded less energy to achieve a given LSC. The intensification of refining caused a negative effect on the LSC, and its values abruptly decreased with increasing pulp xylan content.

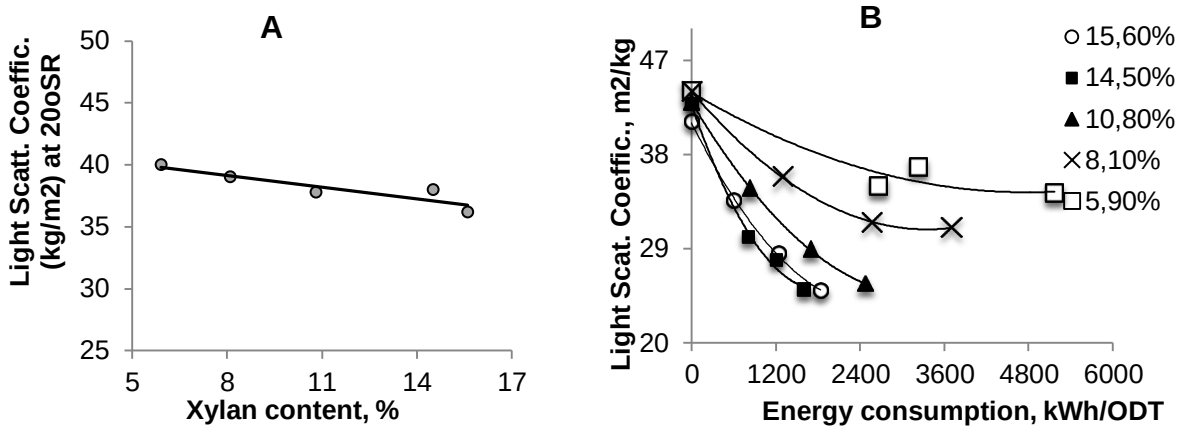


Figure 18. Effect of pulp xylan content on light scattering coefficient, expressed two different ways: (A) at fixed drainage resistance degree of 20°SR, (B) at various beating energy consumptions.

Conclusions of Task 4.3

The main objective of Task 4.3 was assessing the potential of upgrading xylans as paper grade pulp additives. The results obtained were relevant to fine tune the methodology and assess the potential of the technology. Positives result were obtained, such as: (1) the ideal conditions for xylans extraction are: 15 minutes, 15% of consistency and 400 kg/odt of NaOH; (2) Grass xylan deposition on wood pulp was successful, increasing *the* xylan content from 14.4 to 17.3%; (3) The deposited xylans are stable across bleaching and beating; (4) Bleaching yield gains of about 3% due to xylans deposition were achieved; (5) Pulps with improved strength properties and beatability were obtained; (6) The removal of the xylans from pulp using alkali treatment reduced significantly the chlorine demand in the bleaching sequence DPD at 90% ISO brightness; (7) the xylan depleted pulps derived from the xylan extraction treatment showed potential for production of special tissue grade papers with improved drainability, bulk, softness and water absorption capacity, and with acceptable tensile and tear strength.

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3.2. DEVIATIONS FROM THE WORK PLAN

No deviations were produced from the original Work Plan

4. PLANS FOR DISSEMINATION AFTER 36th MONTHS

During the course of the project we have already published several papers regarding the detailed composition of elephant grass and the selected Brazilian eucalypt hybrids. We are now preparing one more paper which is being submitted in the *Bioresources Journal* for publication, and it is undergoing revision following the demand of editors of the journal.

On the other hand, part of the results obtained during the course of the project will be presented in several scientific congresses, including "*6th International Colloquium on Eucalyptus Pulp*", that will be held in Colonia del Sacramento, Uruguay, during November 24-27, 2013; the "13th European Workshop on Lignocellulosic and Pulp", that will be held in Seville, Spain, during June 24-27, 2014; the 2013 International Symposium on Wood Fiber and Pulp Chemistry, in June 2013, Vancouver, Canada.

Also, one PhD Thesis will be presented at the Federal University of Viçosa with the main results obtained during the course of the project: "The biorefinery concept applied to pulp and paper industry" by Fernando José Borges Gomes.

5. DELIVERABLES AND MILESTONES

List the deliverables and milestones you are responsible due during the period covered by the report indicating whether they have been achieved.

DL3.6: Characterisation of black liquors and other side streams (**UFV**; 24th month) has been achieved in due time.

DL4.1: Pulp and papermaking evaluation after optimised pre-treatment (**UFV**, Novozymes; 34th month) has been achieved in due time.

DL4.4: Procedure for improving eucalypt pulp with grass xylan additive (**UFV**; 36th month) has been achieved in due time.

DL6.1: FUNARBE financial report (**UFV**; 36th month) has been achieved in due time.

6. NEW CONTACT PERSONS

In case that any of the responsible persons of any of the beneficiaries is replaced for a particular reason, please explain and indicate the name and contact details of the new contact person.

Not applicable

APPENDIX B

CIB – IRNAS

Grant agreement no: KBBE-2009-3-244362

"Optimized pre-treatment of fast growing woody and nonwoody Brazilian crops by detailed characterization of chemical changes produced in the lignin-carbohydrate matrix"

Second Periodic Report

1-Jul-2011 to 31-Dec-2012

Partner P2 (CSIC): P2a (CIB) + P2b (IRNAS)

Grant agreement no: **KBBE-2009-3-244362**

Project acronym: LIGNODECO

Project title: Optimized pre-treatment of fast growing woody and nonwoody Brazilian crops by detailed characterization of chemical changes produced in the lignin-carbohydrate matrix

Funding Scheme: Collaborative project (small or medium-scale focused research project)

Thematic Priority: KBBE-2009-3

Period covered: From 1 July 2011 to 31 December 2012

Date of preparation: 15 January 2013

Start date of project: 1 January 2010 Duration: 36 months

Partner name: **CSIC (CIB+IRNAS)**
Author 1 *José C. del Río (IRNAS)*
Author 2 *Ana Gutiérrez (IRNAS)*
Author 3 *Jorge Rencoret (IRNAS)*
Author 4 *Pepijn Prinsen (IRNAS)*
Author 5 *Alejandro Rico (IRNAS)*
Author 6 *Angel T. Martínez (CIB)*
Author 7 *Craig B. Faulds (CIB)*
Author 8 *Álvaro Berbis (CIB)*
Author 9 *Jesús Jiménez-Barbero (CIB)*
Author 10 *Marta Pérez-Boada (CIB)*

1. SUMMARY OF THE WORK

During this reporting period, in **WP2** we concluded the study of the delignification of elephant grass and eucalypt wood by the use of a high-redox potential laccase (from *Trametes villosa; TvL*) in the presence of HBT as mediator. The study showed for the first time that woody and nonwoody biomass can be significantly delignified by this laccase-mediator system (with 30-50% lignin removal) by applying a sequence consisting of several alternative laccase-mediator and alkaline extraction stages, directly on the whole lignocellulosic material. The enzymatic pretreatments also resulted in improved cellulase hydrolysis, enabling shorter fermentation times for ethanol production. On the other hand, in **WP2** we studied the delignification elephant grass and eucalypt wood by a commercial low-redox potential laccase (from *Myceliophthora thermophila; MtL*) and using methyl syringate (MeS) as mediator. Four cycles of enzymatic treatment followed by alkaline extraction were assayed, similarly as previously done with the high-redox potential laccase from *Trametes villosa*. The lignin content in elephant grass was not modified by the use of this low-redox laccase-mediator system. However, and interestingly, when applied to eucalypt wood, the lignin content dramatically decreased after the enzymatic treatments, attaining up to 50% lignin removal when using high doses of *MtL* and MeS. Further insights into the lignin structure modification by the enzymatic treatments were achieved by 2D-NMR analyses of the lignocellulosic samples. The results obtained indicate for the first time that the laccase–mediator treatment using a low-redox laccase and a natural phenolic compound as mediator, can be an economically feasible procedure from an industrial point of view, since both the laccase (*MtL*) and the phenolic mediator (MeS) used are commercial and cheap.

Concerning enzymatic pre-treatment with hydrolases, the Novozymes celluloytic enzyme cocktail NS-22086 was found to be the most efficient preparation for the solubilisation of finely milled elephant grass (EG), especially after the biomass has been modified slightly by MSD Pressafiner treatment. An optimum dosage (100 ml/kg biomass), pH of 6 and the reaction to take place in water was recommended for the demonstration trials at CTP (**WP5**). In addition, we demonstrated that NS-22086 was more efficient than Celluclast in solubilising glucan from the initial lignocellulosic matrix and that the arabinoxylan and Klason lignin component of EG remained virtually unaffected. This suggests that the hemicellulosic and lignin components of EG do not form a barrier for the degradation of cellulose in this biomass. The addition of a feruloyl esterase type-C from the filamentous fungi *Talaromyces stipitatus* (TsFaeC), previously shown to remove acetyl groups from both the hemicellulose and lignin components of abaca, also improved slightly the solubilisation of Pressafiner pre-treated EG by NS-22086, together with an increase in reducing group production and acetic acid release. A much smaller increase in these parameters was achieved with the same esterase supplementing Celluclast, while there was no observed benefit with the addition of a type-A feruloyl esterase from *Aspergillus niger*. It is not yet known if TsFaeC is acting on the acetate groups in the biomass or on the hydroxycinnamate linkages present on the hemicellulose and lignin, or on both.

The internal pith of EG is more extensively degraded (>50%) compared to the outer cortex (31%) or the whole EG stem (35%). NS-22086 was the best enzyme cocktail on all these EG-derived material with Ultraflo the worst of the preparations tested. However, reducing sugar release from the cortex was higher than from the pith. The presence of the pith may restrict enzyme accessibility to their substrates in solid stems compared to than published before for hollow stem biomass, such as wheat straw.

In addition of the above studies with hydrolytic enzymes, it was demonstrated that the presence of low concentrations of co-solvents, such as DMSO and methanol has positive effects on the ability of the commercial (Novozymes) low radox potential laccase from *Myceliophthora thermophila* (MtL) to oxidize 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) and 2,6-dimethoxyphenol (2,6-DMP). The same reactions using the high radox potential laccase from *Pycnoporus cinnabarinnus* (PcL) resulted in reaction inhibition by the same solvents. A purified sample of MtL supplied by Novozymes displayed a 4-fold increase in the oxidation of 2,6-DMP in the presence of 30% methanol.

The presence of DMSO reduced the solubility of biomasses, such as EG, wheat straw and abaca, although acetic acid release was increased, complimenting the results found on model esterase

substrates. DMSO at low concentrations was also shown to have a protective effect on xylanase activity within Ultraflo when residual activity in the supernatant was measured in comparison to reactions performed in solely aqueous media. DMSO appears to be solubilising carbohydrate (xylan and glucan) in preference to lignin in the biomass but also leading to reduced solubility of other components in the EG. This carbohydrate solubilisation effect was biomass specific, as with wheat straw, xylose was not solubilised.

In **WP3**, during this period, we fully accomplished and finished the structural characterization of the main components of the feedstocks selected in the project (elephant grass and Brazilian eucalypt hybrid woods) by calculating their molecular weights. In addition, in **WP3** we fully accomplished the chemical characterization of the residual lignins and black liquors produced from elephant grass and eucalypt G1xUGL by different chemical alkaline deconstruction processes (kraft, soda-AQ and soda-O_2 processes) at different kappa numbers. Two different sets of pulps produced from elephant grass and eucalypt hybrid G1xUGL by different cooking processes were received from Suzano: i) Pulps intended for paper production (pulp samples from eucalypt G1xUGL and elephant grass prepared by the kraft and soda-AQ processes at kappa 20 and 15, and their respective black liquors); and ii) Pulps intended for bioethanol and biogas production (pulp samples from eucalypt G1xUGL and elephant grass prepared by the soda-AQ and soda-O_2 processes at kappa 50, 35 and 15).The residual lignins were isolated from the pulps by acidolysis and, together with the lignins precipitated from the black liquors, were analyzed by 2D-NMR and Py-GC/MS. The main linkages observed in the residual lignins from G1xUGL and elephant grass, in both the kraft and soda-AQ pulps, were β–*O*–4 aryl ether, with lower amounts of β–β resinol and β–5 phenylcoumaran structures. A reduction of the main substructures was observed after the cooking, this reduction being more evident in the pulps with lower kappa number (kappa 15) due to the higher extent of delignification. At similar kappa numbers, the content of β–*O*–4 aryl ether linkages in the residual lignins from eucalypt G1xUGL and elephant grass were lower for the soda-AQ process than for the kraft process, indicating a higher efficiency of the soda-AQ process for delignifying these plant feedstocks. In the case of the pulps intended for bioethanol and biogas production, it seems that the soda-O_2 process is more efficient than soda-AQ process for delignification of eucalypt wood and elephant grass.

2. PROJECT OBJECTIVES FOR THE PERIOD

Not applicable.

3. WORK PROGRESS AND ACHIEVEMENTS DURING THE PERIOD

3.1 Work Progress

3.1.2. Progress on WP2. "Optimised pre-treatments for woody and nonwoody materials"

In WP2 we studied the delignification of elephant grass and eucalypt wood by the use of a high-redox potential laccase (from *Trametes villosa; TvL*) in the presence of HBT as mediator. The study showed for the first time that woody and nonwoody biomass can be significantly delignified by this laccase-mediator system (with 30-50% lignin removal) by applying a sequence consisting of several alternative laccase-mediator and alkaline extraction stages, directly on the whole lignocellulosic material. The enzymatic pretreatments also resulted in improved cellulase hydrolysis, enabling shorter fermentation times for ethanol production. On the other hand, we also studied the delignification elephant grass and eucalypt wood by a commercial low-redox potential laccase (from *Myceliophthora thermophila; MtL*) and using methyl syringate (MeS) as mediator. Four cycles of enzymatic treatment followed by alkaline extraction were assayed, similarly as previously done with the *TvL*. The lignin content in elephant grass was not modified by the use of this low-redox laccase-mediator system. However, and interestingly, when applied to eucalypt wood, the lignin content dramatically decreased after the enzymatic treatments, attaining up to 50% lignin removal when using high doses of *MtL* and MeS. In addition, important lignin removal (ca. 40% removal) was also observed when using lower doses of *MtL* (10 U/g) and MeS (1%). Further insights into the lignin structure modification by the enzymatic treatments were achieved by 2D-NMR analyses of the lignocellulosic samples. The results obtained indicate for the first time that the laccase–mediator treatment using a low-redox laccase and a natural phenolic compound as mediator, that produces up to 50% lignin reduction of eucalypt wood feedstocks, can be an economically feasible procedure from an industrial point of view, since both the laccase (*MtL*) and the phenolic mediator (MeS) used are commercial and cheap.

Concerning enzymatic pre-treatment with hydrolases, the Novozymes celluloytic enzyme cocktail NS-22086 was found to be the most efficient preparation for the solubilisation of finely milled elephant grass (EG), especially after the biomass has been modified slightly by MSD Pressafiner treatment. An optimum dosage (100 ml/kg biomass), pH of 6 and the reaction to take place in water was recommended for the demonstration trials at CTP (**WP5**). In addition, we demonstrated that NS-22086 was more efficient than Celluclast in solubilising glucan from the initial lignocellulosic matrix and that the arabinoxylan and Klason lignin component of EG remained virtually unaffected. This suggests that the hemicellulosic and lignin components of EG do not form a barrier for the degradation of cellulose in this biomass. The addition of a feruloyl esterase type-C from the filamentous fungi *Talaromyces stipitatus* (TsFaeC), previously shown to remove acetyl groups from both the hemicellulose and lignin components of abaca, also improved slightly the solubilisation of Pressafiner pre-treated EG by NS-22086, together with an increase in reducing group production and acetic acid release. A much smaller increase in these parameters was achieved with the same esterase supplementing Celluclast, while there was no observed benefit with the addition of a type-A feruloyl esterase from *Aspergillus niger*. It is not yet known if TsFaeC is acting on the acetate groups in the biomass or on the hydroxycinnamate linkages present on the hemicellulose and lignin, or on both. The internal pith of EG is more extensively degraded (>50%) compared to the outer cortex (31%) or the whole EG stem (35%). NS-22086 was the best enzyme cocktail on all these EG-derived material with Ultraflo the worst of the preparations tested. However, reducing sugar release from the cortex was higher than from the pith. The presence of the pith may restrict enzyme accessibility to their substrates in solid stems compared to than published before for hollow stem biomass, such as wheat straw.

In addition of the above studies with hydrolytic enzymes, it was demonstrated that the presence of low concentrations of co-solvents, such as DMSO and methanol has positive effects on the ability of the commercial (Novozymes) low radox potential laccase from *Myceliophthora thermophila* (MtL) to oxidize 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) and 2,6-dimethoxyphenol (2,6-DMP). The same reactions using the high radox potential laccase from *Pycnoporus cinnabarinnus* (PcL)

resulted in reaction inhibition by the same solvents. A purified sample of MtL supplied by Novozymes displayed a 4-fold increase in the oxidation of 2,6-DMP in the presence of 30% methanol.

Finally, the presence of DMSO reduced the solubility of biomasses, such as EG, wheat straw and abaca, although acetic acid release was increased, complimenting the results found on model esterase substrates. DMSO at low concentrations was also shown to have a protective effect on xylanase activity within Ultraflo when residual activity in the supernatant was measured in comparison to reactions performed in solely aqueous media. DMSO appears to be solubilising carbohydrate (xylan and glucan) in preference to lignin in the biomass but also leading to reduced solubility of other components in the EG. This carbohydrate solubilisation effect was biomass specific, as with wheat straw, xylose was not solubilised.

Task 2.3. Enzymatic deconstruction using hydrolases and oxidoreductases

Lignocellulosic samples. Air-dried Elephant grass (*P. purpureum* and eucalypt (*E. globulus*) wood were grounded in a Retsch cutting mill to pass through a 100-mesh screen and then finely ball-milled in a Retsch S100 centrifugal ball at 400 rpm using agate jar and balls. Samples of *P. purpureum* pith and cortex manually separated were milled using a knife mill (Janke& Kunkel, Analysemühle) and subsequently ball-milled in an agate container in a Retsch S100 centrifugal ball mill.

Fungal laccases and mediators. Two fungal laccase preparations, obtained from the basidiomycetes *Trametes villosa* (*TvL*) and *Myceliophthora thermophila* (*MtL*), were provided by Novozymes (Bagsvaerd, Denmark) for this study. Laccase activity was measured as initial velocity during oxidation of 5 mM ABTS from Roche to its cation radical (ε_{436} 29300 $M^{-1}\cdot cm^{-1}$) in 0.1 M sodium acetate (pH 5) at 24ºC. The mediators used were HBT and methyl syringate (MeS) for *TvL* and *MtL*, respectively.

Laccase-mediator treatments. The enzymatic treatments were performed using finely divided (ball-milled) woody (eucalypt) and nonwoody (elephant grass) samples. The high redox potential *TvL* was used in the presence of HBT as mediator, while the low redox potential *MtL* was used with MeS as mediator. Several laccase doses (10, 25 and 50 U/g) were assayed, together with several mediator doses (1-3%). The treatments were carried out in 200-mL pressurized bioreactors (Labomat, Mathis) placed in a thermostatic shaker at 170 $rev\cdot min^{-1}$ and 50 ºC, using 2 g (dry weight) of lignocellulosic samples at 6% consistency (w:w) in 50 mM sodium tartrate (pH 4) as a buffer under oxygen atmosphere (2 bars) for 24 h. In a subsequent step, samples at 6% consistency (w:w) were submitted to a peroxide-reinforced alkaline extraction (Ep) using 1% (w:w) NaOH and 3% (w:w) H_2O_2 (both referred to pulp dry weight) at 80 ºC for 90 min, followed by water washing. Cycles of four successive enzyme-extraction treatments were applied. Controls including laccase without mediator were also performed.

Hydrolytic enzymes. The multienzyme preparation from *Humicola insolens* (Ultraflo), as well as Cellulase NS22086 and Celluclast preparations, and NZ188 were kindly provided by Novozymes. The A-type feruloyl esterase from *Aspergillus niger* (AnFaeA) was heterologously expressed in *Pichia pastoris* as previously described (Juge et al, 2001). The recombinant C-type feruloyl esterase from *Talaromyces stipitatus* was a kind gift from Biocatalysts Ltd.

a) Elephant grass and eucalypt wood delignification with the high-redox potential TvL and HBT

The lignin contents (as Klason lignin) of Elephant grass and eucalypt samples after the laccase-mediator (*TvL*-HBT) treatments were determined and compared with their respective controls (**Table 2.3.1**). The lignin content in both lignocellulosic samples decreased after the enzymatic treatments concomitantly with increasing laccase doses. In Elephant grass, the lignin content decreased about 11, 22 and 32% after the laccase-mediator treatments using enzyme doses of 10, 25 and 50 U/g, respectively. The reduction in lignin content of eucalypt wood samples was more pronounced, attaining 32, 34 and 48% lignin

decrease using laccase doses of 10, 25 and 50 U/g, respectively. The treatments with laccase alone (without mediator) scarcely decreased the lignin content (<5%) in both materials. In addition to assess the lignin content decrease, further insight into the lignin structure modification by the enzymatic treatments was achieved by 2D NMR analyses of the lignocellulosic samples as described below.

Table 2.3.1. Lignin content of Elephant grass and eucalypt samples treated with three doses of laccase (from *T. villosa*) and HBT (2.5%) in a sequence including 4 enzymatic treatments, each of them followed by an alkaline peroxide extraction (using 3% H_2O_2 in 1% NaOH), compared with a control without enzyme and a treatment with laccase alone.

	Elephant grass	Eucalypt
Control	21.1	18.0
Laccase (10 U/g)-HBT	18.8	12.2
Laccase (25 U/g)-HBT	16.4	11.9
Laccase (50 U/g)-HBT	14.3	9.4
Laccase (50 U/g)	20.7	17.5

The enzymatic modification of elephant grass lignin was revealed by 2D NMR, obtained at the gel state in DMSO-d_6. **Fig. 2.3.1** includes the expanded aliphatic oxygenated (δ_C/δ_H 50-110/2.5-5.5) and aromatic (δ_C/δ_H 100-150/5.7-8.3) regions of the spectra. The main lignin substructures identified are shown in **Fig. 2.3.2**, and the different lignin cross-signals assigned on the spectra are listed in **Table 2.3.2**. A semiquantitative analysis of similar NMR signals in the different regions of the HSQC spectra can be performed, as shown in **Table 2.3.3** for the aromatic region of the Elephant grass samples.

The aliphatic oxygenated region of the spectrum of control Elephant grass (**Fig. 2.3.1A**) showed signals of both lignin and carbohydrates (X) that mainly correspond to xylan since crystalline cellulose is nearly "silent" under solution NMR conditions. In this region, cross-signals of methoxyls and side-chains in β-*O*-4' lignin substructures (A), including C_γ-H_γ, C_β-H_β and C_α-H_α correlations (A_γ, A_β and A_α, respectively) were observed. The former widely overlap with other C_γ-H_γ correlations in lignin (and related correlations in other lignocellulose constituents). The C_β-H_β correlations gave two different cross-signals corresponding to β-*O*-4' substructures where the second aromatic units is a G unit or an S units ($A_{\beta(S)}$ and $A_{\beta(G)}$, respectively). The main cross-signals in the aromatic region of the HSQC spectrum of control Elephant grass (**Fig. 2.3.1D**) corresponded to the benzenic rings of the different lignin units, including signals from guaiacyl (G) and syringyl (S) units. The S-lignin units showed a prominent cross-signal for the $C_{2,6}$-$H_{2,6}$ correlation ($S_{2,6}$), while the G-lignin units showed different correlations for C_2-H_2 (G_2), C_5-H_5 (G_5) and C_6-H_6 (G_6). Signals corresponding to $C_{2,6}$-$H_{2,6}$ correlations in C_α-oxidized S-lignin units ($S'_{2,6}$) were hardly observed. On the other hand, signals of *p*-coumaric acid (PCA) structures were prominent in this region including cross-signals corresponding to the $C_{2,6}$-$H_{2,6}$ ($PCA_{2,6}$) and $C_{3,5}$-$H_{3,5}$ ($PCA_{3,5}$) aromatic correlations, and olefinic signals for the correlations of the unsaturated C_α-H_α (PCA_α) and C_β-H_β (PCA_β) of the *p*-coumarate unit.

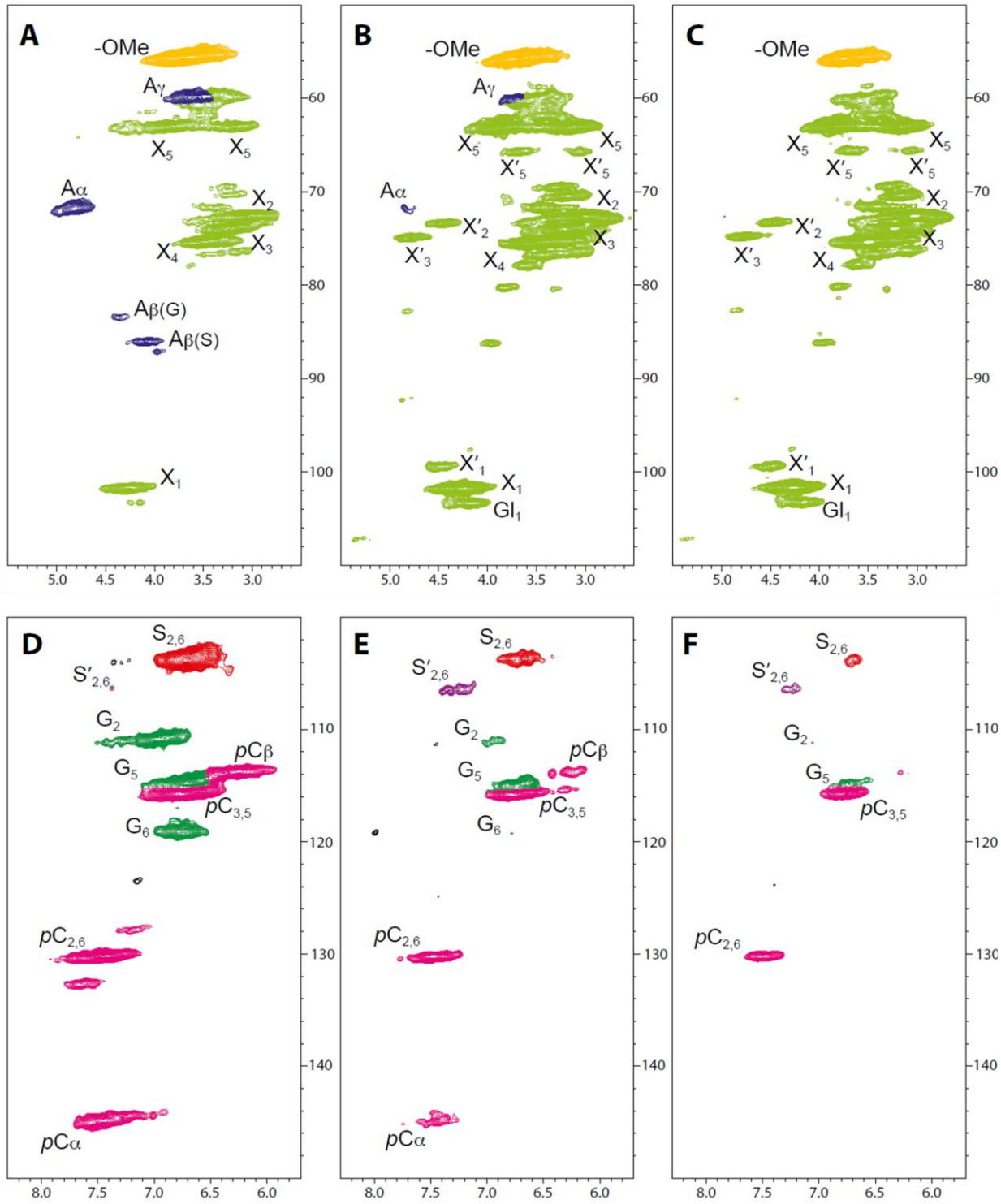


Figure 2.3.1. Expanded aliphatic oxygenated (δ_H-δ_C, 2.5-5.5 and 50-110 ppm; **top**) and aromatic (δ_H-δ_C, 5.7-8.3 and 100-150 ppm; **bottom**) regions of the HSQC NMR spectra of Elephant grass treated with and high doses of *T. villosa* laccase in the presence of HBT: **A** and **D**) Control without enzyme; **B** and **E**) 10 U/g enzyme; and **C** and **F**) 50 U/g enzyme. See **Table 2.3.2** for cross-signal assignment, **Fig. 2.3.2** for the main lignin structures identified, and **Table 2.3.3** for quantification of these lignin structures.

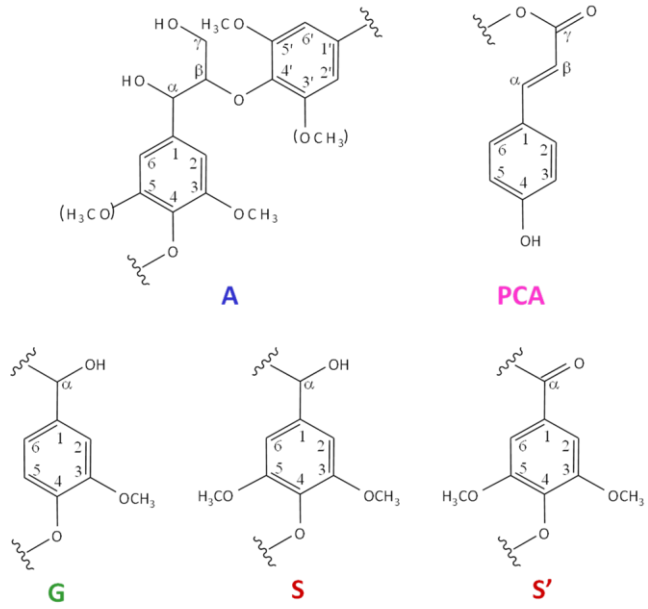


Figure 2.3.2. Main lignin and cinnamic structures identified in the Elephant grass and eucalypt samples analyzed by HSQC NMR: **A**) β-*O*-4' lignin substructures (including a second S or G unit); **PCA**) *p*-coumarate units; **G**) guaiacyl units; **S**) syringyl units; and **S')** oxidized S units.

Table 2.3.2. Assignments of lignin and cinnamic acid main ^{13}C-^{1}H correlation signals in the HSQC NMR spectra of the Elephant grass and eucalypt samples. See **Fig. 2.3.2** for chemical structures.

Label	δ_C/δ_H (ppm)	Assignment
MeO	55.6/3.73	C-H in methoxyls
A_γ	59.4 /3.40 and 3.72	$C_\gamma H_\gamma$ in β-*O*-4' structures (**A**)
A_α	71.8/4.83	C_α-H_α in β-*O*-4' structures (**A**)
$A_{\beta(G)}$	83.4/4.27	C_β-H_β in β-*O*-4' structures (**A**) linked to a G-unit
$A_{\beta(S)}$	85.9/4.10	C_β-H_β in β-*O*-4' structures (A) linked to a S unit
$S_{2,6}$	103.8/6.69	C_2-H_2 and C_6-H_6 in syringyl units (**S**)
$S'_{2,6}$	106.1/7.32	C_2-H_2 and C_6-H_6 in α-oxidized syringyl units (**S'**)
G_2	110.9/6.99	C_2-H_2 in guaiacyl units (**G**)
PCA_β	113.5/6.27	C_β-H_β in *p*-coumaric acid (**PCA**)
G_5	114.9/6.72 and 6.94	C_5-H_5 in guaiacyl units (**G**)
G_6	118.7/6.77	C_6-H_6 in guaiacyl units (**G**)
$PCA_{3,5}$	115.5/6.77	C_3-H_3 and C_5-H_5 in *p*-coumaric acid (**PCA**)
$PCA_{2,6}$	130.1/7.45	C_2-H_2 and C_6-H_6 in *p*-coumaric acid (**PCA**)
PCA_α	144.7/7.41	C_α-H_α in *p*-coumaric acid (**PCA**)

Table 2.3.3. Lignin units (G, S and α-oxidized S) and *p*-coumaric acid (PCA) contents in the HSQC

spectra of the Elephant grass and eucalypt wood treated with three doses of *TvL* and HBT (in a sequence including four enzymatic treatments and four alkaline peroxide extractions) compared with a control without enzyme and a treatment with laccase alone.

	G (%)	S (%)	S_{ox} (%)	PCA (%)
Elephant grass				
Control	30	38	0	33
Laccase-HBT (10 U/g)	20	26	14	40
Laccase-HBT (25 U/g)	17	29	9	45
Laccase-HBT (50 U/g)	0	29	21	51
Laccase alone (50 U/g)	26	35	0	40
Eucalypt				
Control	23	77	0	0
Laccase-HBT (10 U/g)	0	56	44	0
Laccase-HBT (25 U/g)	0	41	59	0
Laccase-HBT (50 U/g)	0	40	60	0
Laccase alone (50 U/g)	9	91	0	0

The HSQC spectra of the Elephant grass samples after the enzymatic treatments with different laccase doses showed important differences compared to the control ones (**Fig. 2.3.1**). The methoxyl cross-signal and most signals of side-chains in β-*O*-4' lignin substructures (A) present in the aliphatic oxygenated region of the spectrum strongly decreased after laccase-mediator treatment (**Figs. 2.3.1B and C**). Likewise, signals of S lignin units present in the aromatic region of the spectrum also strongly decreased after the laccase-mediator treatment (**Figs. 2.3.1E** and **F**) with respect to the control, and the cross-signal of oxidized S-lignin units ($S'_{2,6}$) increased concomitantly. The decrease in G units occurred to a greater extent than S ones and almost disappeared after the enzymatic treatments using higher laccase doses. Indeed, the Elephant grass lignin which has a similar proportion of S and G units with an S/G ratio around 1.2 in control samples, became an S lignin after the enzymatic treatments (**Table 2.3.3**). On the other hand, signals corresponding to the aromatic ring of *p*-coumarate structures ($PCA_{2,6}$ and $PCA_{2,5}$) remain in the spectrum at higher laccase doses, although with lower intensities with respect to the carbohydrate signals. The relative content of the different lignin units and *p*-coumaric acid structures, as molar percentages of total aromatic units, are shown in **Table 2.3.3**, revealing a preferential removal of lignin with respect to *p*-coumaric acid.

A visual inspection of the above spectra revealed the general decrease of lignin signals after the laccase-mediator treatment, and the relative increase of signals assigned to carbohydrates (mainly corresponding to xylan) as mentioned above. The intensities of the aromatic (from lignin and *p*-coumaric acid) and aliphatic (from carbohydrate, etc) cross-signals in the NMR spectra of the Elephant grass samples treated with the different laccase doses (10, 15 and 50 U/g) in the presence of HBT and with laccase (50 U/g) alone, compared with the corresponding control, are shown in **Fig. 2.3.3A**. Although the intensities of aromatic and aliphatic cross-signals cannot be compared (even on a semiquantitative basis) due to their very different $^1J_{CH}$ coupling values, the above figure provides a qualitative picture on the composition changes produced by the different enzymatic treatments. The general tendency at increasing enzyme doses is a decrease of lignin carbon and an increase of polysaccharide carbon, in agreement with the chemical analyses. In particular, a decrease of the aromatic carbon in lignin G and S units and *p*-coumaric acid, and the aliphatic carbon in lignin side-chains and methoxyls (that also include contributions from hemicelluloses), was observed. In addition, the increase of oxidized S units (relatively moderate in the case of treated Elephant grass) and the strong increase of acylated xylan were observed. In the case of laccase alone, the tendency was the same but the changes observed were relatively minor.

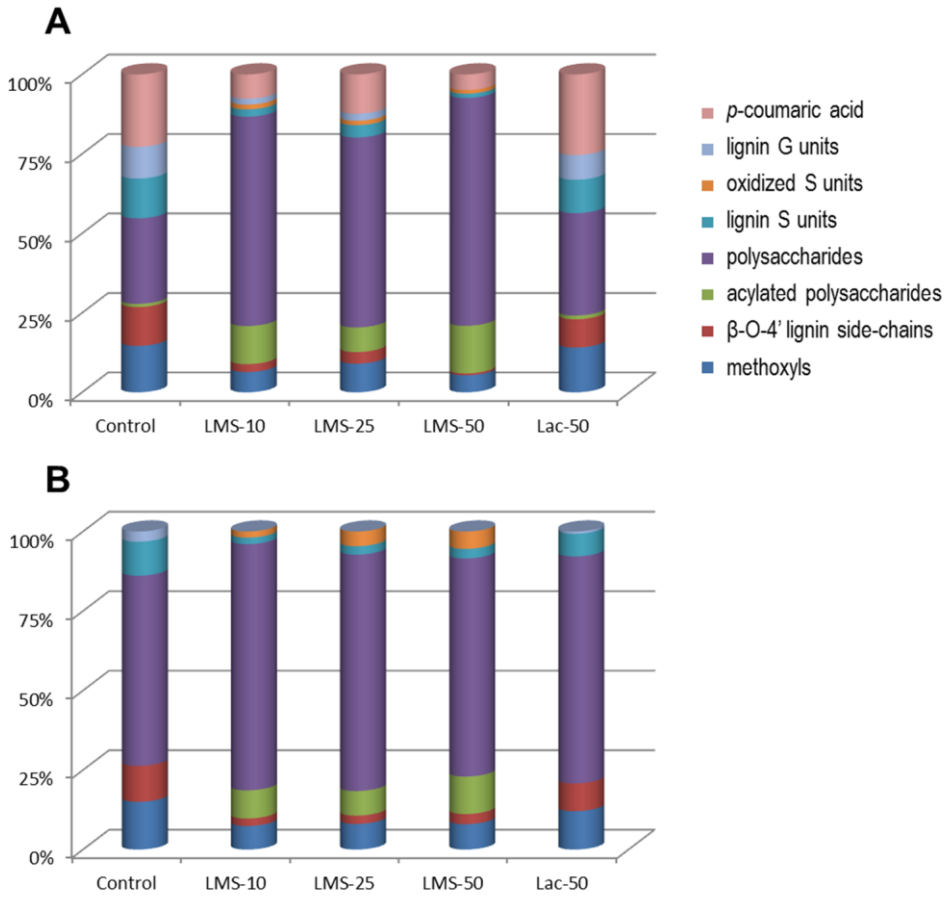


Fig. 2.3.3. Changes in Elephant grass (**A**) and eucalypt (**B**) constituents during treatment with laccase-mediator using different enzyme doses (10, 25 and 50 U/g) as revealed by NMR, compared with a control without enzyme and the treatment with laccase alone (50 U/g).

The enzymatic modification of eucalypt lignin was also shown by 2D NMR obtained at the gel state. The detailed cross-signal assignments are shown in **Fig. 2.3.4**. The main lignin substructures identified are shown in **Fig. 2.3.2** and the different lignin cross-signals assigned are listed in **Table 2.3.2**. As in the case of Elephant grass, **Table 2.3.3** shows the semiquantitative analysis of the different NMR cross-signals in the aromatic region of the eucalypt samples. The aliphatic oxygenated region of the spectrum of control eucalypt sample (**Fig. 2.3.4A**) showed signals of both lignin and carbohydrates (X), the latter corresponding to xylan units, as in Elephant grass spectra. In addition to methoxyl cross-signals, signals of lignin side-chains were observed with lower intensities than found in Elephant grass, the latter corresponding to C_α-H_α correlations in β-*O*-4' alkyl-aryl ether substructures, and C_β-H_β correlations in β-*O*-4' alkyl-aryl ether substructures including a second S-units. The main cross-signals in the aromatic region of the HSQC spectrum of control eucalypt wood (**Fig. 2.3.4D**) corresponded to the benzenic rings of the different lignin units, including the G and S correlations described above for the Elephant grass. The content in S units in eucalypt lignin is higher than in G units, as revealed by the prominent $S_{2,6}$ cross-signal, compared with G_2, G_5 and G_6 signals, with a S/G ratio around 3.3 (**Table 2.3.3**).

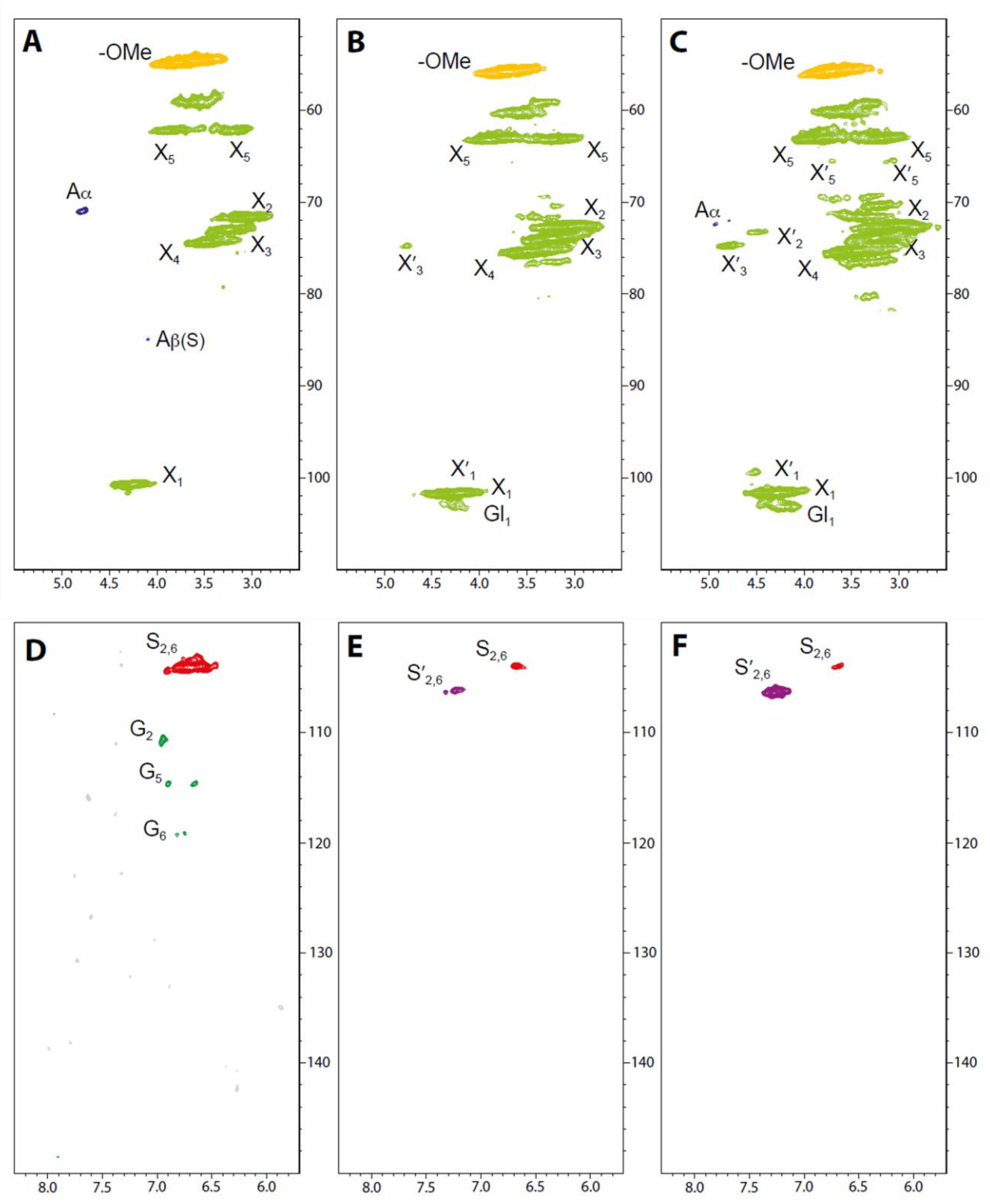


Fig. 2.3.4. Expanded aliphatic oxygenated (δ_H/δ_C, 2.5-5.5/50-110; **top**) and aromatic (δ_H/δ_C, 5.7-8.3/100-150; **bottom**) regions of the HSQC spectra of eucalypt treated with *T. villosa* laccase in the presence of HBT: **A** and **D**) Control without enzyme; **B** and **E**) 10 U/g enzyme; and **C** and **F**) 50 U/g enzyme. See **Table 2.3.2** for cross-signal assignment, **Fig. 2.3.2** for the main lignin structures identified, and **Table 2.3.3** for quantification of these lignin structures.

The HSQC spectra of the eucalypt samples treated with the laccase-mediator system showed important differences compared to the control ones (**Fig. 2.3.4**). The cross-signal of side-chains in β-*O*-4' lignin substructures (A_α) present in the aliphatic oxygenated region of the control spectrum completely disappeared even with the lower enzyme dose (**Figs. 2.3.4B**). Likewise, the G lignin signals, in the aromatic region of the spectrum, also completely disappeared with the lower enzyme dose (**Fig. 2.3.4E**), while the S lignin units were α-oxidized as revealed by the strong increase of the $S'_{2,6}$ cross-signal (to become the most prominent signal in this region) when the enzyme dose was increase, paralleling the decrease of the $S_{2,6}$ signal (**Fig. 2.3.4E** and **F**). Similarly to the Elephant grass enzymatic treatment, the decrease in G units occurred to a greater extent than in the S ones, and completely disappeared even in the treatment with lower laccase doses.

A general picture of the composition changes revealed by the NMR analyses of the eucalypt samples after treatment with the different laccase doses (in the presence of HBT) and with laccase alone is shown in **Fig. 2.3.3B**, where the relative amount of carbon in the different wood constituents is indicated. The general tendency at increasing enzyme doses is a significant decrease of lignin carbon (in aromatic, side-chain and methoxyl structures), although in a lower extant than in the Elephant grass samples, and a concomitant increase of polysaccharides (including both normal and acetylated units). In contrast, the effect of laccase alone was very moderate, being basically reduced to the decrease in lignin G units.

The present study shows for the first time that woody and nonwoody biomass can be significantly delignified by enzymes (with 30-50% lignin removal) by applying a sequence consisting of several alternative laccase-mediator and alkaline extraction stages, directly on the whole lignocellulosic material (i.e. without its previous partial deconstruction). The enzymatic pretreatments also resulted in improved cellulase hydrolysis, enabling shorter fermentation times for ethanol production.

b) Elephant grass and eucalypt wood delignification with the low-redox potential MtL and MeS

During this period we also studied the delignification elephant grass and eucalypt wood by a commercial low-redox potential laccase (from *Myceliophthora thermophile; MtL*) and using methyl syringate (MeS) as mediator. Four cycles of enzymatic treatment followed by alkaline extraction were assayed, similarly as previously done with the high-redox potential laccase from *Trametes villosa*. Different laccase and mediator doses were tested. The decrease in the lignin content by the enzymatic treatments was assessed by measuring the Klason lignin content, while the modifications of the lignin structure were evaluated by 2D-NMR.

The lignin contents (as Klason lignin) of elephant grass and eucalypt wood samples after the laccase-mediator treatments (with *MtL* and MeS) were determined and compared with their respective controls, and the data are shown in **Table 2.3.4**.

Table 2.3.4. Lignin content of elephant grass and eucalypt samples treated with different doses of laccase (from *Myceliophthora thermophila*) and methyl syringate, in a sequence including 4 enzymatic treatments, each of them followed by an alkaline peroxide extraction (using 3% H_2O_2 in 1% NaOH), compared with a control without enzyme and a treatment with laccase alone (in the absence of mediator).

	Elephant grass	Eucalypt
Control	22.0	21.1
Laccase (50 U/g)	21.5	16.8
Laccase (50 U/g)-MeS (3%)	21.5	11.2
Laccase (10 U/g)-MeS (1%)	-	13.3

The lignin content in elephant grass was not modified by the use of this low-redox laccase-mediator system, which contrasts with the high lignin removal obtained when using a high-redox potential laccase. However, and interestingly, when applied to eucalypt wood, the lignin content dramatically decreased after the enzymatic treatments, attaining up to 50% lignin removal when using high doses of *MtL* and MeS. In addition, important lignin removal (ca. 40% removal) was also observed when using lower doses of *MtL* (10 U/g) and MeS (1%). The treatments with laccase alone (without mediator) also produced some decrease in the lignin content in eucalypt wood. The large decrease in lignin observed in eucalypt wood by laccase-mediator treatment with the use of a commercial laccase (*MtL*) and a cheap natural phenol as mediator (MeS) opens up new possibilities to develop and implement an industrially-feasible protocol for the pretreatment of eucalypt wood as feedstocks for the production of bioethanol of second generation.

Further studies on delignification of eucalypt wood with low redox potential laccase (and methyl syringate as mediator) were carried out, including the influence of treatment conditions (reaction time, mediator doses, and presence of organic solvents) and the evaluation of the delignification after each cycle of the enzymatic treatment.

The effect of different parameters of the laccase-mediator treatments in the delignification of eucalypt wood was studied. First of all, we studied the influence of the reaction time on the delignificatoon extent. In **Table 2.3.5** we present the data of *MtL*-MeS treatment after 6 hours and after 24 hours. While after 24 hours treatment, the lignin removal obtained was 50%, however, after reducing the enzymatic treatment to only 6 hours, the lignin removal was still high, up to 27 % Klason lignin reduction, which indicates that it is possible to optimize and reduce the reaction time without losing delignification efficiency.

Table 2.3.5. Lignin content (%) of eucalypt wood treated with different doses of laccase (from *Myceliophthora thermophila*) and methyl syringate, in a sequence including 4 enzymatic treatments, each of them followed by an alkaline peroxide extraction (using 3% H_2O_2 in 1% NaOH), compared with a control without enzyme and a treatment with laccase alone (in the absence of mediator). Effect of reaction time (24 h *vs.* 6 h treatment).

	6 h	24h
Control	19.9	21.1
Laccase (50 U/g)	-	16.8
Laccase (50 U/g)-MeS (3%)	14.6	11.2
Laccase (10 U/g)-MeS (1%)	17.7	13.3

The effect of the increase of mediator doses was studied after 6 hours reaction. However, when increasing the doses of MeS as high as 12%, (and using 50 U/g MtL) only minor lignin removal (16% lignin decrease) was observed. Also, the presence of organic solvents, such as methanol barely modified the lignin removal, even when large reaction times (24 hours) were used for the enzymatic treatments.

The extent of delignification after each cycle of enzymatic treatment was also evaluated. **Table 2.3.6** shows the lignin content of eucalypt wood feedstock after each cycle of enzymatic treatment using *MtL* and MeS as mediator. This study indicates that the delignification increases with the number of sequences used.

Table 2.3.6. Lignin content (%) of eucalypt wood treated with the laccase from *Myceliophthora thermophile* and methyl syringate, in each of the 4 sequences, that include the enzymatic treatment followed by an alkaline peroxide extraction (using 3% H_2O_2 in 1% NaOH), compared with a control without enzyme and a treatment with laccase alone.

	1 cycle	2 cycles	3 cycles	4 cycles
Control	21.6	20.0	20.0	20.9
Laccase (10 U/g)	22.0	-	18.0	18.3
Laccase (10 U/g)-MeS (1%)	20.9	18.2	15.6	13.5

The results obtained indicate, for the first time, that the laccase–mediator treatment using a low-redox laccase and a natural phenolic compound as mediator, that produces up to 50% lignin reduction of eucalypt wood feedstocks, can be an economically feasible procedure from an industrial point of view, since both the laccase (MtL) and the phenolic mediator (MeS) used are commercial and cheap.

In addition to assessing the lignin content decrease, further insight into the lignin structure modification by the enzymatic treatments was achieved by 2D NMR analyses of the lignocellulosic samples. **Figure 2.3.5** shows the HSQC NMR spectra of whole eucalypt wood material treated with laccase-mediator and the corresponding controls, obtained at the gel state in DMSO-d_6. The main lignin substructures identified are also shown in this Figure.

The aliphatic oxygenated region of the spectrum of control eucalypt wood showed signals of both lignin and carbohydrates (X) that mainly correspond to xylan since crystalline cellulose is nearly "silent" under solution NMR conditions. In this region, cross-signals of methoxyls and side-chains in β-*O*-4' lignin substructures (A), including C_γ-H_γ, C_β-H_β and C_α-H_α correlations (A_γ, A_β and A_α, respectively) were observed. The main cross-signals in the aromatic region of the HSQC spectrum of control eucalypt wood corresponded to the benzenic rings of the different lignin units, including signals from guaiacyl (G) and syringyl (S) units. The S-lignin units showed a prominent cross-signal for the $C_{2,6}$-$H_{2,6}$ correlation ($S_{2,6}$), while the G-lignin units showed different correlations for C_2-H_2 (G_2), C_5-H_5 (G_5) and C_6-H_6 (G_6). Signals corresponding to $C_{2,6}$-$H_{2,6}$ correlations in C_α-oxidized S-lignin units ($S'_{2,6}$) were hardly observed.

The HSQC spectra of the eucalypt wood after the enzymatic treatments with different laccase doses showed important differences compared to the control ones (**Fig. 2.3.5**). The signals of side-chains in β-*O*-4' lignin substructures (A) present in the aliphatic oxygenated region of the spectrum strongly decreased after laccase-mediator treatment. Likewise, signals of S lignin units present in the aromatic region of the spectrum also strongly decreased after the laccase-mediator treatment with respect to the control, and the cross-signal of oxidized S-lignin units ($S'_{2,6}$) increased concomitantly. The decrease in G units occurred to a greater extent than S ones and disappeared after the enzymatic treatments. The spectra confirmed the extent delignification of eucalypt wood attained after the enzymatic treatments.

The results obtained indicate that the laccase–mediator treatment using a low-redox laccase and a natural phenolic compound as mediator, that produces up to 50% lignin reduction of eucalypt wood feedstocks, can be an economically feasible procedure from an industrial point of view, since both the laccase (*MtL*) and the phenolic mediator (MeS) used are commercial and cheap.

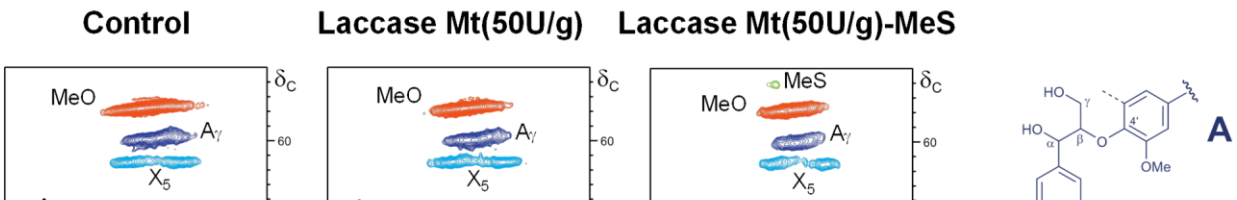


Figure 2.3.5.- HSQC NMR spectra of eucalypt wood treated with MtL in the presence of MeS as mediator (four cycles followed by Ep treatment). The spectra corresponded to control eucalypt wood; control eucalypt wood with MtL (50U/g); euclaypt wood trated with MtL (50U/g) and MeS (3%). Main lignin structures identified in the spectra : **A**) β-O-4 lignin substructures; **G**) guaiacyl units; **S**) syringyl units; **S'**) Cα-oxidized syringyl units.

c) Enzymatic pre-treatment of elephant grass with hydrolases

Comparison of the hydrolysis of whole Elephant grass fibres by Ultraflo, Cellulase NS-22086 and Celluclast: Effect of Pressafiner pre-treatment

In previous experiments carried out in the project the enzymatic preparations Ultraflo and Cellulase NS-22086 were found to have the greatest effect in whole stalk Elephant grass (EG) digestion among all the enzymes tested. To complete this study, a comparison of Ultraflo and Cellulase NS22086 with Celluclast, the commercial preparation commonly used in hydrolytic treatments of fibres before fermentation to bioethanol, was performed at **CIB**. The effect of supplementation of Celluclast with a β-glucosidase (BG; Novozym188) was also compared. The treatments were performed on ball-milled EG as well as a Pressafiner sample (EG-P) which corresponded to whole stalk Elephant grass fibres that were subjected to MSD Pressafiner pre-treatment in water at CTP, dried and balled-milled for 12 h to produce a fine flour. All hydrolysis reactions were performed at 50ºC and pH 6 with 5% dry matter per reaction and 4.5 mg protein/g lignocellulosic biomass enzyme dosage. This corresponds to 1000 U xylanase/g biomass for Ultraflo and NS-22086, and 10 FPU and 200 U xylanase/g biomass for Celluclast. Celluclast was supplemented with BG (5 U against *p*-nitrophenyl-β-D-glucopyranoside/g biomass) where applicable. After 72 h incubation, reactions were terminated by centrifugation and immediate removal of supernatant for determination of reducing sugars (**Fig. 2.3.6b**) and acetic acid

release (**Fig. 2.3.6c**). Residual biomass was dried at 65°C before determining the recovered weight (**Fig. 2.3.6a**).

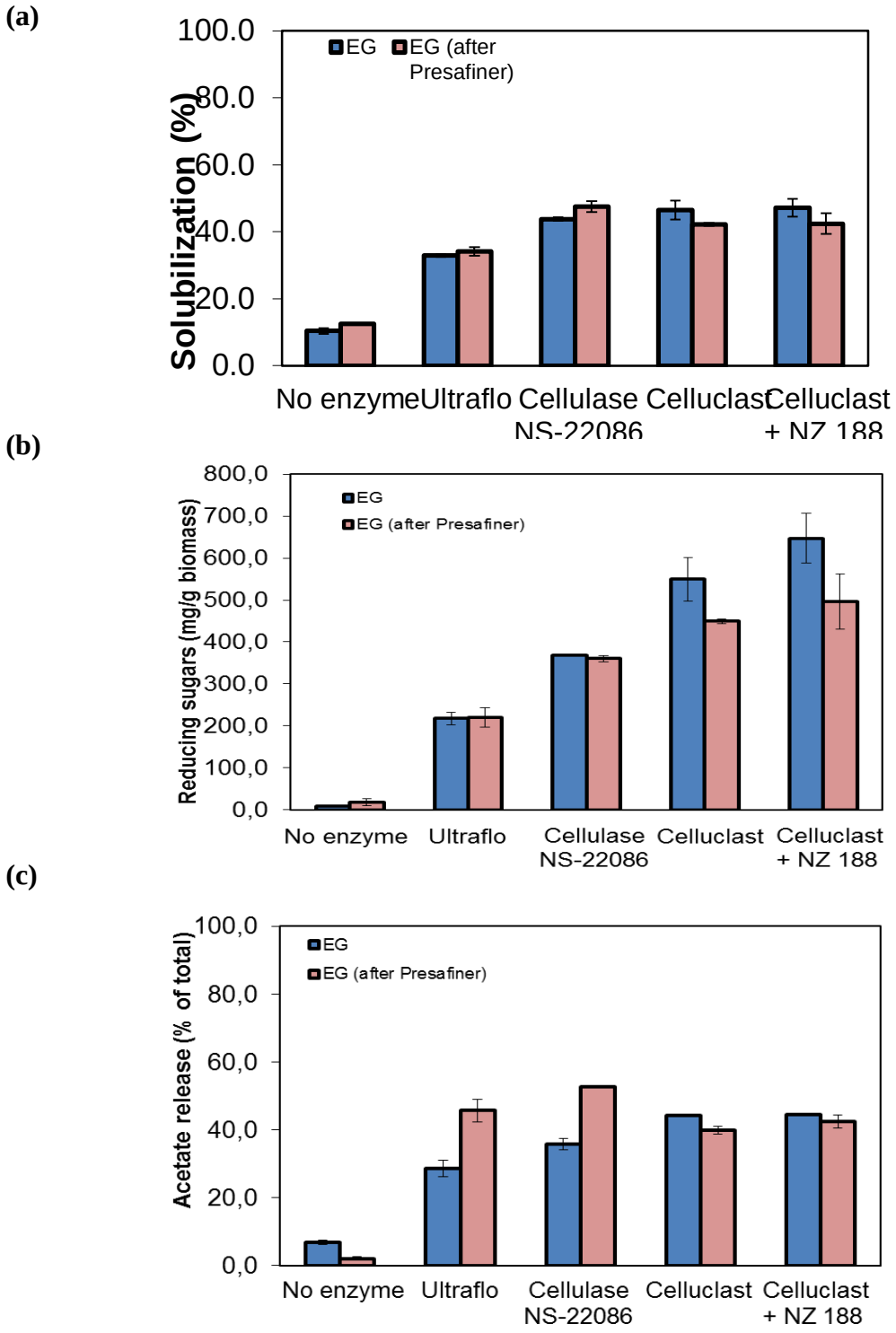


Figure 2.3.6.
Comparison of the effect of several enzymes on **(a)** Solubilisation of Elephant grass (EG) biomass; **(b)** reducing sugar release (expressed as xylose-equivalents); and **(c)** acetate released after 72h at 50ºC, pH 6.

Slightly more buffer-soluble material is produced from EG by the MSD Pressafiner (12%) than is commonly present (10%). The Pressafiner pre-treatment had no apparent effect on fibre solubilisation by

Ultraflo, while with Cellulase NS-22086, 3.5% more EG-P biomass was solubilised compared to the EG sample (**Fig. 2.3.6a**). When Celluclast was used instead of NS-22086 for the hydrolysis, both in the absence and presence of BG, the extent of solubilisation of EG was up to 5% higher than that of EG-P, the converse of that seen with the NS-22086 preparation. NS-22086 has a similar origin to Celluclast but the hydrolytic activity is further boosted by the addition of more cellulolytic and hemicellulolytic activities, making it more suitable for biomass breakdown. While the extent of total solubilisation by the enzymatic treatment remained at 47%, some remaining structural features affected the way the enzymes behave. The extra enzymes present in NS-22086 appear to allow it to degrade more of the EG-P compared to Celluclast, while taking into account the standard error, there is no difference between the enzymes on intact EG. However, the overall increase in biomass solubilisation after pre-treatment is not significantly higher than the enzymatic activity on the intact lignocellulosic biomass to make it a relevant treatment with these enzyme combinations.

Ultraflo was demonstrated to be the poorest performing enzyme preparation on EG of the three preparations examined on an xylanase-equivalent dosage (**Fig. 2.3.6b**). As with biomass solubilisation, there was no increase in reducing group released upon hydrolysis after Pressafiner pre-treatment. There was also no difference in the amount of reducing groups released from the two materials by NS-22086. The increased solubilisation observed in **Fig. 2.3.6a** must therefore be down to the release of non-reducing material or in larger polysaccharides where the number of free reducing ends is comparably low to allow significant changes in the assay response. Therefore, detailed analysis of the residues and solubilised fractions was performed (see section on the effects of enzyme degradation below). With Celluclast, however, the amount of reducing groups generated through hydrolysis of the polysaccharides in the biomass was lower with EG-P compared to the untreated EG**.** It is possible that hydrosolubles present in the Elephant grass are generally easily solubilised and higher reducing group production is a result of further degradation of this soluble material by the enzymes within the Celluclast preparation. Alternatively, pre-treatment with the Pressafiner is generating inhibitory agent(s) of enzyme action. The higher level of reducing ends generated by Celluclast treatment in the presence of BG is due to the further hydrolysis of the reaction products by the endo-acting enzymes.

A significant increase of released acetate levels was observed in the EG-P digestions compared to EG digestion with Ultraflo and NS-22086 (**Fig. 2.3.6c**), whereas there was little difference in deacetylation with Celluclast. This suggests that the Pressafiner treatment enabled some modifications in the fibres that affect enzyme accessibility and, in the case of NS-22086 and Ultraflo, the higher esterase activities present in the preparation with respect to Celluclast are helping in the material solubilisation and acetate release. Considering the increased solubilisation of the pre-treated biomass observed with NS-22086 (**Fig. 2.3.6a**), the comparable lack of increased reducing end generation suggests that NS-22086 either is releasing more non-carbohydrate material or that the material released is resistant to further hydrolysis by the enzymes present in this enzyme preparation. Acetylation of the xylan and lignin does not appear to impart an insurmountable barrier to biomass hydrolysis by these enzyme mixtures.

Analyses of the residues recovered from these treatments were performed to determine the amount of Klason lignin, acid-soluble lignin, and residual glucose (**Table 2.3.7**). It is clear that NS-22086 is the most effective of these enzyme preparations in the removal of glucan from the lignocellulosic matrix, and that surprisingly, the addition of the β-glucosidase preparation (NZ-188) resulted in a poorer solubilisation of the available glucose in EG, as well as the poorest degree of solubilisation (49.5%). The residual Klason lignin was not significantly different between the four samples, demonstrating that these enzymes do not act upon this polymer, but the amount of recovered acid-soluble lignin (ASL) was reduced by the action of NS-22086 and Celluclast. It is possible that other material removed through enzymatic activity from EG was affecting what is measured using the spectrophotometric method as ASL, thus giving the lower recovery values expressed in the table.

Table 2.3.7 Total recoveries in the residues of EG treated with buffer, NS-22086, Celluclast or Celluclast+NZ188 at 50°C for 24h.

Treatment	Initial amount EG before treatment (mg)	Initial Solubilisation (%)	Residual EG after treatment (mg)	Initial Lignin (mg)	Recovered lignin (mg)	Initial acid-soluble lignin (mg)	Recovered acid soluble lignin (mg)	Initial glucose (mg)	Recovered glucose (mg)
no enzyme	750	19,3	605,0	135	145,2	11,5	7,3	291	244,3
Cellulase (NS22086)	750	54,0	344,7	135	135,6	11,5	5,5	291	65,7
Celluclast	750	53,7	347,6	135	128,6	11,5	5,2	291	80,1
Celluclast + NZ188	750	49,6	378,2	135	133,6	11,5	6,8	291	79,8

The sugar composition of the residual material was also determined by gas chromatography (**Fig. 2.3.7**). The main sugar removed through the action of these cellulolytic cocktails was not surprisingly glucose, but what was surprising was that arabinose- and xylose-containing polysaccharides within EG were most resistant to the action of these enzymes. This demonstrates that the hemicellulosic material present within the EG matrix does not form a barrier to the removal of glucan. Another surprising observation was with the removal of mannose-containing material during hydrolysis.

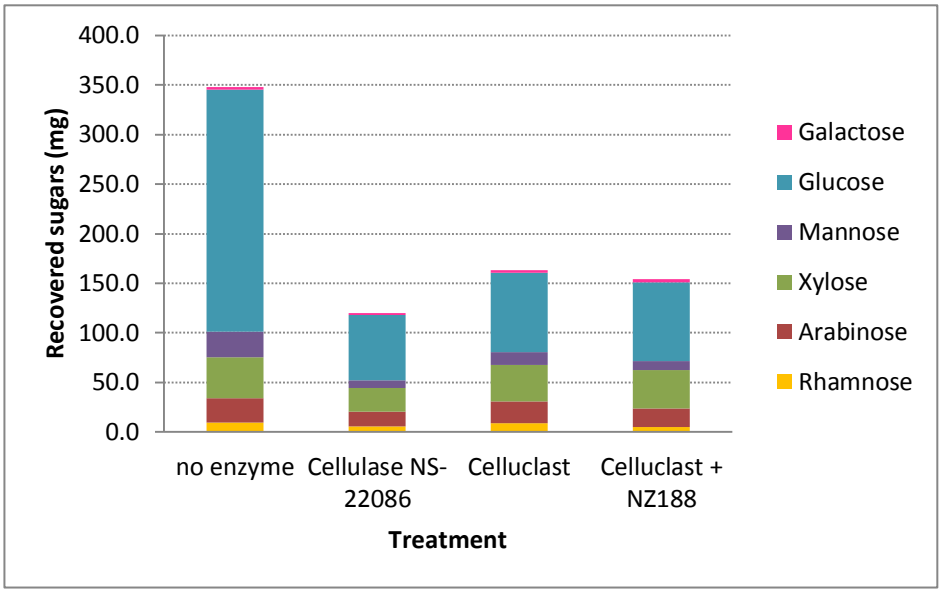


Fig. 2.3.7. Monosaccharide analysis of the recalcitrant EG recovered after 24 h hydrolysis with NS-22086, Celluclast, Celluclast+NZ188 or only buffer.

Effect of pH on the hydrolysis of Elephant grass fibres by Cellulase NS-22086

In order to check for the optimum pH to perform these hydrolysis reactions, NS-22086 treatment of EG and EG-P was compared at different pH values (**Fig. 2.3.8**) in 72 h reactions at 50ºC. The results show a suitable enzyme performance at pH 5-6 values, with a significant decrease in reducing end production and solubilisation at values higher than pH 6.5. Therefore, the experiment confirms that the reactions performed at pH 6 in the previous and present trials can be considered as optimum for this enzyme preparation. In this experiment, it is clear that EG-P is hydrolysed to a much greater extent by NS-22086 compared to EG, but the trends with respect to pH remained the same irrespective of the substrate.

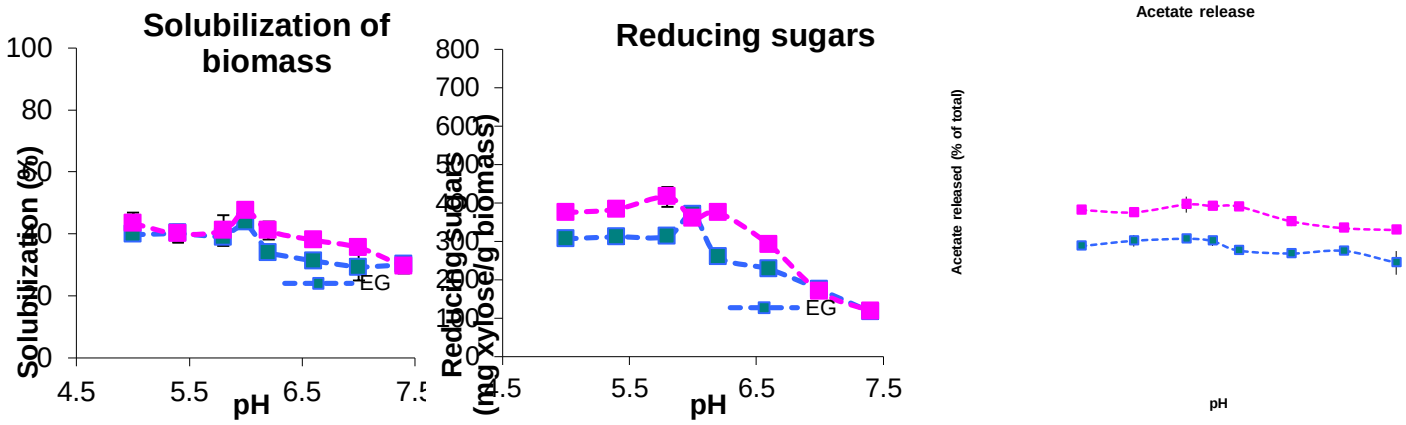


Figure 2.3.8. Effect of pH on EG and EG-P hydrolysis by Cellulase NS-22086 after 72 h at 50ºC: Solubilization of biomass, and acetate and reducing sugar release.

Time-course of hydrolysis of Elephant grass by Celluclast and Pressafiner-treated Elephant grass by Cellulase NS-22086

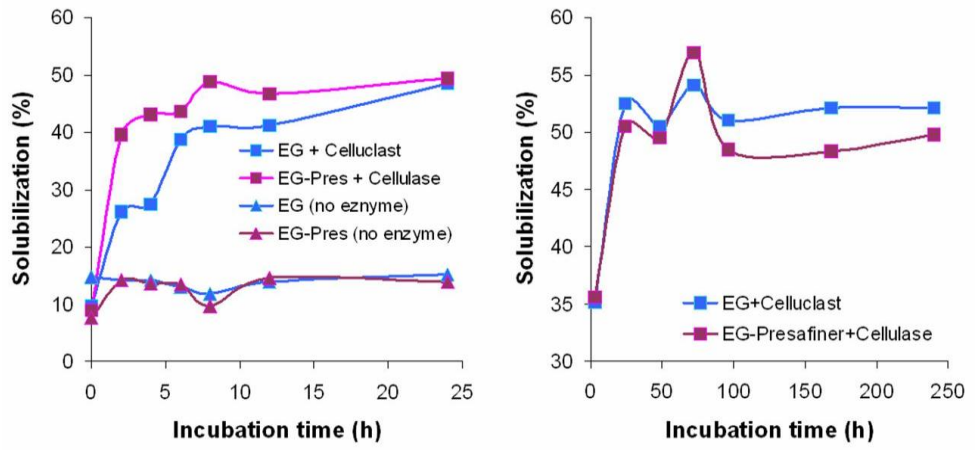
CIB evaluated the extent of biomass hydrolysis over time using the most effective preparations identified so far, i.e. Celluclast for EG and Cellulase NS-22086 for the Pressafiner pre-treated Elephant grass (EG-P) from CTP. Biomass solubilisation (**Fig. 2.3.9a**), reducing sugars release (**Fig. 2.3.9b**) and acetate release (**Fig. 2.3.9c**) were determined at 2, 4, 6, 8, 12 and 24 h (left) time points during the initial day of hydrolysis and subsequently after 2, 3, 4, 7 and 10 days (right). All reactions were performed at 50ºC and pH 6.

The results show a rapid increase in biomass solubilisation (**Fig. 2.3.9a**) and reducing sugar release (**Fig. 2.3.9b**) during the first 3-10 h. After 10 h, little change is observed in all measured parameters for both substrates (EG and EG-P), over the 10-d time course. The increase in reducing sugar levels was higher for the EG-P sample digestion with NS-22086 during the first 12 h, but after 2-3 days similar values were observed for both sample-enzyme treatments. The slight increase in reducing groups measured is probably due to subsequent reduction of solubilised oligosaccharides. Released acetic acid levels (**Fig. 2.3.9c**) reached a more or less constant value after 12-h incubation of both samples, the levels being higher for the EG-P sample treated with NS-22086 in accordance to what was described above.

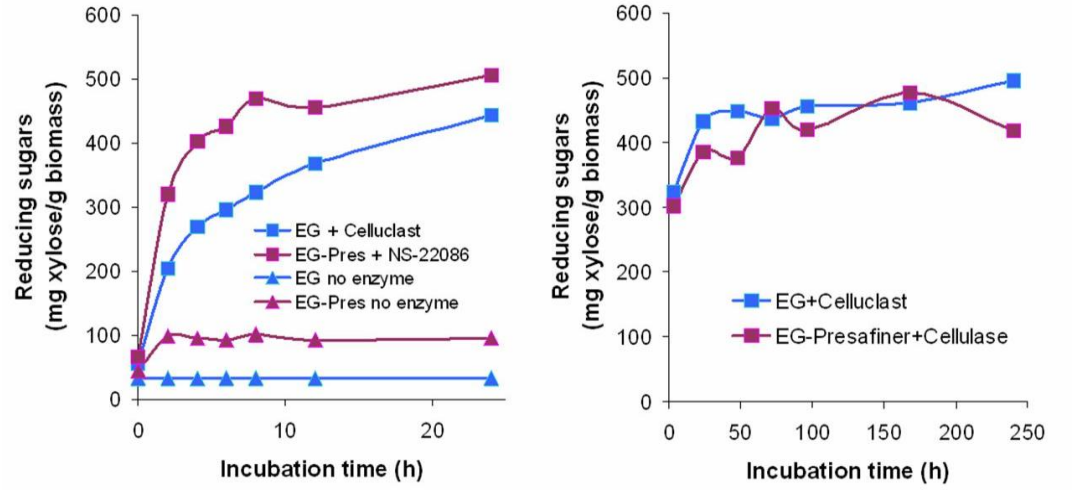
To examine further the long-term effect of incubation on enzyme activity, residual xylanase activity was determined in the EG+Celluclast and EG-P+NS22086 combinations at 24 h periods (**Fig. 2.3.10**). It is important to note that these enzymes were not dosed on xylanase activity. NS-22086 has over 5-fold more xylanase than Celluclast (communication with Novozymes). What is important to point out is that after 24 h of incubation of NS-22086 at 50ºC with EG-P, under 60% of the initial activity remains in solution. The incubations were within Novozymes product specifications for temperature optimization, so either the enzyme is associating with the substrate or it is inactivating either through denaturation or binding soluble inhibitors. Further work is needed to determine this effect. With EG+Celluclast however, measurable xylanase activity increased after 24-h incubation. As this is whole Elephant grass, it is possible that the initial enzymatic hydrolysis has released residual endogenous xylanase, and that the

more intact nature of the substrate prevents non-specific interactions between the Elephant grass fibres and the Celluclast enzymes.

(a)



(b)



(c)

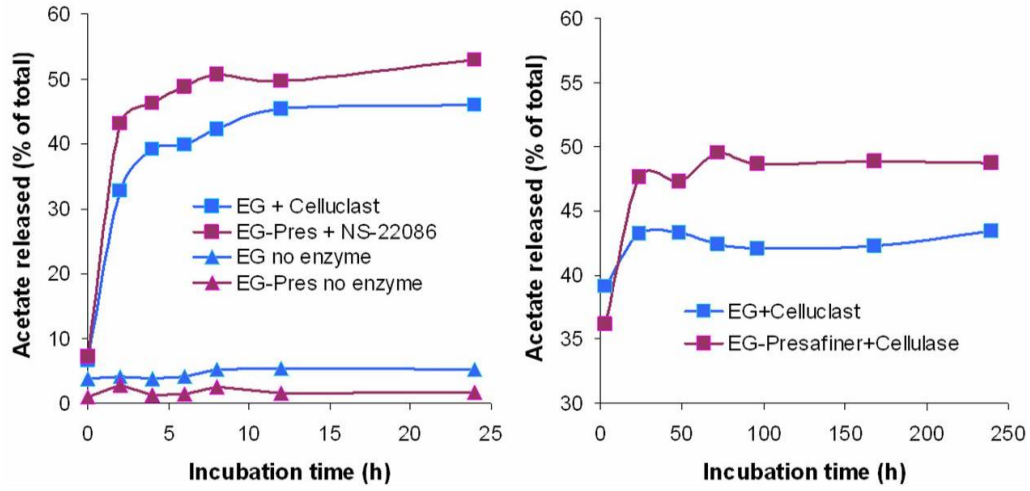


Figure 2.3.9. Time-course (0-24 h, **left**, and 0-10 d, **right**) of hydrolysis of Elephant grass by Celluclast and Pressafiner-Elephant grass by Cellulase NS-22086: **(a)** Biomass solubilisation; **(b)** Reducing sugars (as xylose-equivalents); and **(c)** Acetate release.

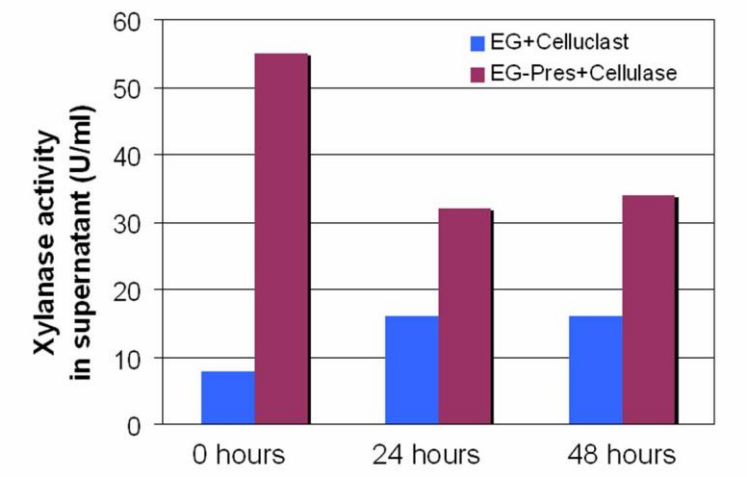


Figure 2.3.10. Residual xylanase activity in reaction supernatants after 24-h and 48-h incubation at 50ºC in the presence of Elephant grass biomass.

Effect of supplementation with feruloyl esterases

The effect of supplementing NS-22086 and Celluclast with an A-type feruloyl esterase from *Aspergillus niger* (AnFaeA) or a C-type feruloyl esterase from *Talaromyces stipitatus* (TsFaeC) was examined at **CIB**. Whole stalk EG or EG-P was enzymatically hydrolysed with the above mixtures at 50°C for 24h as before and the effect on biomass solubilisation, reducing sugar release and acetic acid release determined (**Fig. 2.3.11**). The addition of either of the feruloyl esterases had no significant effect on any of the measured parameters with the non-pre-treated EG. The higher amount of reducing sugars produced by NS-22086 is concurrent with its improved activities compared to Celluclast, as described by the manufacturer, **Novozymes**. However, with EG-P, TsFaeC does display a positive effect over and above that of NS-22086 or Celluclast. The increased solubilisation, reducing sugar release and acetic acid release is more pronounced when TsFaeC is added with NS-22086. It is not yet known if TsFaeC is acting on the acetate groups in the biomass or on the hydroxycinnamate linkages present on the hemicellulose and lignin, or on both. The addition of AnFaeA to the enzymes had no measurable effect on the hydrolysis.

Effect of enzymatic hydrolysis on EG whole stalk and the separated pith and cortex fractions

Samples of ball-milled manually-separated EG pith and cortex were received from **IRNAS**. Similar treatments as those performed before with the different enzymatic preparations available from **Novozymes** were carried out in order to compare the effect of the enzymes in the two fractions and the whole material. Initially, a time-course of the hydrolysis of the three materials using Ultraflo was performed measuring biomass solubilization, reducing sugars and acetic acid release after 0, 24, 48, 72 and 96 hours (**Fig. 2.3.12**). Overall, the treatment was more efficient in the pith fraction than in the cortex or the whole material as revealed by the higher values observed in the three measured parameters.

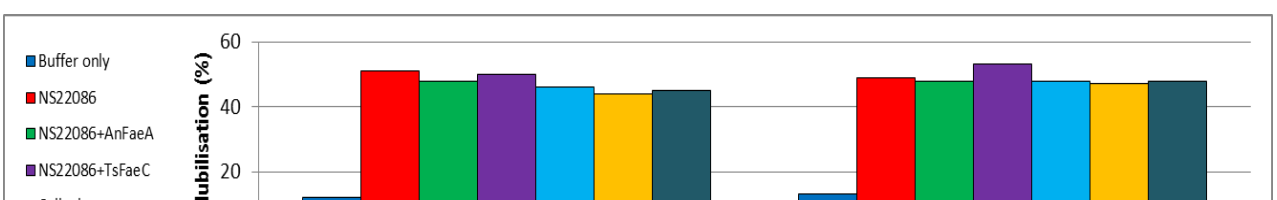


Figure 2.3.11. The influence on the (a) solubilisation, (b) reducing sugar release, and (c) acetic acid release from EG and EG-P by supplementation of commercial carbohydrase cocktails with feruloyl esterases.

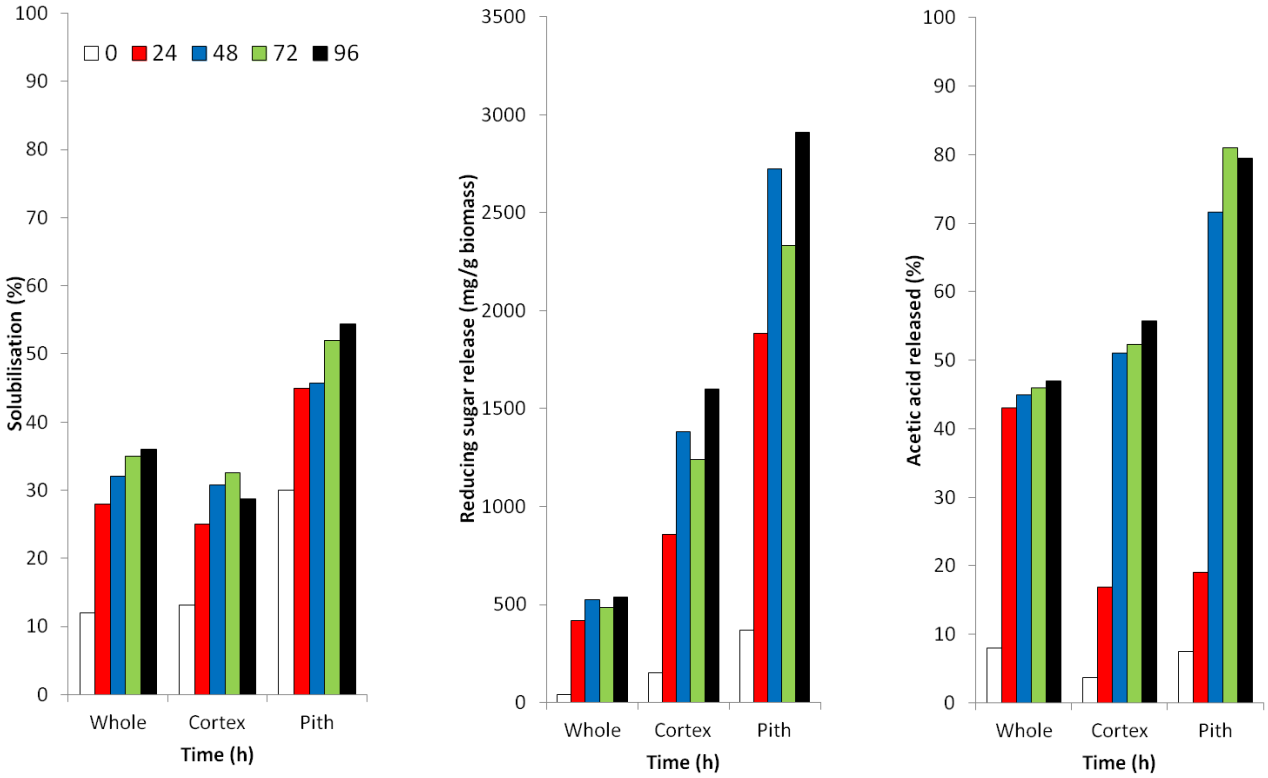


Fig. 2.3.12. Effect of enzymatic treatment of whole stalk EG, cortex and pith with Ultraflo on (a) biomass solubilization, (b) reducing sugars and (c) acetic acid release as a function of time.

A reaction time of 72 hours was chosen for the comparison with NS-22086 and Celluclast action on both EG fractions as well as in the whole material (**Fig. 2.3.13**).

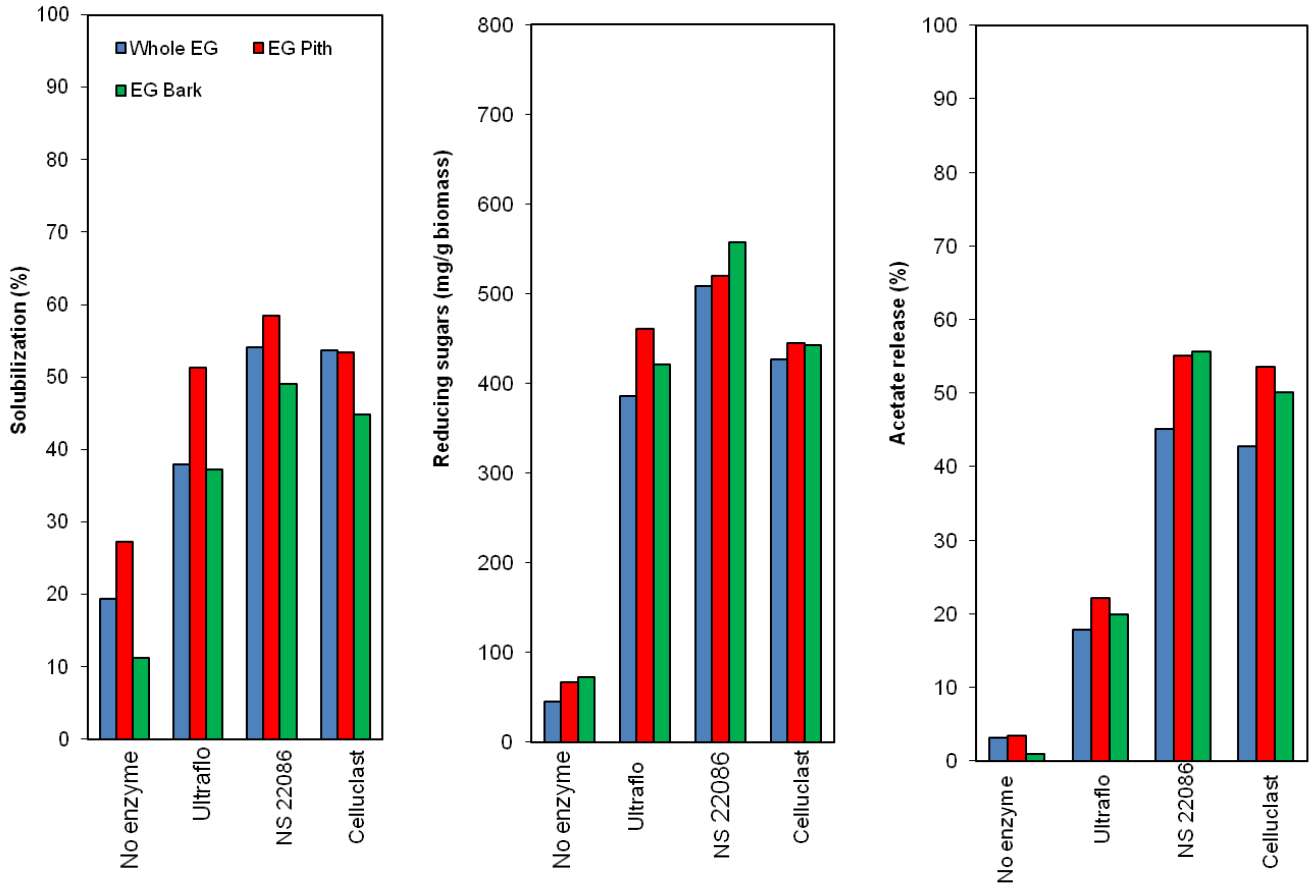


Fig. 2.3.13. Comparison of the enzymatic treatment of EG whole, pith and cortex with Ultraflo, NS22086 and Celluclast. Effect on biomass solubilization, reducing sugars and acetic acid release.

As in previous experiments, the highest values of solubilization, reducing sugars an acetic acid release were obtained with NS22086 preparation. Overall, extracted EG pith is easier to degrade than the whole stem or cortex/bark as shown by the increased biomass solubilization observed specially with Ultraflo and NS22086 compared to the whole fibre although the effect was not so evident when using Celluclast (yielding similar solubilization values for whole material and pith) (**Fig. 2.3.13**, left). In all cases the cortex was the material less solubilised. When comparing reducing sugars released by NS22086 or Celluclast similar values can be observed, the release being even higher for the cortex fraction with NS22086 (**Fig. 2.3.13**, middle) suggesting that the solubilised sugars may be further degraded by the type of enzymes in NS22086, but that this has no effect on reducing the amount of recalcitrant material. The levels of released acetate were higher for the pith or cortex treated with either enzyme preparation in comparison to the whole fibre (**Fig. 2.3.13**, right). It is possible that the compact structure of the whole stalk material restricts access of the glycoside hydrolases in the enzyme preparations to both the pith and cortex fractions and hence the rate of enzyme degradation is poorer than with the separated material. It has been shown on wheat straw that degradation occurs from inside to outside and the vascular bundles located at the outer edge of the cortex together with the cuticular epidermis are not significantly affected by the enzymes. The presence of the pith in EG will reduce this accessibility to the cortex further resulting in poor degradation. The presence of less accessible areas on the pith in the whole stalk material will also reduce degradation compared to the exposed pith samples.

Enzymatic hydrolysis of EG by Ultraflo in the presence of DMSO

Organic co-solvents can expand the use of enzymes in lignocellulose deconstruction through making hemicelluloses and lignin more soluble or at least less compact due to disruption of hydrophobic and electrostatic bonds between the polymers and thus more accessible to enzymatic degradation (Quesada-Medina et al., 2010). During the first 18-month period we looked at the effect of DMSO on EG whole fibres hydrolysis by Ultraflo. These studies were completed in this reporting period through examining at the effect of the presence of DMSO on the pith and cortex fractions of EG. The whole stalk EG or pith and cortex material (50 mg) was mixed with 20% DMSO in 100 mM MOPS buffer, pH 6. Ultraflo was then added and the samples incubated at 37°C for 72 h under agitation. Hydrolysis was terminated by centrifugation as before, the resultant pellets being dried at 65°C and the supernatants being tested for reducing group and acetic acid release (**Fig. 2.3.14**).

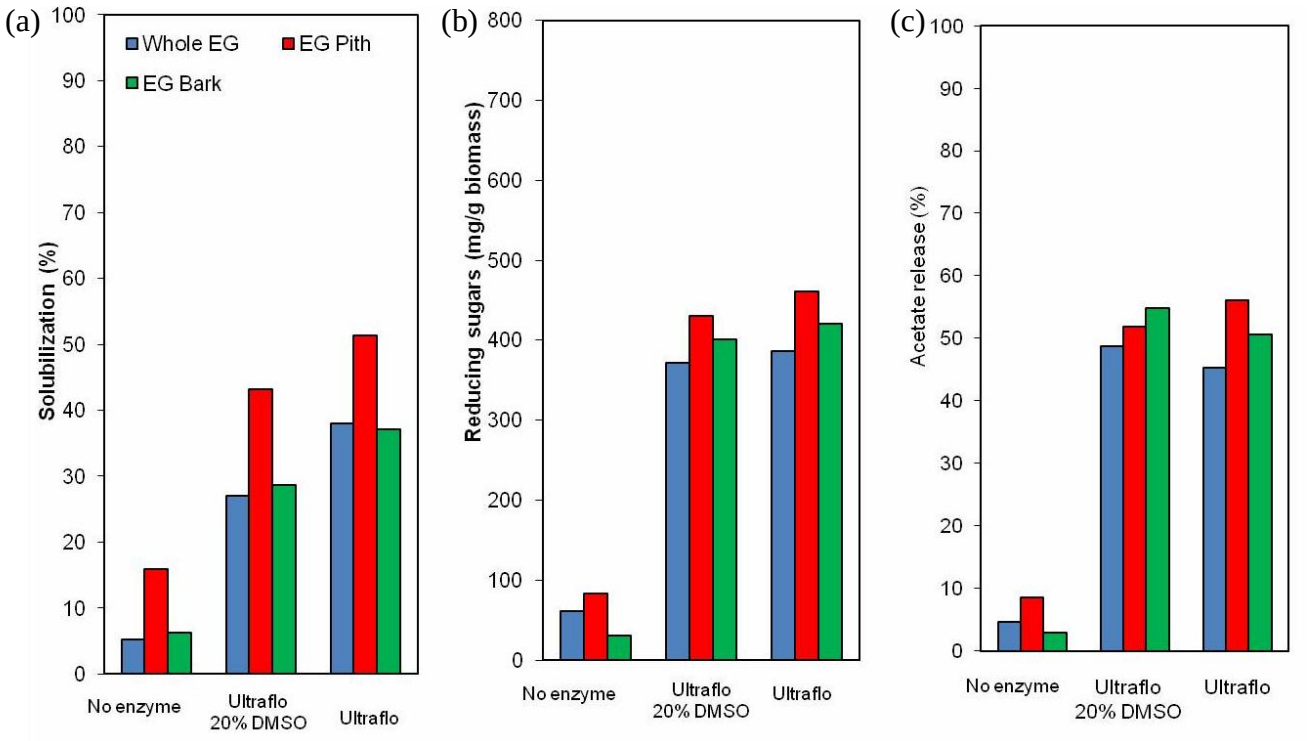


Fig. 2.3.14. The influence of DMSO on (a) solubilisation, (b) production of reducing groups and (c) acetic acid release from whole elephant grass, pith and cortex by Ultraflo.

The presence of DMSO led to a decrease in solubilisation of the three substrates (**Fig. 2.3.14a**) as we have shown in our previous experiments with the whole fibre. Thus it appears that the use of DMSO does not improve biomass solubility, and generally leads to a reduction in degradation. However, DMSO addition did not significantly affect the reducing sugar or acetic acid release by Ultraflo (**Fig. 2.3.14b** and **c**), the latter values being slightly enhanced in whole EG and cortex fraction indicating that xylanase and esterase activities in Ultraflo could be stimulated by the presence of DMSO, which compliments the results reported previously on the stimulation of feruloyl esterase activity on model compounds by the presence of 20% DMSO, as well as the protective effect of DMSO on xylanase stability throughout the reaction time course, also reported in the first periodic report.

To understand the effect DMSO was having on the substrate, comparative analysis regarding the levels of Klason lignin, acid-soluble lignin, and residual glucose was performed on the initial substrate and on the residues recovered from Ultraflo treatment on whole EG, pith or cortex in the absence and presence of 20% DMSO (**Table 2.3.8**). In addition, we also examined the specific effect of DMSO on EG sugars (**Table 2.3.9**).

Table 2.3.8. Total recoveries in the residues of whole EG, pith and cortex treated with buffer or Ultraflo in the absence or presence of 20% DMSO at 37°C for 72 h. (--) signifies value not determined.

Treatment	Initial amount EG before treatment (mg)	Initial Solub (%)	Residual EG (mg)	Final lignin (mg)	Final acid-soluble lignin (mg)	Final glucose (mg)
Buffer+DMSO (whole)	750	5	711	--	28	--
Buffer (whole)	750	19	605	--	--	--
Ultraflo+DMSO (whole)	750	27	547	345	21	47
Ultraflo (whole)	750	38	465	324	18	73
Buffer+DMSO (pith)	750	16	630	357	22	14
Buffer (pith)	750	27	546	342	15	20
Ultraflo+DMSO (pith)	750	43	427	256	17	73
Ultraflo (pith)	750	51	365	237	12	81
Buffer+DMSO (cortex)	750	6	703	452	24	3
Buffer (cortex)	750	11	666	450	18	7
Ultraflo+DMSO (cortex)	750	29	535	351	20	12
Ultraflo (cortex)	750	37	471	327	17	59

DMSO appears to be aiding the solubilization of the carbohydrate in preference to the lignin in the whole stalk material as shown with the same Klason lignin content with and without DMSO in all samples and the reduction of glucose in the presence of the co-solvent. However the removal of glucose with DMSO does to correspond to the lower degree of solubility in the presence of DMSO, suggesting that the co-solvent is restricting more the removal of extractives during the hydrolysis treatment. It is also interesting to point out that Ultraflo is removing material which normally is incorporated in the Klason lignin. It is probably unlikely that lignin is being broken down by this preparation and suggests that the cocktail acts on proteinaceous or lipid-type compounds which are entrapped within the matrix and hence not so easily extractable in an aqueous environment without the aid of enzymes.

Table 2.3.9. Specific effect on EG sugars after treatment with Ultraflo in the absence or presence of 20% DMSO at 37^{o}C for 72 h.

Treatment	Glucose (mg)		Xylose (mg)	
	-DMSO	+DMSO	-DMSO	+DMSO
Whole EG+buffer	5	-	69	113
Whole EG+Ultraflo	73	47 (64%)	161	80 (50%)
Pith+ buffer	20	14 (70%)	230	156 (68%)
Pith+ Ultraflo	81	73 (90%)	105	95 (90%)
Cortex+ buffer	7	3 (43%)	128	70 (55%)
Cortex+ Ultraflo	59	12 (20%)	119	28 (24%)

Table 2.3.9 shows that DMSO is removing xylose and glucose from the walls of EG even in the absence of Ultraflo. The values in parenthesis indicate the percentile of the sugar present in the recalcitrant material without the addition of DMSO. The low difference in the pith signifies the efficiency of Ultraflo in the initial solubilisation of the sugars from the matrix and that the xylan and glucan remaining is thus impervious to removal even by DMSO. The co-solvent appears very efficient on the cortex-derived material, and more so in the presence of the enzymes, suggesting that the DMSO is indeed either solubilising the polysaccharides or at least swelling the matrix allowing better enzyme accessibility to their substrates. So in conclusion, while the presence of DMSO aids in the solubilisation

of xylan and glucan as well as increasing the activity of enzymes, other material are less soluble in the presence of DMSO and remain in the recalcitrant matrix. It could be possible to perform an initial aqueous extraction to remove such material before performing the hydrolytic reaction in the presence of the co-solvent, thus potentially solubilising even more of the biomass.

d) Enzyme degradation of wheat straw (WS) and Abaca (Aba)

During the first 18-month reporting period, we also looked at the effect of Ultraflo hydrolysis on wheat straw (WS) and Abaca (Aba) fibres. The results showed that Ultraflo was more effective on abaca, solubilising almost 45% of the biomass after 5 days, and that most reducing groups were generated from the hydrolysis of abaca. To examine whether the esterases present in Ultraflo were insufficient for the ester linkages present, 50 µl of the enzyme preparation was supplemented with an A-type feruloyl esterase from *Aspergillus niger* (AnFaeA) or a C-type feruloyl esterase from *Talaromyces stipitatus* (TsFaeC) and the effect on biomass solubilisation, reducing sugar release and acetic acid release was determined as well as acid soluble lignin (ASL), Klason lignin and total glucose (**Fig. 2.3.15**).

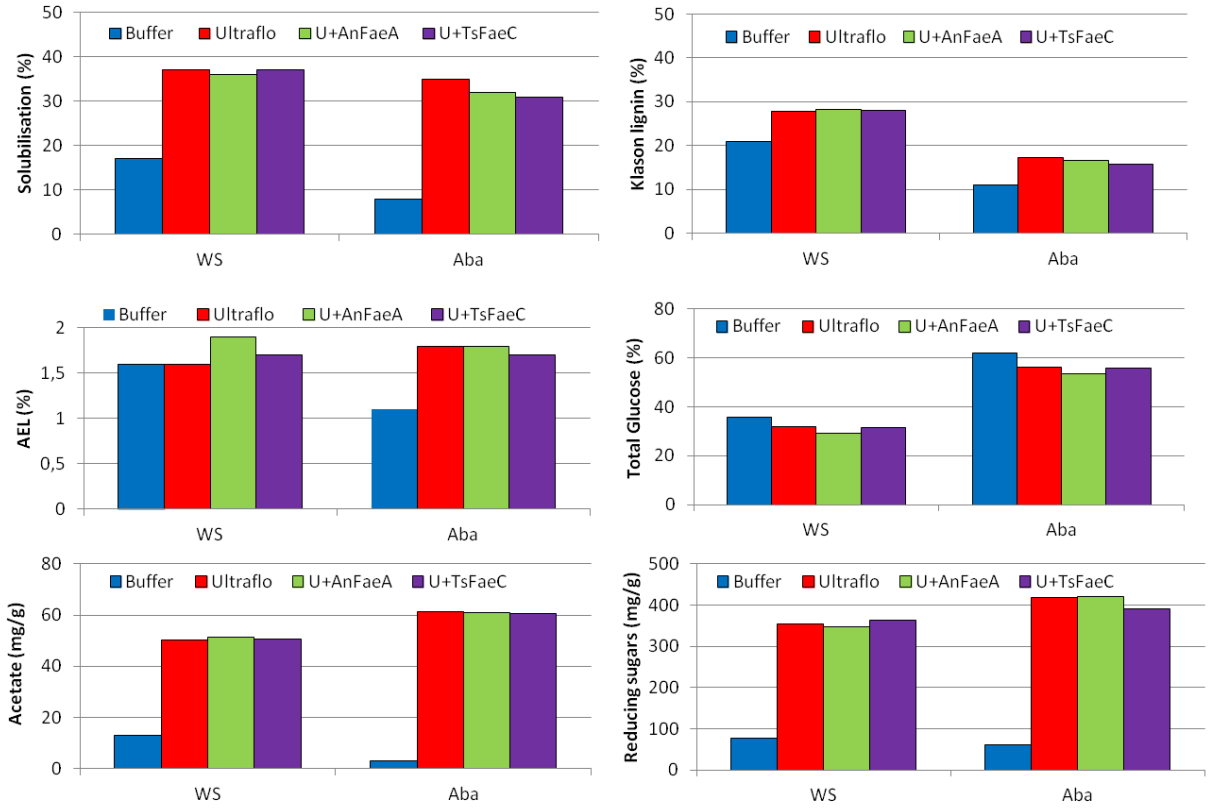


Fig. 2.3.15. Treatment of wheat straw and Abaca fibres with Ultraflo and feruloyl esterases. Effect on solubilization, Klason lignin, acid soluble lignin (ASL), total glucose, acetic acid release and reducing sugar release.

The first noticeable effect was that the enzymes behaved differently on the 2 plant-derived substrates. While more WS was solubilised than abaca, supplementation with FAE led to a decrease in biomass solubilisation in abaca tissues. This decrease was not due to an effect on global reducing sugar and acetic acid release. As expected, Klason lignin levels increase in the residues after Ultraflo treatment, and with WS, the addition of the feruloyl esterases increased slightly the amount of non-lignin removed while with abaca the opposite was observed.

Furthermore, gas chromatography analysis was carried out to investigate the residual sugars in wheat straw (**Fig. 2.3.16a**) and Abaca (**Fig. 2.3.16b**) after enzymatic hydrolysis with Ultraflo alone or supplemented with AnFaeA or TsFaeC. The y-axis scales of the graph are different to take into account the higher level of sugars in the abaca sample. With WS, very little xylose is being removed by Ultraflo

compared to glucose and in particular arabinose and mannose. Mannose appears to being selectively removed when AnFaeA is present. It is also apparent that Ultraflo treatment used in the experiment is only removing 33% of the total sugar. The addition of the feruloyl esterase does not have a significant effect on total sugar release. With abaca, 71% of the total sugar appears to be removed with Ultraflo treatment, and the addition of AnFaeA removed the remaining mannose and arabinose. TsFaeC was not supplementing saccharification of abaca by Ultraflo.

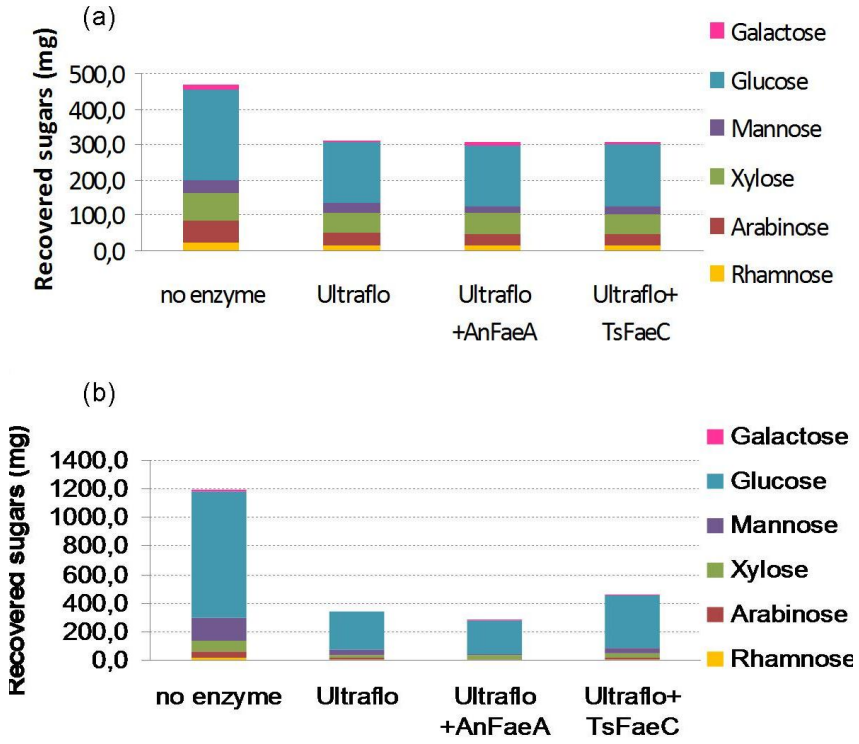


Fig. 2.3.16. Residual sugars after enzyme treatment of (a) wheat straw and (b) Abaca with buffer, Ultraflo or Ultraflo supplemented with AnFaeA or TsFaeC feruloyl esterases.

Similar gas chromatography analyses were performed to investigate the effect of DMSO on sugar solubilization from wheat straw and Abaca in the Ultraflo treatments (**Fig. 2.3.17)**. There was a problem in analysing the sugars after DMSO-buffer treatment in the absence of enzyme, and the sugar recovery is very low. DMSO treatment of WS is selectively removing mannose and arabinose, leaving a residue enriched in glucan and xylose. The same effect was seen with Ultraflo although the recovered sugar levels are lower. It is also interesting to note that Ultraflo is not a suitable preparation to remove WS arabinoxylan, even though there is high xylanase activity present. This technique helps to indicate shortcomings in commercial cocktails on specific substrates. With the abaca sample, the presence of DMSO decreased sugar solubilisation, especially xylose removal, although again mannose is preferentially removed by the addition of DMSO.

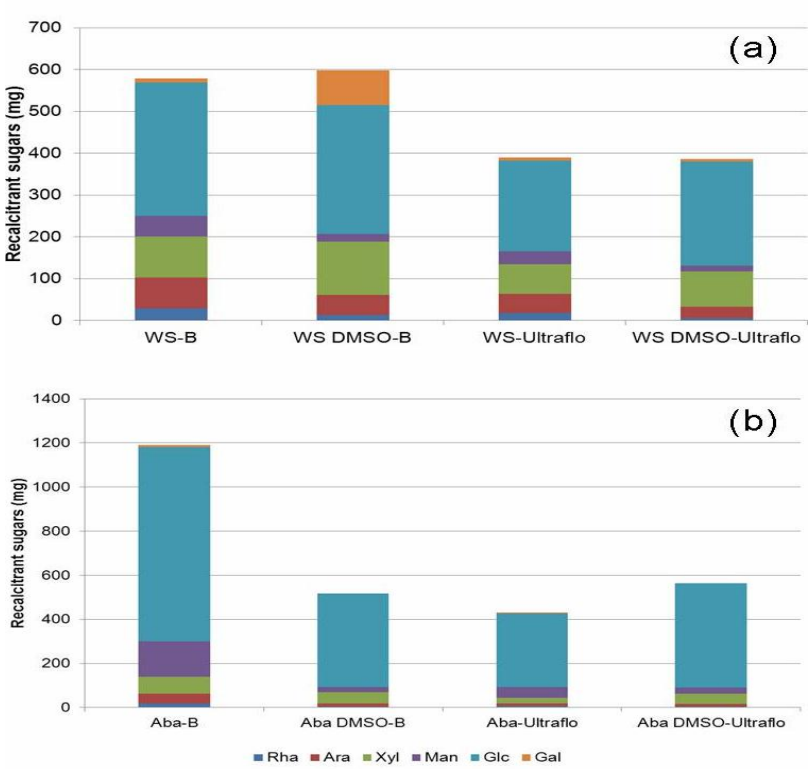


Fig. 2.3.17. Residual sugars after enzyme treatment of (a) wheat straw and (b) Abaca with buffer (B), 20% DMSO in buffer (DMSO-B), Ultraflo or Ultraflo in the presence of 20% DMSO (DMSO-Ultraflo).

e) Effect of co-solvents on* Myceliophthora thermophila *laccase

The possibility of using certain amounts (10-20%) of organic solvents in some enzymatic treatments to increase substrate (for example, lignin) solubilisation and hence improve the chances of a further biomass deconstruction by the enzymes is of high interest, but depends on the ultimate effect that these solvents produce on the enzyme catalytic properties. In this frame, **CIB** studied and already reported the influence of organic co-solvents on the activity of feruloyl esterases and the effect of dimethylsulfoxide (DMSO) on biomass hydrolysis by Ultraflo, as well as the solvents effect on the high redox potential laccase from the basidiomycete *Pycnoporus cinnabarinus* (PcL). We have now completed the activity measurements for the low redox potential laccase from the thermostable ascomycete *Myceliophthora thermophila* (MtL) in the presence of organic co-solvents.

MtL activity was assayed by following the oxidation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) and 2,6-dimethoxyphenol (DMP) at pH 5.0 and 25ºC (Herpoël et al, *FEMS Microbiol. Lett.* 183:301, 2000) in the absence of mediators. In all cases the solvent was slowly added to the buffer, mixed to insure homogeneity and then enzyme was added. After brief equilibrium at 25ºC, substrate was added to initiate the reaction. Initial activity was calculated and referred to the activity in the absence of solvent to determine the residual activity. As shown in **Fig. 2.3.18**, 90-100% of the activity was retained in the presence of 10-20% 1,4-dioxane or acetone (depending on substrate and solvent) but higher concentrations led to the gradual inactivation of the enzyme. While the hydrolysis of both substrates was equally inhibited by 1,4-dioxane, oxidation of DMP was inhibited by a lower concentration of acetone compared to the oxidation of ABTS, with corresponding $IC_{50\%}$ values of approx. 18% and 35% acetone, respectively. Conversely, an activation effect seemed to occur upon incubation in DMSO or methanol in concentrations up to 20-30% (depending on substrate and solvent).

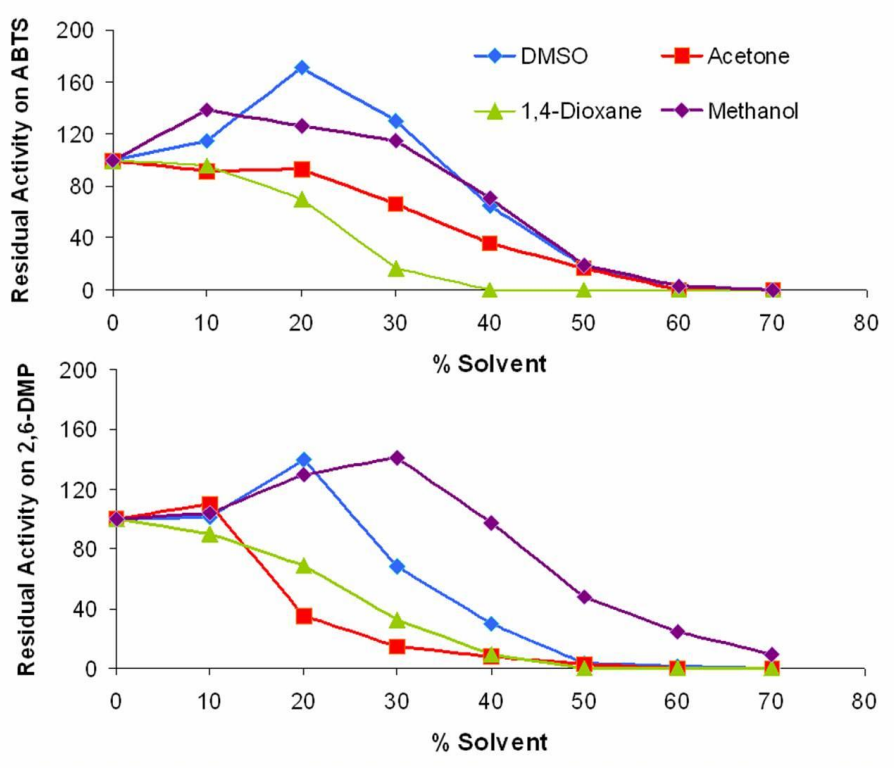


Figure 2.3.18. Effect of organic solvents in the activity of *Myceliophthora thermophila* laccase measured by the oxidation of ABTS (**top**) and DMP (**bottom**) at pH 5.

In order to further investigate this activation/stabilization effect, MtL was incubated for 5 min or 1 hour in increasing concentrations of acetone, methanol or DMSO, and initial activity on both ABTS and DMP substrates was subsequently determined (**Fig. 2.3.19**).

There was a clear positive effect of methanol and DMSO on MtL oxidation of both substrates, the initial activity being 40% and 20% higher on ABTS and DMP, respectively, after 1 hour pre-incubation in 30% methanol (**Fig. 2.3.19b**) and 20-30% higher on both substrates with 20% DMSO (**Fig. 2.3.19c**). In the case of methanol, the initial activity on ABTS measured after 1 hour incubation at 20% solvent content is double the value obtained in the absence of solvent, and 60% higher on DMP in the same conditions. In addition, 100% of the activity on ABTS and 80% on DMP was retained after 1 hour incubation at 40% solvent concentration. The effect of acetone was less significant (**Fig. 2.3.19a**), but still 100% of the activity was retained on ABTS and DMP after 1 hour incubation in 20% and 10% solvent concentration, respectively. Incubation with 10% acetone also resulted in a 60% increase of activity on ABTS.

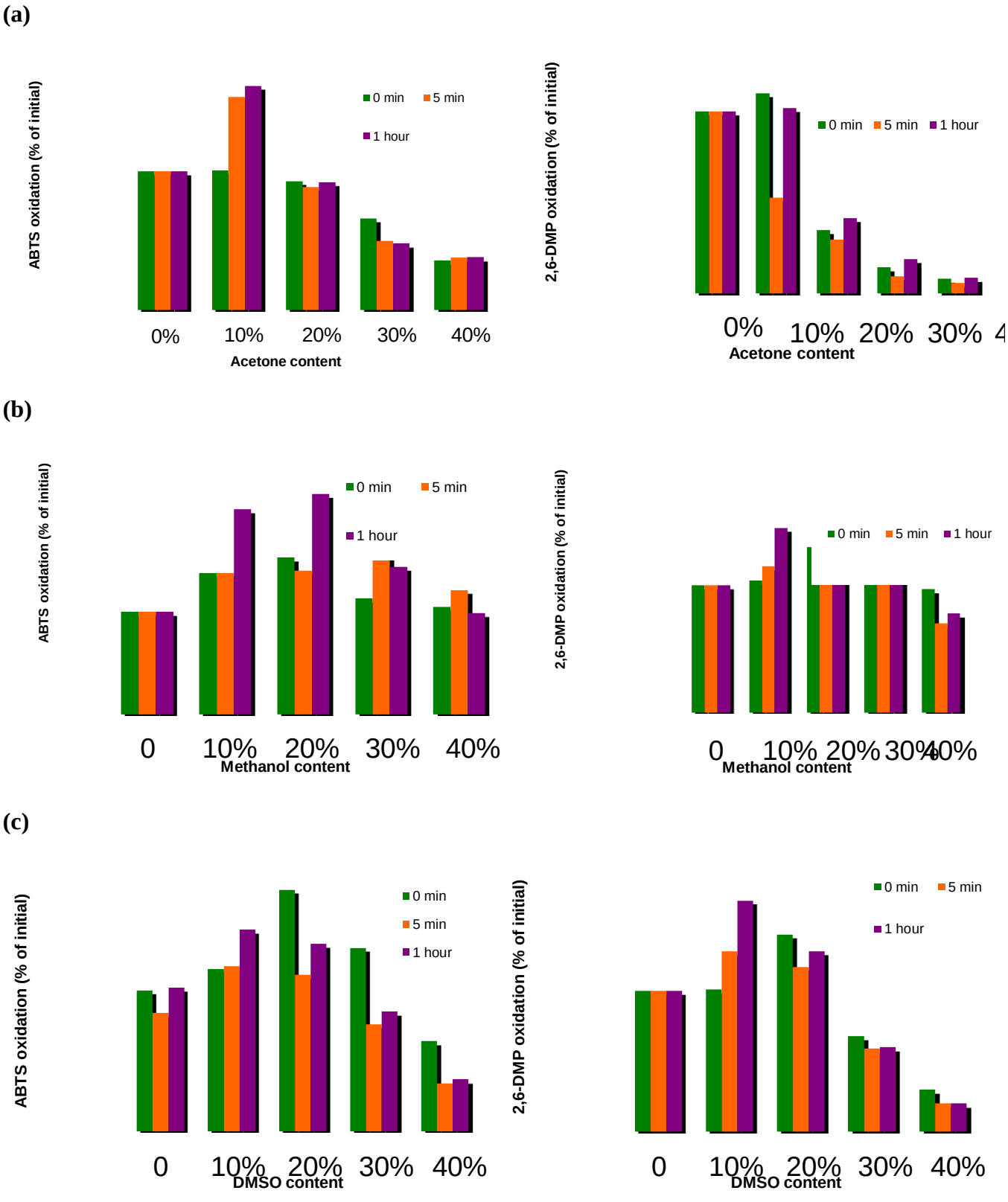


Figure 2.3.19. Effect of organic co-solvents on *Myceliophthora thermophila* laccase stability measured as the initial activity on ABTS **(left)** and DMP **(right)** after 0, 5 and 60 min incubation in **(a)** acetone, **(b)** methanol and **(c)** DMSO at 25ºC (as percentages of the initial activity in the absence of co-solvent).

The *Myceliophthora thermophila* laccase used in these trials is a commercial enzyme formulation, most probably including additives aimed to stabilize and support enzyme performance. To check if this

stability and activation effect was due to the enzyme itself or driven by the other components of the preparation, a pure sample of MtL was received from **Novozymes** to repeat the incubation with the organic co-solvents. As a comparison, we include here the activity of high redox potential laccase from *Pycnoporus cinnabarinus* (PcL) in the presence of the same mixtures (**Fig. 2.3.20**). Surprisingly, the activation effect observed for pure MtL was more than double the activation of commercial MtL preparation, the best results being obtained for the oxidation of both substrates in the presence of 30% methanol (3.5 and 4.3-fold increased activity on ABTS and DMP, respectively). As observed in previous experiments, for PcL, no activation effect was observed in any case, with methanol being the less affecting solvent, but still producing enzyme inactivation with increased solvent concentrations. A similar activation effect as with MtL, although to a much lesser extent, has been recently described for the laccase produced by another ascomycete, *Chaetomium thermophilum* (Maijala et al (2011) J Mol Catalys Enz B) although in the same paper other ascomycete laccases and one basidiomycete laccase were also investigated, all of them resulting in inactivation by the organic co-solvents. MtL as well as the laccase from *Chaetomium thermophilum* are thermostable enzymes, and the same structural properties that confer thermal stability could influence the general robustness of the enzyme making it more stable to, for example, organic co-solvents. However more studies are required to elucidate what enzyme properties are being affecting by the solvent (i.e. stability, substrate binding, electron transfer, environmental effects) and to determine if this effect is enzyme specific or specific to a certain laccase family, and then to determine if this effect can be transferred to biomass treatment with the laccases.

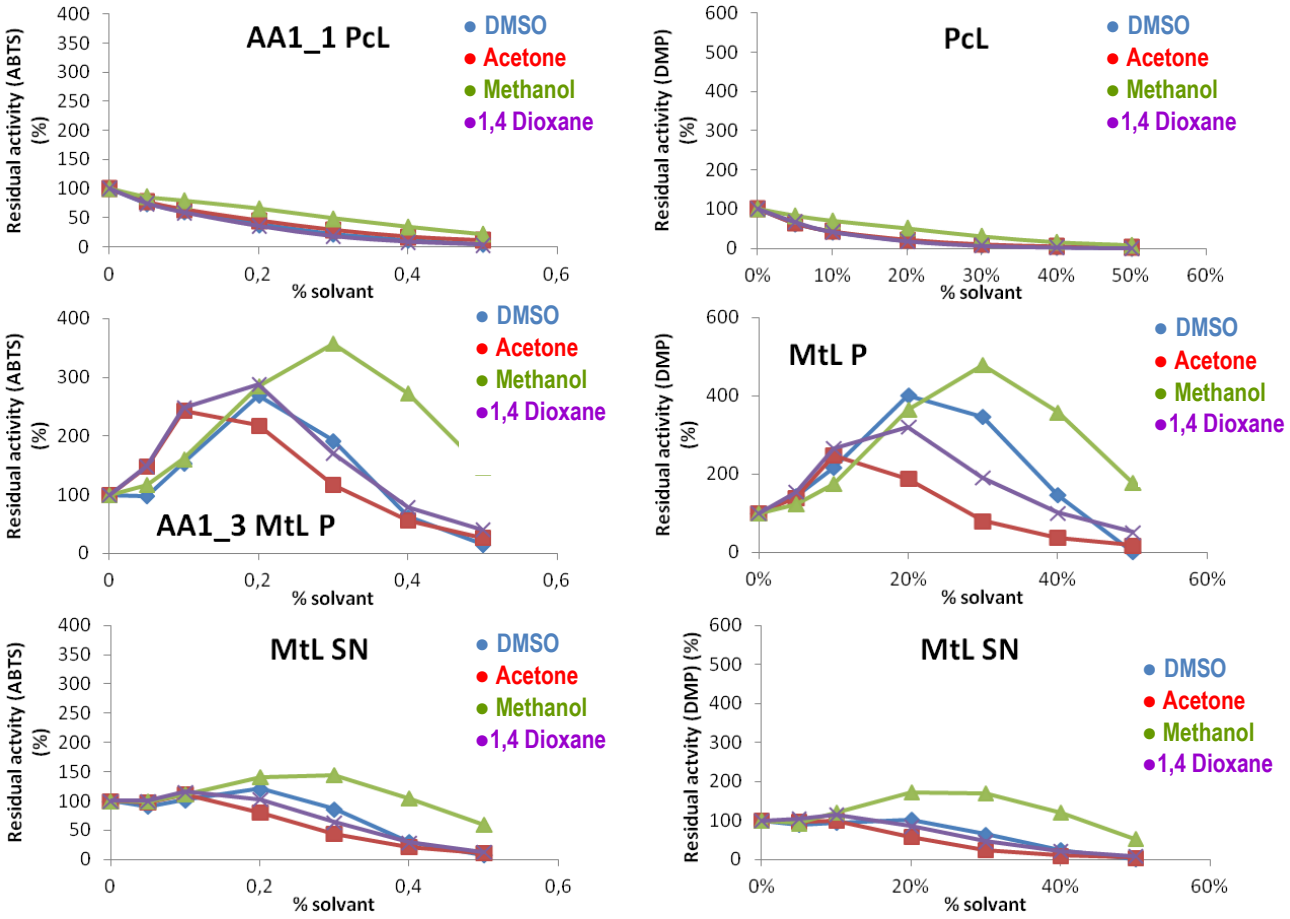


Figure 2.3.20. Effect of organic co-solvents on the activity of *Pycnoporus cinnabarinus* laccase (PcL), pure *Myceliophthora thermophila* laccase (MtL P) and *Myceliophthora thermophila* laccase commercial preparation (MtL SN) measured by the oxidation of ABTS (**left**) and DMP (**right**) at pH 4.

3.1.3. Progress on WP3. "Physical/chemical characterisation of the pre-treated materials"

In this period, in WP3, we completed the characterization of the selected samples of elephant grass and eucalypt woods by calculating the molecular weights (M_w) of their lignins. In addition, we accomplished the characterization of the residual lignins and black liquors produced from elephant grass and eucalypt wood by different chemical alkaline deconstruction processes (kraft, soda-AQ and soda-O_2 processes) at different kappa numbers. The residual lignins were isolated from the pulps by acidolysis and, together with the lignins precipitated from the black liquors, were analyzed by 2D-NMR and Py-GC/MS.

Task 3.3. Analysis of lignin and minor components

a) Structural characterization of sound woody and non-woody feedstocks

We have completed the characterization of the samples of elephant grass and eucalypt woods by calculating the molecular weights (M_w) of their lignins. In the case of elephant grass, the cortex and the pith were separated manually and analysed independently. For this purpose, the milled wood lignins were isolated according to classical procedures and the M_w estimated by Gel permeation Chromatography (GPC).

Gel Permeation Chromatography. GPC analyses of the isolated MWLs were performed on a Shimadzu LC-20A LC system (Shimadzu, Kyoto, Japan) equipped with a photodiode array (PDA) detector (SPD-M20A; Shimadzu) using the following conditions: TSK gel α-M + α-2500 (Tosoh, Tokyo, Japan) column; 0.1 M LiBr in dimethylformamide (DMF) as eluent; 0.5 mL min-1 flow rate; 40 °C oven temperature; PDA detection at 280 nm. The data acquisition and computation used LCsolution version 1.25 software (Shimadzu). The molecular weight calibration was via polystyrene standards.

The values of the weight-average (M_w) and number-average (M_n) molecular weights of the MWL isolated from the cortex and pith fractions of elephant grass stems, estimated from the GPC curves (relative values related to polystyrene standards), and the polydispersity (M_w/M_n), are indicated in **Table 3.3.1**. The two lignins exhibited similar molecular weight distributions, in the range 6920-6720 g mol-1, being slightly higher in the case of the lignin from the cortex. In addition, both lignins exhibited relatively narrow molecular weight distributions, with $M_w/M_n < 3$. Those values are comparable to literature values for various isolated lignins.

Table 3.3.1. Weight-average (*Mw*) and number-average (*Mn*) molecular weights (g mol-1), and polydispersity (*Mw/Mn*) of the MWLs isolated from the cortex and pith of elephant grass (*P. purpureum*).

	MWL cortex	MWL pith
M_w	6920	6720
M_n	2390	2490
M_w/M_n	2.9	2.7

The values of the weight-average (M_w) and number-average (M_n) molecular weights, estimated from the GPC curves (relative values related to polystyrene), and the polydispersity (M_w/M_n) of the MWL from the selected eucalypt hybrids, are indicated in **Table 3.3.2**. The MWLs exhibited similar molecular weight distributions, in the range 11300-15040 g mol-1, being slightly higher in the case of the MWL

from IP and lower for the MWL from DG×U2. In addition, all the MWL exhibited relatively narrow molecular weight distributions, with $M_w/M_n < 4$.

Table 3.3.2. Weight-average (*Mw*) and number-average (*Mn*) molecular weights (g mol-1), and polydispersity (*Mw*/*Mn*) of the MWL from the woods of the different eucalypt hybrids selected in this study

	IP	U1×U2	G1×UGL	DG×U2
M_w	15000	12900	13300	11300
M_n	4300	3900	3500	3000
M_w/M_n	3.5	3.3	3.8	3.8

b) Characterization of residual lignins from kraft, soda-AQ pulps and soda-O_2 pulps (intended for paper and/or bioethanol and biogas production)

Different sets of pulps produced from elephant grass and eucalypt hybrid G1xUGL by different cooking processes were received from **Suzano** (partner 5):

- Pulps intended for paper production: pulp samples from eucalypt G1xUGL and elephant grass prepared by the kraft and soda-AQ processes at kappa 20 and 15. The respective black liquors were also received and analyzed, and the main results are reported in the next section (**Task 3.4**).

- Pulps intended for bioethanol and biogas production: pulp samples from eucalypt G1xUGL and elephant grass prepared by the soda-AQ and soda-O_2 processes at kappa 50, 35 and 15 (these pulps include the rejects).

The residual lignins from the different pulps were isolated by acidolysis and subsequent analyzed by 2D-NMR in HSQC experiments, and by Py-GC/MS.

Isolation of the residual lignins. The isolation of the residual lignins was performed by acid hydrolysis. The extractives-free pulp sample (100 g dry weight) was refluxed for 2 h with 150 ml of 0.1 M HCl in dioxane–water 82:18 (v/v) under nitrogen. The pulp was the filtered and washed with dioxane–water 82:18. The filtrate was evaporated at 40 °C and then the lignin was precipitated in water.

2D-NMR spectroscopy. NMR spectra of isolated lignins were recorded at 25 ºC using a Bruker AVANCE 600 MHz instrument equipped with a cryogenically-cooled z-gradient triple resonance probe. Around 40 mg of lignin were dissolved in 0.75 mL of deuterated dimethylsulfoxide (DMSO-d_6) and 2D-NMR spectra were recorded in HSQC (heteronuclear single quantum coherence) experiments using Bruker's 'hsqcetgp' pulse program with spectral widths of 5000 Hz and 13200 Hz for the ^{1}H- and ^{13}C-dimensions. The number of collected complex points was 2048 for the ^{1}H-dimension with a recycle delay of 1 s. The number of transients was 64, and 256 time increments were recorded in 13C-dimension. The $^{1}J_{CH}$ used was 140 Hz. Processing used typical matched Gaussian apodization in ^{1}H and a squared cosine-bell in^{13}C. Prior to Fourier transformation, the data matrices were zero-filled up to 1024 points in the 13C-dimension. The central solvent peak was used as an internal reference (δ_C 39.5; δ_H 2.49). A semiquantitative analysis of the volume integrals of the HSQC cross-correlation signals was performed. As the volume integral depends on the particular $^{1}J_{CH}$ value, as well on the T_2 relaxation time, absolute quantitation is impossible but relative integrals (between spectra) allow valid comparisons. Thus, the integration of the cross-signals was performed separately for the different regions of the HSQC spectrum, which contain signals that correspond to chemically analogous carbon-

proton pairs. In the aliphatic oxygenated region, the relative abundances of side-chains involved in inter-unit linkages or present in terminal units were estimated from the C_α–H_α correlations to avoid possible interference from homonuclear ^1H–^1H couplings. In the aromatic region, C_2-H_2 correlations from H, G and S lignin units and from *p*-coumarate and ferulate were used to estimate their relative abundances.

Py-GC/MS. Pyrolysis of isolated residual lignins (approximately 100 μg) was performed with a 2020 micro-furnace pyrolyzer (Frontier Laboratories Ltd.) connected to an Agilent 6890 GC/MS equipment using a DB-1701 fused-silica capillary column (30 m x 0.25 mm i.d., 0.25 μm film thickness) and an Agilent 5973 mass selective detector (EI at 70 eV). The pyrolysis was performed at 500 ºC. The oven temperature was programmed from 50 ºC (1 min) to 100 ºC at 30 ºC min-1 and then to 300 ºC (10 min) at 10 ºC min-1. He was the carrier gas (1 mL min-1). The compounds were identified by comparing their mass spectra with those of the Wiley and NIST libraries and those reported in the literature. Peak molar areas were calculated for the lignin-degradation products, the summed areas were normalized, and the data for two repetitive analyses were averaged and expressed as percentages.

b1) Structural characteristics of the residual lignins isolated from pulps intended for paper production (kraft and soda-AQ processes).

The residual lignins isolated from eucalypt hybrid G1xUGL and elephant grass pulps produced by the kraft and soda-AQ pulps at kappa 20 and 15, as well as the lignins precipitated from their respective black liquors, were analysed by 2D-NMR (in HSQC experiments). The data regarding the lignins from black liquors belongs to ***Task 3.4*** and will be explained in detail latter in that section.

The HSQC spectra (δ_C/δ_H 50-125/2.5-8.0) of a representative residual lignin (isolated from the eucalypt wood G1xUGL kraft pulp at kappa 20), and the precipitated lignin from its respective black liquor, are shown in **Fig. 3.3.1**. The spectrum of the MWL isolated from G1xUGL is also shown for comparison. The main substructures found are also depicted in **Fig. 3.3.1**. Likewise, the HSQC spectra (δ_C/δ_H 50-150/2.5-8.0) of a representative residual lignin isolated from the elephant grass kraft pulp at kappa 20, and the precipitated lignin from its respective black liquor, are shown in **Fig. 3.3.2**. The spectrum of the MWL isolated from elephant grass is also shown for comparison. The main substructures found are also depicted in **Fig. 3.3.2**.

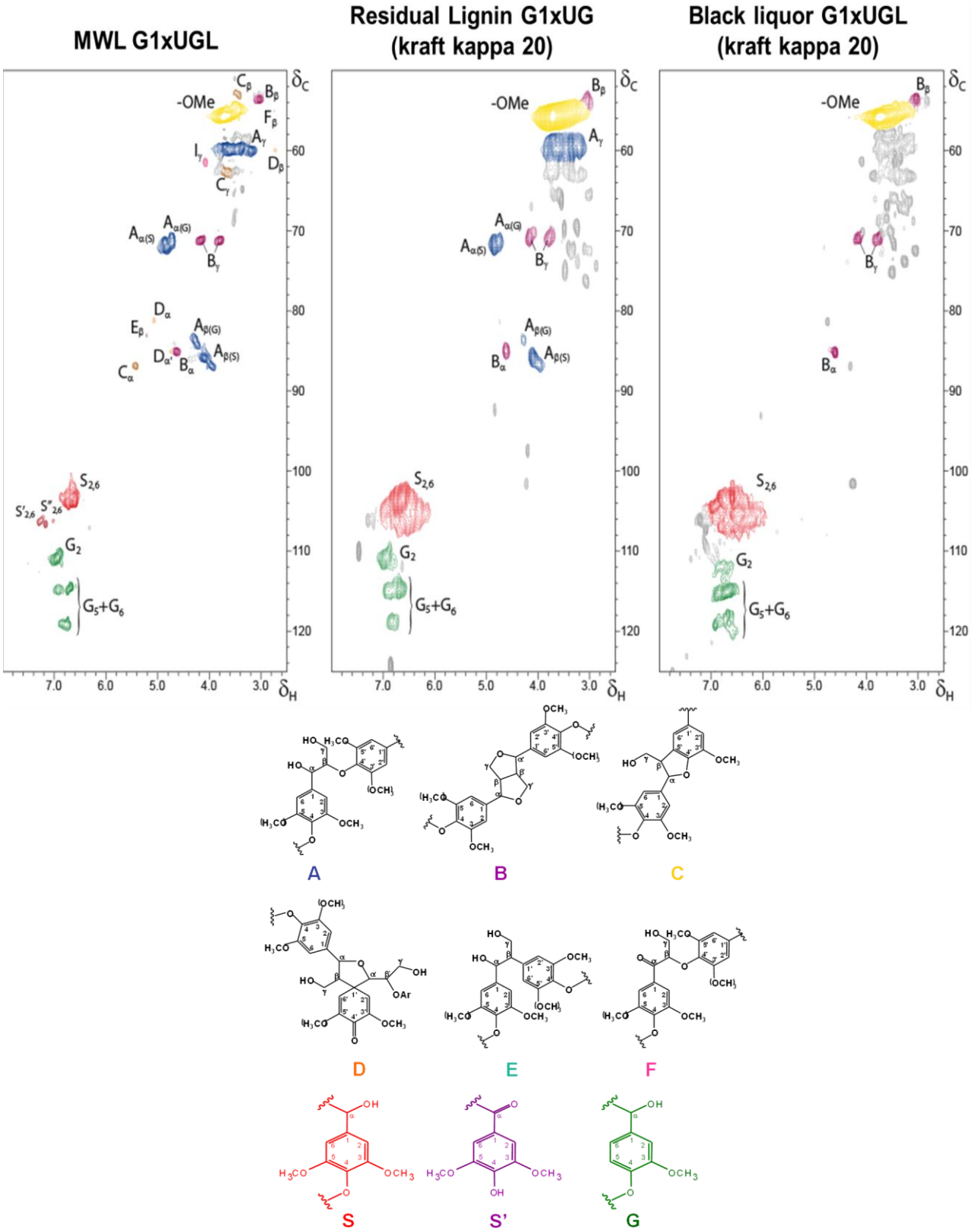


Fig. 3.3.1. 2D-NMR spectra of a selected residual lignin (isolated from eucalypt G1xUGL kraft pulp at kappa 20) and the lignin precipitated from the respective black liquor. The spectrum of the MWL from eucalypt G1xUGL is also shown for comparison. The main inter-units linkages and lignin structures are also depicted here.

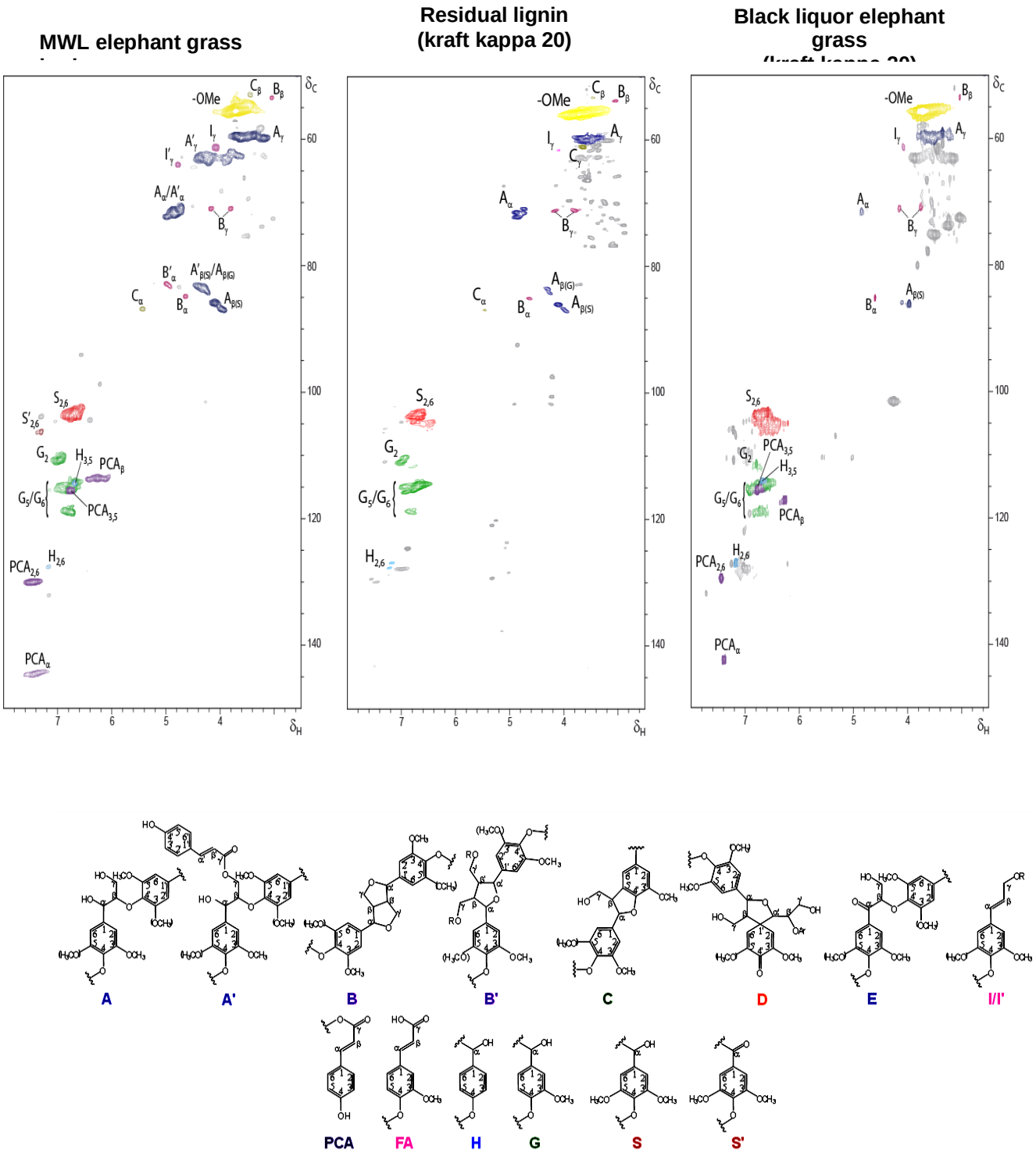


Fig. 3.3.2. 2D- NMR spectra of a selected residual lignin (isolated from elephant grass kraft pulp kappa 20) and the lignin precipitated from the respective black liquor. The spectrum of the MWL from elephant grass is also shown for comparison. The main inter-units linkages and lignin structures are also depicted here.

A quantitation of the abundance of the main lignin inter-unit linkages present in the different residual lignins from eucalypt G1xUGL pulps, as well as the abundance of the G and S lignin units was performed by integration of the volume contours of their cross-signals and was referred to as per 100 aromatic units (**Table 3.3.1**). The main linkages observed in the residual lignins from G1xUGL were β–*O*–4 aryl ether, β–β resinol and β–5 phenylcoumaran structures, in both the kraft and soda-AQ pulps. No oxidized lignin moieties were observed by 2D-NMR in these residual lignins. The distributions of the different inter-unit linkages in the pulp residual lignins is similar to that observed in the native lignin in wood, with a predominance of β–*O*–4 alkyl-aryl ether linkages, followed by lower amounts of resinols and phenylcoumaran, although with a drastic reduction in their content. This reduction in the content of linkages was more evident in the pulps with lower kappa number (kappa 15) than in pulps with higher kappa number (kappa 20) due to the more drastic pulping conditions at lower kappa numbers. Moreover, at similar kappa number, the content of β–*O*–4 aryl ether linkages in the residual lignin were lower for the soda-AQ process that for the kraft process, indicating a higher efficiency of the soda-AQ process for delignifying the G1xUGL eucalypt wood.

Table 3.3.1. Main lignin structural characteristics (linkages per 100 aromatic units, and S/G ratio) of the residual lignins isolated from the pulps produced from eucalypt G1xUGL after kraft and soda-AQ processes at kappa 20 and 15. The composition of the MWL isolated from eucalypt hybrid G1xUGL is shown for comparison.

Linkages (per 100 aromatic units)	MWL	Kraft process		Soda-AQ process	
		Kappa 20	Kappa 15	Kappa 20	Kappa 15
β–*O*–4 alkyl-aryl ether	90.0	16.0	14.0	18.0	12.0
β–β resinols	10.5	7.4	7.4	6.5	6.7
β–5 phenylcoumarans	5.3	0.3	0.1	0.4	0.4
S/G ratio	2.8	3.0	3.0	2.8	2.8

In the case of elephant grass pulps, the structural characteristics (obtained from the HSQC spectra) of the residual lignins are reported in **Table 3.3.2**. In the case of the MWL from elephant grass, 2D-NMR showed a predominance of alkyl aryl ether (β–*O*–4) linkages, with low amounts of the so-called 'condensed' substructures, such as resinols (β–β) and phenylcoumarans (β–5). Moreover, the NMR spectra indicated that these lignins are extensively acylated at the γ-carbon of the side-chain, and predominantly with *p*-coumarate groups. The main lignin substructures present in the residual lignins were β–*O*–4 aryl ether, with lower amounts of β–β resinol and β–5 phenylcoumaran structures, in both the kraft and soda-AQ pulps, with a similar distribution as observed in the native lignin of elephant grass. However, a reduction of the main substructures was observed after the cooking, this reduction being more evident in the pulps with lower kappa number (kappa 15) due to the higher extent of delignification. At similar kappa numbers, the content of β–*O*–4 aryl ether linkages in the residual lignin were lower for the soda-AQ process than for the kraft process, as already observed in the case of eucalypt wood shown above, indicating a higher efficiency of the soda-AQ process for delignifying the elephant grass. As already observed in the eucalypt pulps, no oxidized lignin moieties were observed by 2D-NMR in the residual lignins from elephant grass. On the other hand, both kraft and soda-AQ cooking produced a complete hydrolysis of the *p*-coumarate groups that are acylating the γ-carbon of the lignin side-chain, and only minor amounts of *p*-coumarates are found in the pulps.

Table 3.3.2. Main lignin structural characteristics (linkages per 100 aromatic units, percentage of γ-acylation, S/G and H/G ratios, and percentage of *p*-coumaric acid) of the residual lignins isolated from the pulps produced from elephant grass after kraft and soda-AQ processes at kappa 20 and 15. The composition of the MWL isolated from elephant grass is shown for comparison.

Linkages (per 100 aromatic units)	MWL	Kraft process		Soda-AQ process	
		Kappa 20	Kappa 15	Kappa 20	Kappa 15
β–*O*–4 alkyl-aryl ether	73.0	41.0	11.0	15.0	11.0
β–β resinols	2.4	2.5	0.7	1.8	1.5
β–5 phenylcoumarans	2.9	3.6	0.2	0.3	0.5
% of γ-acylation	39.0	14.0	0.0	0.0	0.0
S/G ratio	1.7	1.1	0.9	1.1	0.8
H/G ratio	0.1	0.1	0.2	0.2	0.2
p-coumaric acid	25.5	13.3	0.3	0.5	0.4

On the other hand, the isolated residual lignins, as well as the lignins precipitated from the black liquors were also analyzed by Py-GC/MS (the data regarding the black liquors are described in *Task 3.4*).

The Py-GC/MS of a selected residual lignin from eucalypt G1xUGL (from kraft pulp at kappa 20) and the lignin precipitated from the respective black liquor are shown in **Figure 3.3.3**. The Py-GC/MS of the MWL from eucalypt G1xUGL is also shown for comparison. The main lignin structural characteristics obtained from the Py-GC/MS data (percentage of H, G and S units, and S/G ratio, and percentage of short-chain pyrolysis products) of the residual lignins isolated from the pulps produced from eucalypt G1xUGL after kraft and soda-AQ processes at kappa 20 and 15, are shown in **Table 3.3.3**. Interestingly, and as already observed by NMR, there is a similarity between the residual lignins from kraft and soda-AQ processes and the native lignin (as reflected by the MWL), observed upon Py-GC/MS. However, the residual lignin is being depleted in S-lignin units and enriched in G- and H-lignin units, with decreasing kappa number (with increasing the extent of delignification), resulting in a decrease of the S/G ratio, as a consequence of the preferential removal of S-lignin during alkaline delignification. A small increase of the amounts of pyrolysis compounds with shorter chain indicates a partial degradation of the residual lignins, which is more evident at lower kappa numbers.

The residual lignins isolated from elephant grass after kraft and soda-AQ processes, were also studied by Py-GC/MS. The Py-GC/MS of a selected residual lignin from elephant grass (from kraft pulp at kappa 20) and the lignin precipitated from the respective black liquor are shown in **Figure 3.3.4**. The Py-GC/MS of the MWL from elephant grass is also shown for comparison. The main lignin structural characteristics obtained from the Py-GC/MS data (percentage of H, G and S units, and H/G and S/G ratio, and percentage of short-chain pyrolysis products) of the residual lignins isolated from the pulps produced elephant grass after kraft and soda-AQ processes at kappa 20 and 15, are shown in **Table 3.3.4**. As occurred with the residual lignins from eucalypt wood, the residual lignins from elephant grass are similar to the native lignin, although being depleted in S-units with decreasing kappa number. In addition, the pyrogram showed important amounts of 4-vinylphenol, that indicates the presence of *p*-coumarates in these residual lignins; most probably *p*-coumarates are linked by ether bonds since ester bonds are supposedly hydrolyzed during alkaline cooking. The small increase of the amounts of pyrolysis compounds with shorter chain indicates a partial degradation of the residual lignins, which is more evident at lower kappa numbers, as also occurred in the case of eucalypt pulps.

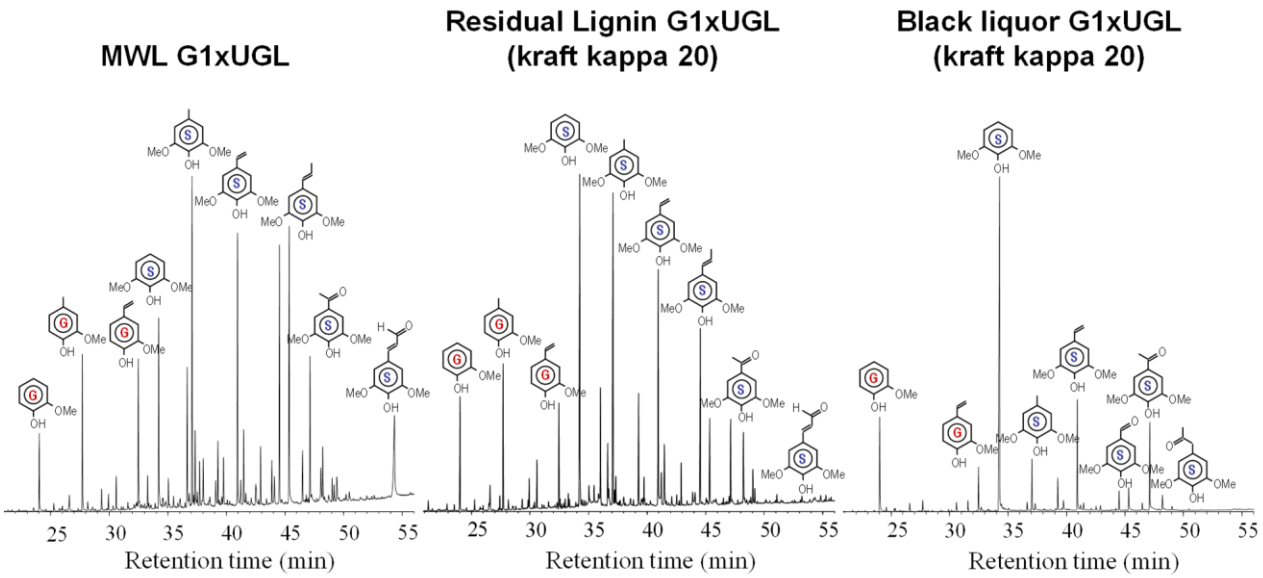


Figure 3.3.3.- Py-GC/MS of a selected residual lignin (from eucalypt G1xUGL kraft pulp at kappa 20) and the lignin precipitated from the respective black liquor. The Py-GC/MS of the MWL from eucalypt G1xUGL is also shown for comparison. The main lignin structures are also depicted here.

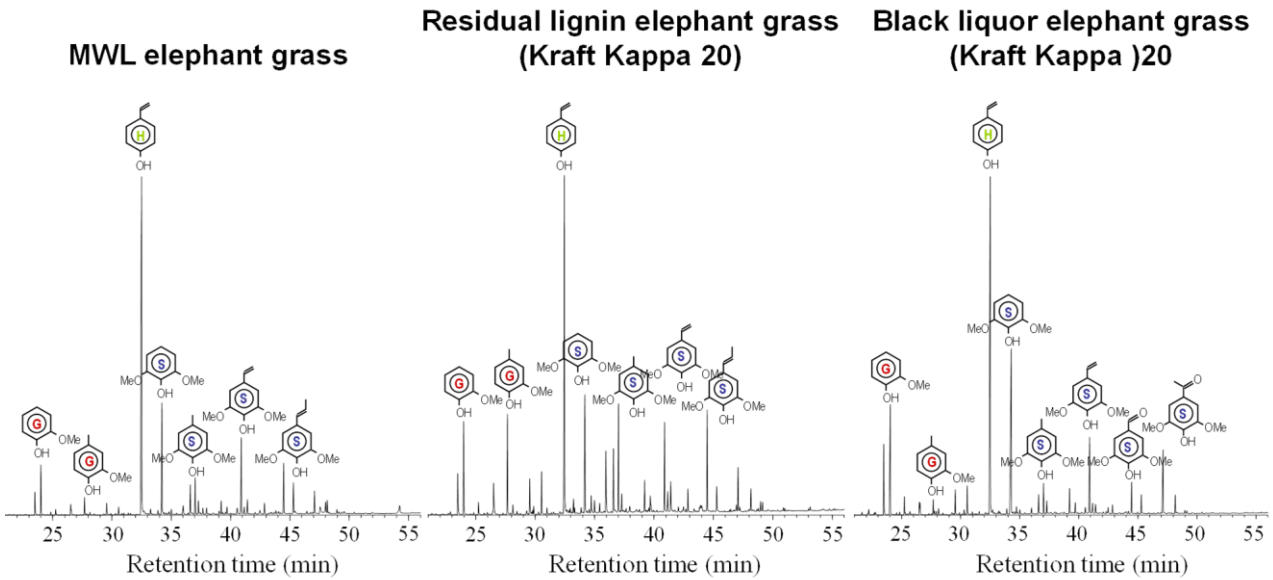


Figure 3.3.4.- Py-GC/MS of a selected residual lignin (from elephant grass kraft pulp kappa 20) and the lignin precipitated from the respective black liquor. The Py-GC/MS of the MWL from elephant grass is also shown for comparison. The main lignin structures are also depicted here.

Table 3.3.3. Main lignin structural characteristics (percentage of H, G and S units, and S/G ratio, and percentage of short-chain pyrolysis products) of the residual lignins isolated from the pulps produced from eucalypt G1xUGL after kraft and soda-AQ processes at kappa 20 and 15. The composition of the MWL isolated from eucalypt hybrid G1xUGL is shown for comparison.

Aromatic units	MWL	Kraft process		Soda-AQ process	
		Kappa 20	Kappa 15	Kappa 20	Kappa 15
H	1.7	2.7	3.6	2.6	4.1
G	33.1	30.3	33.5	31.3	35.6
S	65.2	67.0	62.8	66.1	60.4
S/G ratio	2.0	2.1	1.9	2.1	1.7
% short chains (C_{0-2})	64	76	85	81	84

Table 3.3.4. Main lignin structural characteristics (percentage of H, G and S units, and H/G and S/G ratios, and percentage of short-chain pyrolysis products) of the residual lignins isolated from the pulps produced from elephant grass after kraft and soda-AQ processes at kappa 20 and 15. The composition of the MWL isolated from elephant grass is shown for comparison.

Aromatic units	MWL	Kraft process		Soda-AQ process	
		Kappa 20	Kappa 15	Kappa 20	Kappa 15
H	49.8	30.4	40.2	34.7	37.0
G	19.4	34.8	31.2	33.1	35.4
S	30.8	34.8	28.5	32.1	27.6
S/G ratio	1.6	1.0	0.9	1.0	0.8
H/G ratio	2.6	0.9	1.3	1.0	1.0
% short chains (C_{0-2})	86	83	88	88	90

b2) Structural characteristics of the residual lignins isolated from pulps intended for bioethanol and biogas production (soda-AQ and soda-O_2 processes)

Eucalypt hybrid G1xUGL and elephant grass pulps were prepared by the soda-AQ and soda-O_2 processes at kappa 15, 35 and 50 by **Suzano** (**partner 5**) and sent to **IRNAS** for subsequent analysis of the residual lignins. For this, the residual lignins were isolated by acidolysis and subsequently analyzed by 2D-NMR (in HSQC experiments), and by Py-GC/MS, as shown above.

The structural characteristics (obtained from the HSQC experiments) of the residual lignins isolated from eucalypt G1xUGL pulps prepared by the soda-AQ and soda-O_2 processes are reported in **Table 3.3.5**. The main linkages observed in the residual lignins isolated from the soda-AQ and soda-O_2 pulps from G1xUGL were β–*O*–4 aryl ether, β–β resinol and β–5 phenylcoumaran structures, as also observed in the native lignin. A reduction in these linkages was observed as the kappa number decreases, due to the increasing extent of delignification. The pulps with higher kappa number (kappa 50 and 35) still presented high contents of β–*O*–4 aryl ether linkages, which were drastically reduced in the pulps with the lowest kappa number (kappa 15). Comparing both processes, it is apparent that, at the same kappa number, the soda-O_2 process produces pulps with lower content of β–*O*–4 aryl ether linkages for the pulps with higher kappa. Therefore, it seems that the soda-O_2 process is more efficient than soda-AQ process for delignification of eucalypt wood, at least at high kappa numbers.

Table 3.3.5. Main lignin structural characteristics (linkages per 100 aromatic units, and S/G ratio) of the residual lignins isolated from the pulps produced from eucalypt G1xUGL after soda-AQ and soda-O_2 processes at kappa 15, 35 and 50. The composition of the MWL isolated from G1xUGL is shown for comparison.

Linkages (per 100 aromatic units)	MWL	Soda-AQ process			Soda-O_2 process		
		K50	K35	K15	K50	K35	K15
β–*O*–4 alkyl-aryl ether	90.0	56.0	47.0	12.0	41.0	29.0	12.0
β–β resinols	10.5	11.0	11.0	6.7	13.0	10.3	8.2
β–5 phenylcoumarans	5.3	1.0	0.9	0.4	0.9	1.0	0.9
S/G ratio	2.8	5.9	5.9	4.8	4.6	4.5	4.6

On the other hand, the structural characteristics (obtained from HSQC spectra) of the residual lignins isolated from elephant grass pulps prepared by the soda-AQ and soda-O_2 processes are reported in **Table 3.3.6**. The main lignin substructures present in the residual lignins were β–*O*–4 aryl ether, with lower amounts of β–β resinol and β–5 phenylcoumaran structures, as also observed in the native lignin. A reduction in the content of these linkages was observed as the kappa number decreases, due to the higher extent of delignification. At similar kappa numbers, the content of β–*O*–4 aryl ether linkages in the residual lignins from elephant grass were lower for the soda-O_2 than for the soda-AQ process, as already observed in the case of eucalypt wood shown above, indicating a higher efficiency of the soda-O_2 process for delignifying the elephant grass. Both, soda-AQ and soda-O_2 cooking produced a complete hydrolysis of the *p*-coumarate groups that are acylating the γ-carbon of the lignin side-chain, and only minor amounts of free *p*-coumaric acid are found in the soda-AQ pulps.

Table 3.3.6. Main lignin structural characteristics (linkages per 100 aromatic units, percentage of γ-acylation, S/G and H/G ratios and percentage of *p*-coumaric acid) of the residual lignins isolated from the pulps produced from elephant grass after soda-AQ and soda-O_2 processes at kappa 15, 35 and 50. The composition of the MWL isolated from elephant grass is shown for comparison.

Linkages (per 100 aromatic units)	MWL	Soda-AQ process			Soda-O_2 process		
		K50	K35	K15	K50	K35	K15
β–*O*–4 alkyl-aryl ether	73.0	54.0	52.0	11.0	56.0	56.0	24.0
β–β resinols	2.4	4.6	3.3	1.5	4.5	5.0	3.0
β–5 phenylcoumarans	2.9	4.0	3.0	0.3	4.0	4.0	1.5
% of γ-acylation	39.0	6.0	8.0	0.0	4.0	2.0	0.0
S/G ratio	1.7	1.8	1.1	0.8	1.4	1.4	0.8
H/G ratio	0.1	0.0	0.1	0.1	0.1	0.1	0.2
p-coumaric acid	25.5	9.0	10.0	0.0	8.0	5.0	1.0

The residual lignins isolated from eucalypt G1xUGL and elephant grass pulps obtained from the soda-AQ and soda-O_2 processes (at kappa 50, 35 and 15), were also analyzed by Py-GC/MS (**Fig. 3.3.5** and **Fig. 3.3.6**, respectively). The main structural characteristics of these residual lignins from eucalypt G1xGL and elephant grass pulps, obtained upon Py-GC/MS, are shown in **Table 3.3.7** and Table **3.3.8**, respectively.

In the case of the eucalypt wood, the residual lignins are enriched in H- and G-lignin units, and depleted in S-units with decreasing kappa number. Moreover, there is also an increase in the amounts of lignin

pyrolysis compounds with shorter chain, indicating a partial lignin degradation with decreasing kappa number. In addition, and in agreement with the NMR data shown above, soda-O_2 process seems to be more efficient than soda-AQ process to delignify eucalypt wood, at least at higher kappa numbers.

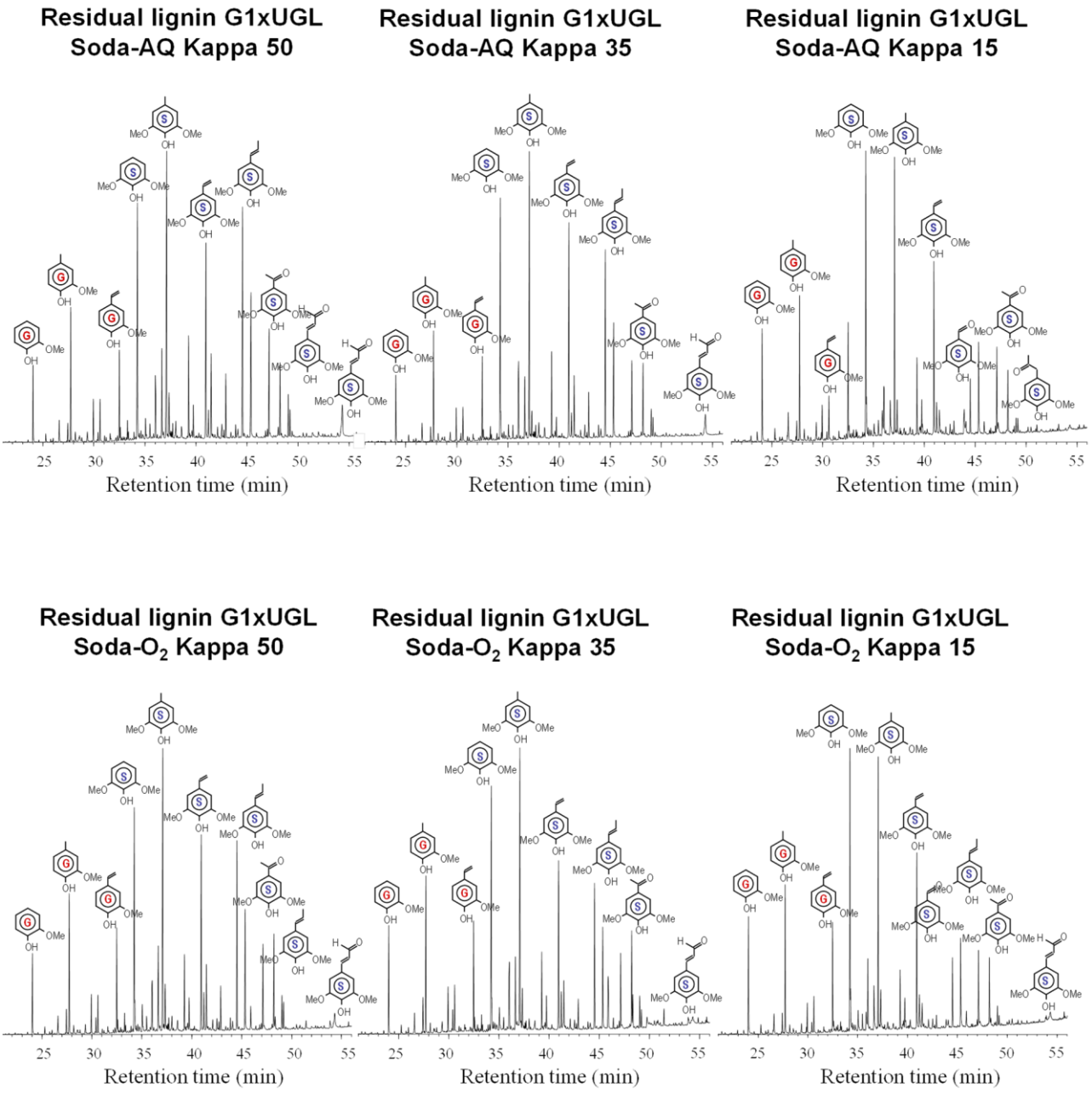


Figure 3.3.5.- Py-GC/MS of the residual lignins isolated from eucalypt G1xUGL after soda-AQ and soda-O_2 at kappa 50, 35 and 15.

In the case of the elephant grass, the residual lignins are also enriched in H- and G-lignin units, and depleted in S-units with decreasing kappa number. Moreover, there is also an increase in the amounts of lignin pyrolysis compounds with shorter chain, indicating a partial lignin degradation with decreasing kappa number. In addition, and in agreement with the NMR data shown above, there is a similarity between the soda-O_2 and soda-AQ processes to delignify elephant grass.

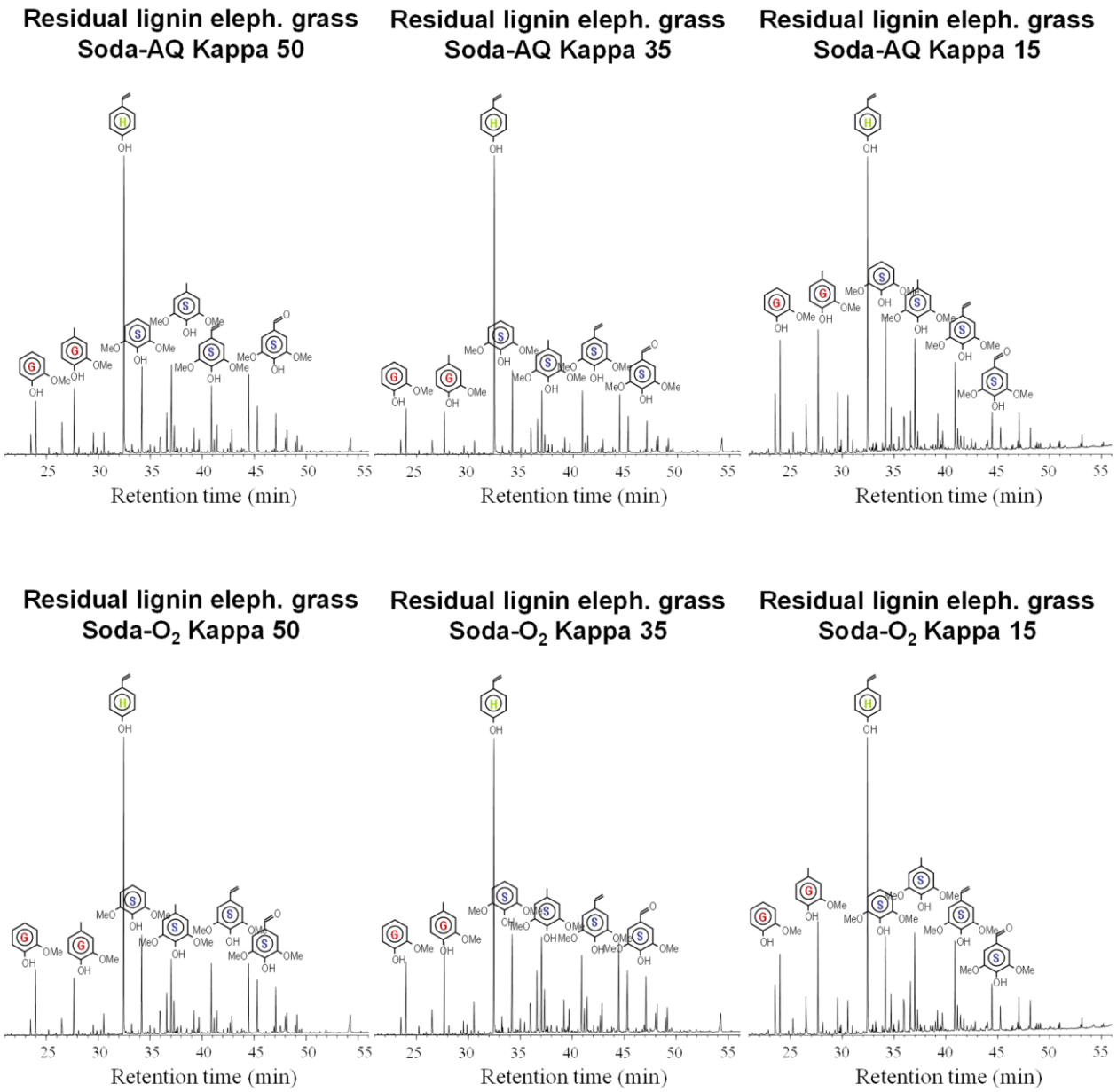


Figure 3.3.6.- Py-GC/MS of the residual lignins isolated from elephant grass after soda-AQ and soda-O_2 at kappa 50, 35 and 15.

Table 3.3.7. Main lignin structural characteristics (percentage of H, G and S units, and S/G ratio, and percentage of short-chain pyrolysis products) of the residual lignins isolated from the pulps produced from eucalypt G1xUGL after soda-AQ and soda-O_2 processes at kappa 15, 35 and 50. The composition of the MWL isolated from G1xUGL is shown for comparison.

Aromatic units	MWL	Soda-AQ process			Soda-O_2 process		
		K50	K35	K15	K50	K35	K15
H	1.7	1.6	1.8	4.0	1.8	2.2	2.8
G	33.1	27.5	27.6	35.6	33.3	35.3	36.2
S	65.2	70.9	70.5	60.4	64.9	62.5	61.0
S/G ratio	2.0	2.6	2.5	1.7	2.0	1.8	1.7
% short chains (C_{0-2})	64	70	73	84	72	76	84

Table 3.3.8. Main lignin structural characteristics (percentage of H, G and S units, and H/G and S/G ratios, and percentage of short-chain pyrolysis products) of the residual lignins from the pulps produced from elephant grass after soda-AQ and soda-O_2 processes at kappa 15, 35 and 50. The composition of the MWL isolated from elephant grass is shown for comparison.

Linkages (per 100 aromatic units)	MWL	Soda-AQ process			Soda-O_2 process		
		K50	K35	K15	K50	K35	K15
H	49.8	35.0	34.8	37.0	27.3	23.2	33.9
G	19.4	29.4	29.1	35.4	32.9	35.6	37.1
S	30.8	35.7	36.1	27.6	39.7	41.2	29.0
S/G ratio	1.6	1.2	1.2	0.8	1.2	1.2	0.8
H/G ratio	2.6	1.2	1.2	1.0	0.8	0.8	0.9
% short chains (C_{0-2})	86	80	81	90	79	80	86

Task 3.4. Analysis of black liquors and other side streams

The lignins precipitated from the black liquors of eucalypt wood G1xUGL from the kraft and soda-AQ processes, at kappa 20 and kappa 15, were also analyzed by 2D-NMR (see **Fig. 3.3.1**). The quantitation of the main inter-unit linkages and lignin units is shown in **Table 3.4.1**. It is clear from the NMR spectra that these precipitated lignins are enriched in β–β resinol structures, while the other linkages (β–*O*–4 alkyl-aryl ether and β–5 phenylcoumarans), if present, are in much lower amounts. An increase of the S/G ratio is observed, that indicates that S-lignin units, which are predominantly forming β–*O*–4 alkyl-aryl ether structures, are preferentially removed from the eucalypt during pulping and are being enriched in the black liquors. It is interesting to note that the lignins from soda-AQ process are more enriched in S-lignin units that the lignins from kraft process. In addition, while minor amounts of β–*O*–4 alkyl-aryl ether structures are still present in the lignins from kraft process, they were completely absent in the lignins from soda-AQ process. Therefore and as already observed in the analysis of the residual lignins from these pulps, the comparison between the kraft and soda-AQ process indicates that the latter process seems to be more efficient to depolymerize the lignin from eucalypt wood than the kraft process.

Table 3.4.1. Main lignin structural characteristics (linkages per 100 aromatic units, and S/G

ratio) of the lignins precipitated from the black liquors produced from eucalypt G1xUGL after kraft and soda-AQ processes at kappa 20 and 15. The composition of the MWL isolated from eucalypt hybrid G1xUGL is shown for comparison.

Linkages (per 100 aromatic units)	MWL	Kraft process		Soda-AQ process	
		Kappa 20	Kappa 15	Kappa 20	Kappa 15
β–*O*–4 alkyl-aryl ether	90.0	1.0	0.0	0.0	0.0
β–β resinols	10.5	9.7	9.8	9.4	9.3
β–5 phenylcoumarans	5.3	0.0	0.0	0.0	0.0
S/G ratio	2.8	8.9	8.2	8.9	8.5

On the other hand, the lignins precipitated from the black liquors from the pulping of elephant grass by the kraft and soda-AQ processes at kappa 20 and kappa 15 were also studied by 2D-NMR (in HSQC experiments) (see **Fig. 3.3.2**). A quantitation of the abundance of the main lignin inter-unit linkages present in the lignins precipitated from the different black liquors, as well as the abundance of the H, G and S lignin units and *p*-coumaric acid, and the percentage of acylation of the γ-carbon, was performed by integration of the volume contours of their cross-signals and was referred to as per 100 aromatic units (**Table 3.4.2**). The composition of the lignins precipitated from the black liquors was completely different from that of the MWL. First of all, the *p*-coumarate esters in the γ-carbon have been completely hydrolyzed, and the *p*-coumaric acid has been liberated. However, only in the black liquor from kraft kappa 20, important amounts of *p*-coumaric acid could be detected. β–*O*–4 linkages are only present in low amounts, and its abundance are reduced with decreasing kappa number due to the more drastic pulping conditions. Comparing kraft and soda-AQ processes, the abundance of β–*O*–4 linkages is lower, in the latest, which seems to indicate that soda-AQ process performs more efficiently in elephant grass, as also occurred with eucalypt woods. Condensed structures, such as β–β resinols and β–5 phenylcoumarans, are present in relatively more abundance in the black liquor compared to the MWL, and especially the resinols, as also occurs in eucalypt wood, despite its very low abundance in the native lignin. Finally, the S/G ratio is higher in the lignins precipitated from the black liquors, which indicates that S-lignin units, that form predominantly β–*O*–4 linkages are preferentially removed during cooking. The higher S/G value observed in the liquors of the soda-AQ process compared to the kraft process is another indication of the better performance of soda-AQ for delignifying elephant grass, as already observed in eucalypt wood.

Table 3.4.2. Main lignin structural characteristics (linkages per 100 aromatic units, percentage of g-acylation, S/G and H/G ratios and percentage of *p*-coumaric) of the lignins precipitated from the black liquors produced from elephant grass after kraft and soda-AQ processes at kappa 20 and 15. The composition of the MWL isolated from elephant grass is shown for comparison.

Linkages (per 100 aromatic units)	MWL	Kraft process		Soda-AQ process	
		Kappa 20	Kappa 15	Kappa 20	Kappa 15
β–*O*–4 alkyl-aryl ether	73.0	4.0	0.0	1.0	1.0
β–β resinols	2.4	2.0	1.8	3.0	3.0
β–5 phenylcoumarans	2.9	0.0	0.0	0.0	0.0
% of γ-acylation	39.0	0.0	0.0	0.0	0.0
S/G ratio	1.7	2.0	1.9	2.0	1.9
H/G ratio	0.1	0.3	0.3	0.3	0.3
p-coumaric acid	25.5	12.0	0.0	0.0	0.0

The lignins precipitated from the black liquors were also analysed by Py-GC/MS (see **Fig 3.3.3** and **Fig. 3.3.4**). The main structural characteristics obtained upon Py-GC/MS are shown in **Table 3.4.3** (for the case of eucalypt G1xUGL) and in **Table 3.4.5** (for elephant grass). The data indicate that the lignin in black liquors is completely different from the native lignin and from the residual lignin in the pulp. The lignin in black liquors is highly enriched in S-lignin units (due to the preferential solubilisation of S-lignin units) and depleted in G-units. In addition, the higher amounts of pyrolysis compounds with shorter chain and/or oxidized, indicates that this lignin is heavily degraded and oxidized. In the case of elephant grass, important amounts of H-units, arising from *p*-coumaric acid, can also be observed, indicating that *p*-coumaric acid is present in free from or etherified to the lignin.

Table 3.4.3. Main lignin structural characteristics (percentage of H, G and S units, and S/G ratio, and percentage of short-chain pyrolysis products) of lignins precipitated from the black liquors produced from eucalypt G1xUGL after kraft and soda-AQ processes at kappa 20 and 15. The composition of the MWL isolated from eucalypt hybrid G1xUGL is shown for comparison.

Aromatic units	MWL	Kraft process		Soda-AQ process	
		Kappa 20	Kappa 15	Kappa 20	Kappa 15
H	1.7	1.1	1.9	2.0	2.3
G	33.1	22.9	24.1	26.1	25.7
S	65.2	76.0	74.0	72.0	72.0
S/G ratio	2.0	3.3	3.1	2.8	2.8
% short chains (C_{0-2})	64	94	95	93	93

Table 3.4.5. Main lignin structural characteristics (percentage of H, G and S units, and H/G and S/G ratios, and percentage of short-chain pyrolysis products) of the lignins precipitated from the black liquors produced from elephant grass after kraft and soda-AQ processes at kappa 20 and 15. The composition of the MWL isolated from elephant grass is shown for comparison.

Aromatic units	MWL	Kraft process		Soda-AQ process	
		Kappa 20	Kappa 15	Kappa 20	Kappa 15
H	49.8	39.4	41.8	38.9	39.2
G	19.4	27.0	27.5	27.4	28.6
S	30.8	33.8	30.7	33.7	32.2
S/G ratio	1.6	1.4	1.2	1.3	1.2
H/G ratio	2.6	1.5	1.5	1.4	1.4
% short chains (C_{0-2})	86	94	96	95	96

3.1.5. Progress on WP5. “Demonstration activities”

Task 5.2. CTP enzymatic pre-treatment and bleaching pilot plant trials

From the previous report from **CIB**, we had concluded that NS-22086 would be a suitable enzyme preparation to investigate further for incorporation into whole EG through the use of the MSD Pressafiner available at **CTP**. In order to establish some reaction parameters, the effect of pH and hydrolysis medium was investigated at **CIB**.

The solubilisation, reducing sugar release and acetic acid release from whole EG and Pressafiner pre-treated EG was investigated over a pH range of 5-7.4 (**Fig. 5.2.1**). As reported before at a single pH value, the hydrolysis of pre-treated EG by NS-22086 was slightly more extensive than the hydrolysis of the initial whole material. pH 6 was the optimum value for hydrolysis, the degree of solubilisation dropping as the pH was increased further. As this was the same for both substrates, it shows that the Pressafiner pre-treatment did not change the physico-chemical properties of EG and the optimum reflects the enzymes present in the cocktail. The reducing group levels in the hydrolysates did show differences with respect to reaction pH. While the initial material has a pH optimum of 6, the pre-treated material has a broad high level of reducing groups between pH 5 and 6.2, dropping thereafter as the pH increased. At pH 7.4, the amount of reducing groups generated through the action of the enzymes was 3 to 4-fold less than the optimum value. The differences in degree of solubilisation was not so drastic and suggests that the enzymes responsible for matrix loosening and solubilisation of poly and oligomeric material still function at the more neutral pHs, while the ability of this material to be broken down into small oligos and monosaccharides has been lost at this higher pH. The release of acetic acid from both EG substrates remained high across the pH range, but the highest level was recorded between pH 5.8 and 6.0 for the initial EG.

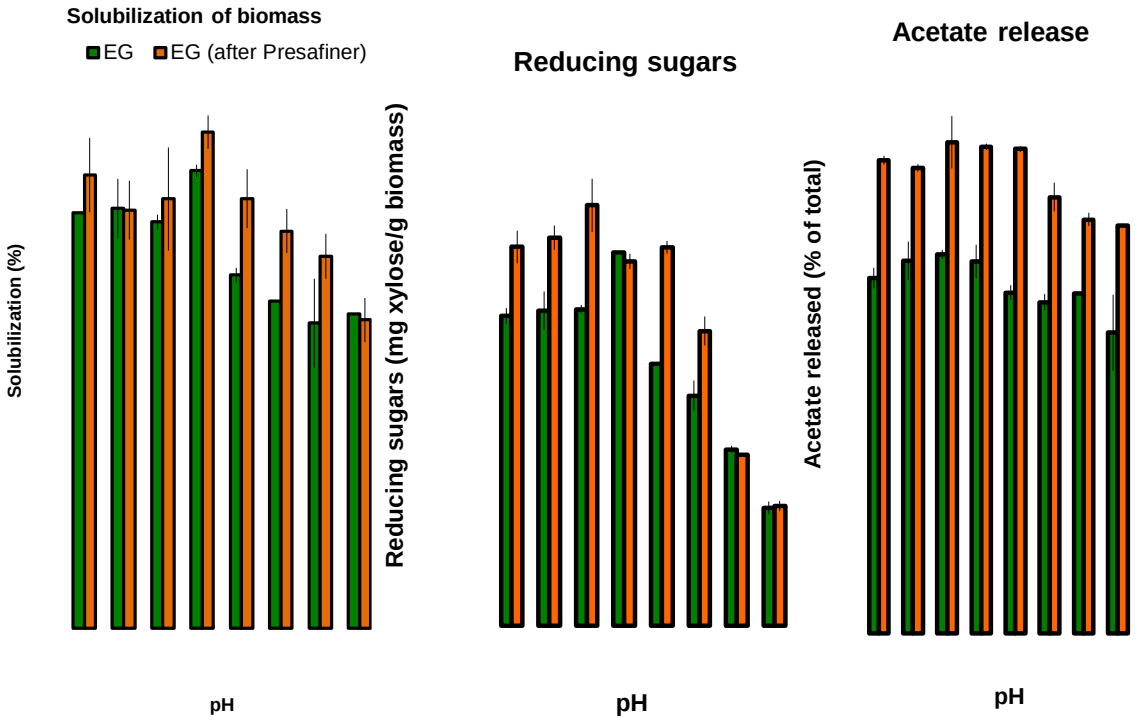


Fig. 5.2.1. The effect of pH on the hydrolysis of EG by NS-22086 at 50^{o}C.

The next selection criterion was that of a suitable solvent. EG at 5% (w/v) consistency, was incubated with or without NS-22086 at 50°C in the presence of water, 50 mM phosphate (pH 6.0) or 50 mM MOPS (pH 6.0). The results are shown in **Fig. 5.2.2**.

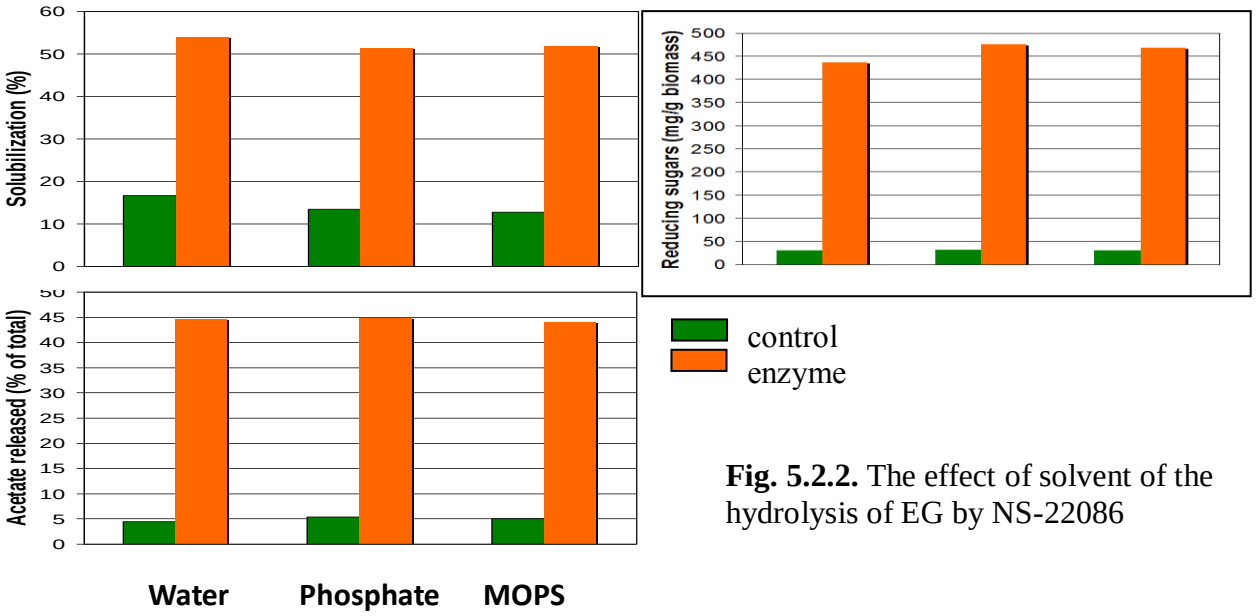


Fig. 5.2.2. The effect of solvent of the hydrolysis of EG by NS-22086

There was no difference between the 3 solvents examined, with or without enzyme. So it is suggested that CTP should use the following conditions for the Pressafiner trials:

- **Enzymatic phase-1** (impregnation with cellulases using MSD-pressafiner): Elephant grass (2 kg, o.d.) passed through the MSD-pressafiner and the "extruded" material directly drop (for impregnation) on the Cellulase cocktail (Cellulase NS22086 from **Novozymes**; 100 mL/kg) (if required for mixing in phase-2, some extra water could be added to the enzymes)

- **Enzymatic phase-2**: The cellulase impregnated grass incubated at 50ºC for 24 h in storage chest or other reactor with mixing (consistency as high as possible but enabling mixing). No azide to be added.

- **Controls**: 1) Initial Elephant grass (without any treatment); and 2) Treatment (MSD-pressafiner and incubation) without enzyme

- **End**: Whole (wet) material to be sent to **VTT** (1.5 kg dry weight/each treatment) for (additional) saccharification and fermentation, and **CIB** (50 g dry weight/each treatment)

- **Materials** needed: i) Elephant grass (8 kg); and ii) Cellulase NS22086 (500 mL sent by **Novozymes**)

3.2. Deviations from the Work Plan

No deviations were produced from the original Work Plan

4. PLANS FOR DISSEMINATION AFTER 36th MONTHS

During the course of the project we have already published several papers regarding the detailed composition of elephant grass and the selected Brazilian eucalypt hybrids. In addition, we also published a paper regarding the enzymatic pretreatment of elephant grass and eucalypt wood with the high redox potential laccase. We are now preparing three more papers that we intend to submit for publication during the next couple of months. Among these, one of the papers will be dealing with the very promising results obtained regarding the enzymatic pretreatment of eucalypt wood with the commercial low-redox potential laccase from *Myceliophthora thermophila*, and using methyl syringate as mediator. The second paper will be devoted to the structural characterization of the residual lignins and black liquors produced from elephant grass and eucalypt G1xUGL by different chemical alkaline deconstruction processes (kraft, soda-AQ and soda-O_2 processes) at different kappa numbers. Finally, a third manuscript is under preparation including the results on the enzymatic degradation of elephant grass stems by hydrolases and the influence of the pith and bark in the total hydrolysis.

On the other hand, part of the results obtained during the course of the project will be presented in several scientific congresses, including "*The 17th International Symposium on Wood, Fibre and Pulping Chemistry*", that will be held in Vancouver, Canada, during June 12-14, 2013; the "*6th International Colloquium on Eucalyptus Pulp*", that will be held in Colonia del Sacramento, Uruguay, during November 24-27, 2013; the "13th European Workshop on Lignocellulosics and Pulp", that will be held in Seville, Spain, during June 24-27, 2014.

Also, two PhD Thesis will be presented at the University of Seville with the main results obtained during the course of the project: "Study of the chemical composition of fast growing lignocellulosic crops and modification of their lignins during alkaline deconstruction" by Pepijn Prinsen, and "Optimization of laccase-based pre-treatments for the enzymatic deconstruction of woody and nonwoody lignocellulosic feedstocks" by Alejandro Rico.

5. DELIVERABLES AND MILESTONES

List the deliverables and milestones you are responsible due during the period covered by the report indicating whether they have been achieved.

DL2.7.- Pre-treatment conditions using selected enzymes (**CIB**, IRNAS, Novozymes, CTP; month 24) has been achieved in due time.

DL2.8.- Pre-treatment materials for characterisation and evaluation – 2 (UFV, **CIB**, IRNAS, VTT, Novozymes, Suzano, CTP; month 24) has been achieved in due time.

DL3.5.- Characterisation of pre-treated woody materials using advanced analytical tools – 1 (UFV, IRNAS, **VTT,** CTP; month 24) has been achieved in due time.

DL2.9.- Conditions for scaling up the enzymatic deconstruction (**CIB**, IRNAS, Novozymes; month 28) has been achieved in due time.

DL3.7.- Characterisation of pre-treated nonwoody materials using advanced analytical tools – 2 (UFV, **CIB**, IRNAS, VTT, CTP; month 32) has been achieved in due time and submitted to UFV.

DL3.8.- Characterisation of pre-treated woody materials using advanced analytical tools – 2 (UFV, **CIB**, IRNAS, VTT, CTP; month 32) has been achieved in due time and submitted to UFV.

6. NEW CONTACT PERSONS

In case that any of the responsible persons of any of the beneficiaries is replaced for a particular reason, please explain and indicate the name and contact details of the new contact person.

Not applicable

APPENDIX C

VTT

Grant agreement no: KBBE-2009-3-244362

Optimized pre-treatment of fast growing woody and nonwoody Brazilian crops by detailed characterization of chemical changes produced in the lignin-carbohydrate matrix

Second Periodic Report

1-Jul-2011 to 31-Dec-2012

Partner P3 (VTT)

Grant agreement no: **KBBE-2009-3-244362**

Project acronym: LIGNODECO

Project title: Optimized pre-treatment of fast growing woody and nonwoody Brazilian crops by detailed characterization of chemical changes produced in the lignin-carbohydrate matrix

Funding Scheme:	Collaborative project (small or medium-scale focused research project)		
Thematic Priority:	KBBE-2009-3		
Period covered:	From 1 July 2011 to 31 December 2012		
Date of preparation:	15 January 2012		
Start date of project:	1 January 2010	Duration:	36 months

Partner name:	**VTT**
Author 1	*Tiina Liitiä (VTT)*
Author 2	*Tarja Tamminen (VTT)*
Author 3	*Dorothee Barth (VTT)*
Author 4	*Harri Heikkinen (VTT)*
Author 5	*Jaakko Pere (VTT)*

1. SUMMARY OF THE WORK

The LGF organosolv process developed at VTT was studied as a potential pre-treatment method for deconstruction of fast growing Brazilian eucalyptus clones and non-woody lignocellulosics feedstocks for bioethanol production. The LGF organosolv cooking with ethanol solvent and phosphinic acid catalyst produced well hydrolysable pulp with high ethanol yield, and the hydrolysability was further improved by alkaline extraction of the LGF fibers. This was demonstrated also at pilot scale, and after alkaline extraction of residual lignin and xylan, nearly 90% of the theoretical ethanol yield was reached. The cooking time required at relatively low cooking temperature of 130°C is still rather long for industrial applications, and should be further reduced. This could be possible by better impregnation, mixing and increased temperature, as well as using fresh non-dried chips.

The LGF organosolv process was also compared with the other deconstruction methods, *i.e.* alkaline cooking (NaOH-O_2, Soda-AQ) and enzymatic (laccase, hydrolytic) pretreatments. The oxidative alkaline pretreatment (NaOH-O_2) provided well hydrolysable pulp already at high kappa levels of 35-50, whereas soda-anthraquinone (Soda-AQ) cooking gave comparable results only at lower kappa levels of 15. The NaOH-O_2 treatment probably opens up the fiber ultrastructure better at the same lignin content, and even at high kappa levels the reject can be hydrolysed more efficiently. The alkaline oxidation is thus another potential pretreatment method for bioethanol production from eucalyptus and elephant grass. Bioethanol yield after LGF and NaOH-O_2 cooking was rather similar, but when the LGF organosolv pretreatment was followed by alkaline extraction clearly higher bioethanol yield was obtained compared to the alkaline oxidation. The enzymatic laccase and hydrolytic pretreatments alone were not sufficient to open up the fiber ultrastructure for efficient enzymatic hydrolysis and ethanol fermentation.

No clear correlation between LGF or alkaline pulp hydrolysability and cellulose crystallinity was detected. Other factors, *e.g.* efficient lignin and xylan removal, thus probably contribute more to the pulp hydrolysability.

2. PROJECT OBJECTIVES FOR THE PERIOD

The main objectives of VTT for the 19-36 months period were:

- To investigate the applicability of the VTT's novel LGF organosolv process as a potential pre-treatment technique for bioethanol production (**WP2**).
    - LGF cooking time required for efficient pulp hydrolysis for bioethanol production was optimised.
    - LGF organosolv pretreatments at lab scale for all selected raw materials in optimised conditions were also finalised.
- Characterisation of the pretreated woody and non-woody materials using advanced analytical tools **(WP3, DL3.5, DL 3.6, DL3.7, DL3.8).**

  - The effect of LGF organosolv and alkaline (Soda-AQ, Soda-O_2) cooking on cellulose crystallinity was investigated using solid-state NMR spectroscopic methods implemented previously.
  - Xylan distribution on the fibre surface after reprecipitation was evaluated by microscopy after immonolabeling.
  - Previously produced organosolv spent liquor lignins, as well as the residual lignins of alkaline pulps and the corresponding black liquor lignins were characterised.
- Evaluation of pretreated materials and residues for bioethanol production **(WP4, DL 4.2).**
  - The bioethanol production potential of selected feedstocks after LGF organosolv cooking and following alkaline extraction was evaluated.
  - The LGF cooking was also compared with the other deconstruction methods, *i.e.* alkaline deconstruction (NaOH-O_2, Soda-AQ) and enzymatic (laccase, hydrolytic) pretreatments.
  - The enzymatic hydrolysis was optimised for best performing pulps, i.e. after LGF organosolv and alkaline oxidation pretreatments.
- To demonstrate the LGF organosolv process at pilot scale, and also to produce material for optimisation of enzymatic hydrolysis and comparison with other pilot pulps **(WP5, DL 5.1).**

3. WORK PROGRESS AND ACHIEVEMENTS DURING THE PERIOD

3.1 Work Progress

3.1.1 Progress on WP2 Optimised pretreatments for woody and non woody materials

Summary of the WP2

The aim of VTT in WP2 was to investigate the applicability of the novel LGF organosolv process, using ethanol solvent and phosphinic acid catalyst, as a potential pretreatment technique for the production of bioethanol by saccharification and fermentation of lignocellulosics. Previously in this project, optimal conditions for the deconstruction of Brazilian fast growing eucalyptus and elephant grass have been defined (3.5-5% phosphinic acid, 15% water, 130˚C), but relatively long cooking time of 48 h was used. Optimisation of cooking time was continued to define the shortest possible cooking time allowing highest glucose yield in enzymatic hydrolysis for bioethanol production. Another aim was to rank the selected feedstocks according to their suitability for bioethanol production in optimised conditions.

Task 2.2 Chemical deconstruction by solvent process

Experimental

The optimal cooking time was screened for a triplet hybrid of *E. grandis x [E. globulus x urophylla] (G1xUGL)* and *Suzano* clone, aiming to produce hydrolysable pulp with sufficient delignification and defibration without compromising cellulose yield. The cooking experiments were carried out under 3 bar pressure in 2L oil heated Juccheim

reactor, using 3.5% phosphinic acid charge at 130 °C and water content of 15%. The alkaline extraction of LGF pulps was performed in constant conditions (1M NaOH, 2.5% consistency, room temperature, 24h with continuous agitation). For comparison of raw materials, the same conditions with optimized cooking time of 20h were used.

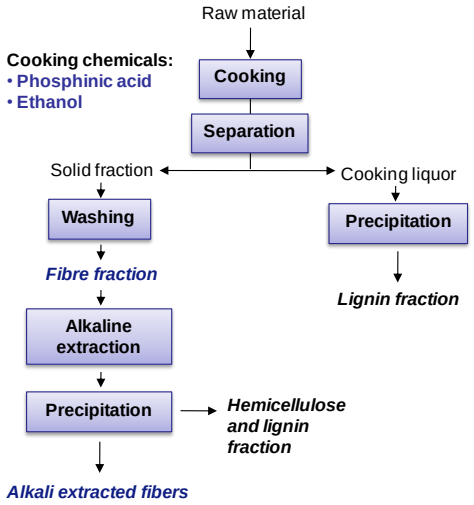


Figure 1. Two-stage fractionation procedure based on LGF organosolv cooking and alkaline extraction of fibre fraction.

The delignification efficiency of LGF cooking and the following alkaline extraction was evaluated according to the total lignin content of the unscreened pulps after acetone extraction (NREL/TP-510-42618). The pulp total carbohydrate content was determined using total lignin and acetone extractives contents: Carbohydrate, % = 100% - total lignin, % - extractives, %. Pulp carbohydrate composition was determined after acid hydrolysis using ion exchange chromatography (AEC-PAD), and the polysaccharide composition was calculated according to Janson (1970) using constants of birch. As unscreened pulps were used, relatively large deviation in the chemical compositions of the samples was detected, making comparison of the samples rather difficult especially when alkaline extraction was not performed.

Optimisation of cooking time

Pulp yield, and especially cellulose yield, is one of the most important factors affecting how efficiently the original raw material can be converted to bioethanol. As expected, higher pulp yields could be obtained with shorter cooking times with both clones. To be able to evaluate the cellulose yields available for the enzymatic hydrolysis and fermentation and thus the degree of wood utilization, the pulp chemical compositions were normalized to pulp yields (Figure 2). With shorter cooking times the delignification became less efficient but the carbohydrates were better preserved as expected. After alkaline extraction the differences were minor, and lignin content still remained relatively low with cooking time of 16 h. With the alkaline extracted *G1xUGL* pulps, the lignin content started to increase steadily when cooking time was shorter than 20h (not shown, reported earlier). However, no clear gain in polysaccharide yield was obtained when cooking time was reduced below 16-20h. The shortest cooking time of 10h preserved especially xylan in LGF pulps of *Suzano* clone (Figure 3), but for *G1xUGL* the effect of cooking time on xylan content was

not so clear. After alkaline extraction the differences in cellulose or xylan content were in both clones minor. No clear indication on optimal cooking time for maximal cellulose yield could be identified either.

Based on chemical composition, and especially lignin content, sufficient deconstruction of Eucalyptus clones should be reached with cooking time of 16-20h. This is well in accordance with the hydrolysis results obtained in WP4.

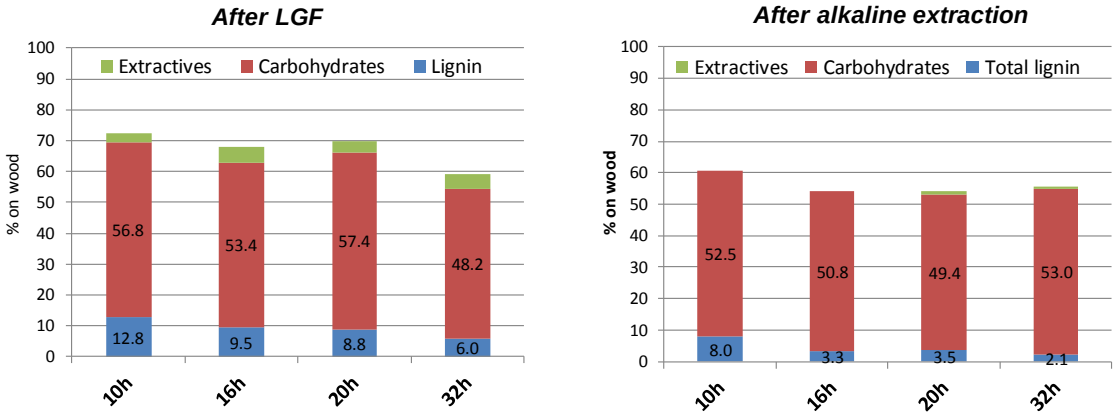


Figure 2. Chemical compositions after LGF organosolv cooking and the following alkaline extraction (scaled to pulp total yield) for Suzano's eucalyptus clone.

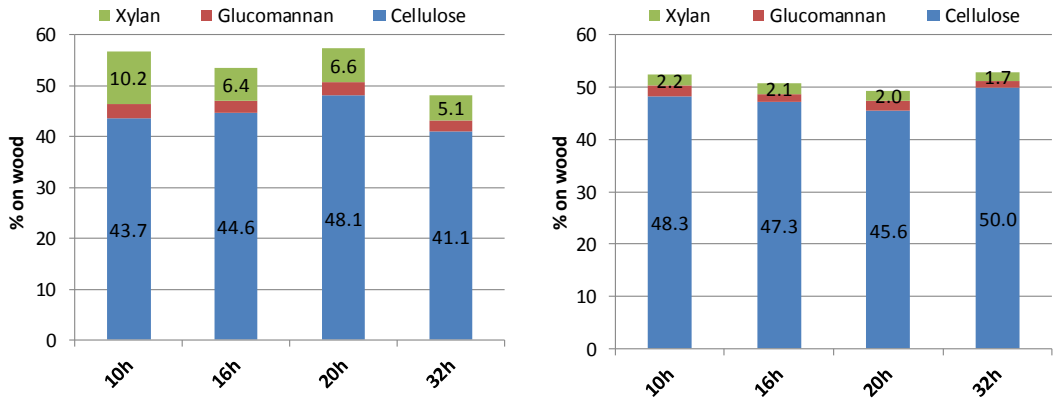


Figure 3. Polysaccharide contents after LGF organosolv cooking and the following alkaline extraction (scaled to the pulp total yields) for the Suzano's clone, describing the recovery of original wood raw material as polysaccharides. (after LGF=left, after alkaline extraction=right)

Comparison of feedstock

To rank the raw materials in respect of bioethanol production potential, the available feedstocks were cooked in the optimised conditions (130°C, 3.5% H_3PO_2, 15% water content) using cooking time of 20h.

After 20 h cooking and alkaline extraction, the DGxU2 clone gave the best yield (figure 4), whereas the lowest lignin contents were reached for *Suzano*, *G1xUGL* and *U1xU2* clones

(Figure 5). These seem to cook easier than the other clones. After LGF cooking and alkaline extraction, the *E. globulus* provided the lowest lignin content as assumed based on the low initial wood lignin content. For some reason, the delignification was not effective for the elephant grass (*EG1*). The polysaccharide yield remained highest in *DGxU2* and *IP* clones, and the pulps with highest total polysaccharide content also had the highest cellulose yields (Figure 6). In elephant grass (EG1) pulp, the xylan content remained higher compared to the eucalyptus pulps even after alkaline extraction, resulting in lowest cellulose yield.

According to the chemical composition, the *DGxU2* clone seemed best for the bioethanol production. This is well in accordance with the pulp hydrolysability determined at WP4. In overall, the differences in pulp compositions were relatively small and obscured by the inaccuracy of unscreened pulp results.

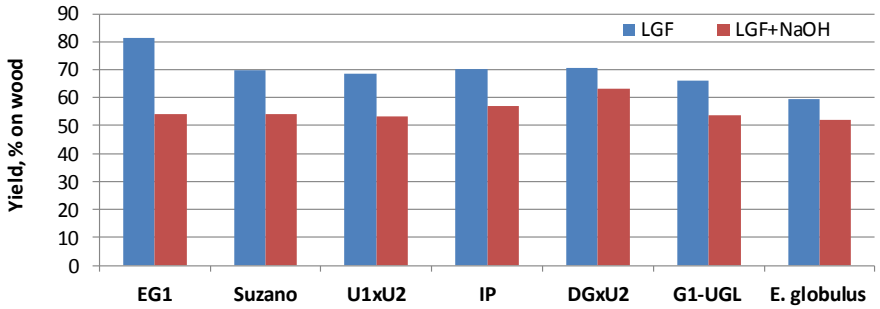


Figure 4. Pulp total yields (unscreened) after LGF cooking and the following alkaline extraction for selected raw materials.

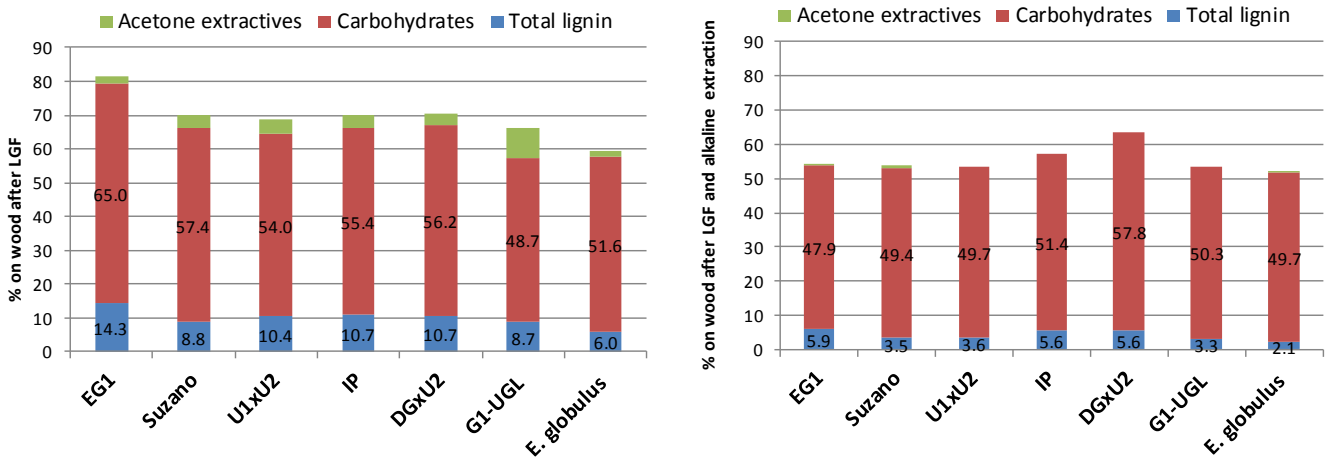


Figure 5. Chemical compositions after 20h LGF cooking (left) and alkaline extraction (right). The results scaled to pulp total yield. (Ash content not taken into account, which probably has largest effect on EG1).*

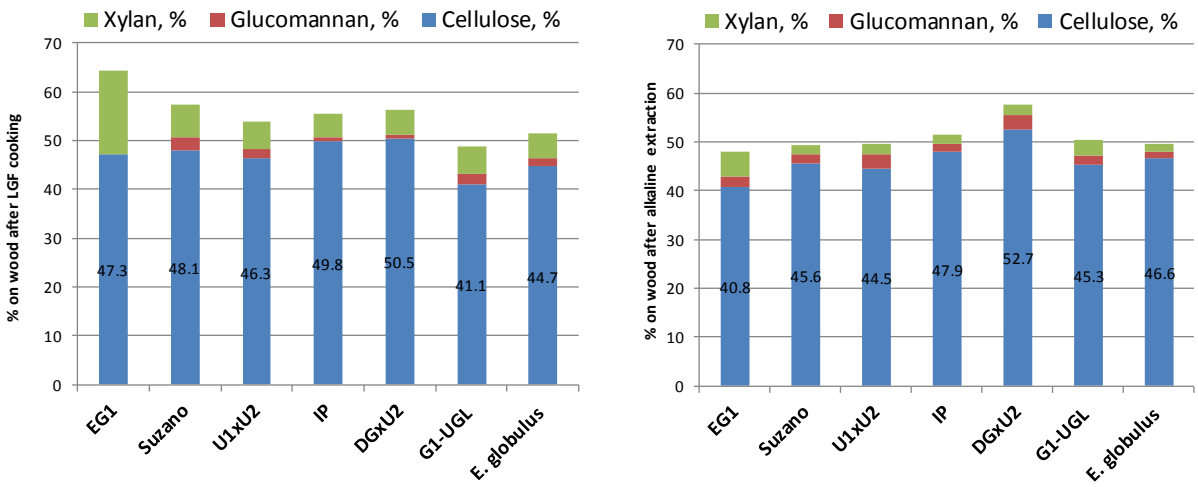


Figure 6. Polysaccharide contents after 20h LGF cooking (left) and alkaline extraction (left). Results scaled to the pulp total yields.

Conclusions of WP2

In optimised LGF cooking conditions (3.5% H_3PO_2, 15% water, 130˚C), sufficient delignification with higher polysaccharide yield could be obtained already during 20h cooking. To reach industrially applicable process, further reduction in LGF cooking time could be possible by impregnation, more efficient mixing/liquor circulation, higher temperature or using fresh chips. After 20h cooking and alkaline extraction, the DGxU2 clone with highest pulp yield seemed most potential raw material for bioethanol production.

References of WP2

Janson, J (1970). Calculation of the polysaccharide composition of wood and pulp, Pap.Puu, 52:5, 323-329.

3.1.2 Progress on WP3 Chemical and physical characterization of pretreated materials

Summary of the WP3

The main task of VTT in WP3 during the reporting period was to follow the structural changes in cellulose crystallinity after LGF and alkaline pretreatments by solid state NMR spectroscopy, and evaluate the effect of cellulose crystallinity on pulp hydrolysability. The produced LGF organosolv, as well as residual lignins of alkaline pulps and spent liquor lignins were also purified and characterised. Distribution of xylan on alkaline pulp fibers was evaluated by microscopy after the immunolabellig.

Task 3.2 Polysaccharide (cellulose and hemicellulose) analyses

Cellulose crystallinity by solid state NMR spectroscopy

The cellulose crystallinity (CrI) was determined from the areas of crystalline and amorphous C4 signals of ^{13}C CPMAS NMR spectra by deconvolution:

$$CrI = A_{86\text{-}92ppm} / (A_{79\text{-}86ppm} + A_{86\text{-}92ppm}) \cdot 100\%.$$

For pure cellulose the method gives comparable results with X-ray (Teeäär *et al.* 1987). However, in wood and pulp samples, also hemicelluloses and lignin side-chains contribute to the amorphous C4 signal, and the CrI values obtained rather describe the crystallinity of the whole pulp/wood. To determine the actual cellulose crystallinity, the interfering signals of other amorphous components must be removed either chemically or spectroscopically before determination of CrI. For spectroscopic removal of hemicelluloses and lignin from cellulose spectra, a proton spin-relaxation based spectral edition (PSRE) method was implemented at VTT, as described previously. In the PSRE method the differences in the proton spin-relaxation times ($T_{1\rho H}$) of crystalline cellulose and amorphous lignin and hemicelluloses are utilized to separate the components into subspectra of their own (Newman and Hemmingson 1990; Newman 1999, Liitiä et al 2003). From the cellulose subspectrum, the crystallinity of cellulose (CrI_{PSRE}) was determined without the interference of the less ordered pulp/wood components.

For NMR measurements the dry wood chips and elephant grass were wiley milled (2 mm screen), and re-wetted with deionised water (~50 wt %). The LGF pulp samples were equally pretreated. All the measurements were performed either with a Chemagnetics CMX 270 MHz or 400 MHz NMR spectrometer. The spinning speed was 5000 Hz with 270 MHz equipment and 8000 Hz at higher field of 400 MHz to improve the resolution. In all cases, the acquisition time was 20 ms, contact time 1 ms and delay between pulses 2 s. The delayed contact measurements were conducted with spin-lock times of t_{sl} =0 and 3-7 ms, and the subspectra of components were obtained by linear combination using intensities of crystalline cellulose C4 and lignin methoxyl signals at 89 and 56 ppm, respectively (Newman and Hemmingson 1990; Newman 1999). This way better linear combinations and cellulose subspectra could be obtained than using signal intensities at 89 and 80 ppm, as described earlier. The biomass crystallinity (CrI_{CPMAS}) was determined using the ordinary CPMAS spectra measured with t_{sl}=0 ms and the cellulose crystallinity (CrI_{PSRE}) was determined from the subspectra of cellulose.

Crystallinity of raw materials and corresponding LGF pulps

Crystallinities determined for the available raw materials before and after the LGF cooking and alkaline extraction are given in Table 1. Cellulose crystallinity in eucalyptus clones varied between 40-57 %, being highest in *G1xUGL* and *Suzano* clones. The cellulose crystallinity of elephant grass was clearly lower compared to eucalyptus, and remained lower also after the LGF cooking and alkaline extraction. During LGF cooking and alkaline extraction, the **pulp crystallinity** determined from the ordinary CPMAS spectra (CrI_{CPMAS}) increased when more amorphous lignin and xylan was removed. In most cases, also the **cellulose crystallinity** determined from the cellulose subspectra after spectral edition (CrI_{PSRE}) increased, as reported also for kraft pulps during cooking (Liitiä 2002). This is due to the removal of more amorphous cellulose, and also some cellulose ordering/aggregation, taking place when amorphous lignin and hemicelluloses are removed between the fibrils.

Table 1 The biomass crystallinity (CrI_{CPMAS}) determined using the ordinary CPMAS spectra and the cellulose crystallinity (CrI_{PSRE}) determined from the subspectra of cellulose.

	Crystallinity of raw materials		Crystallinity of LGF pulps after NaOH extraction	
Feedstock	**Wood CrI_{cpmas} %**	**Cellulose CrI_{PSRE} %**	**Pulp CrI_{cpmas} %**	**Cellulose CrI_{PSRE} %**
G1xUGL	37	57	45	56
Suzano	40	56	43	59
IP	37	49	43	54
U1XU2	33	40	-	-
DGXU2	33	42	48	50
E.Globulus	-	-	48	61
EG1	24	33	36	39

No clear correlation between the hydrolysability of the LGF pulps and crystallinity of the raw materials or LGF pulps could be detected. Although cellulose crystallinity would affect the cellulose hydrolysability, there are clearly also other factors, *e.g.* lignin and hemicellulose content, contributing more.

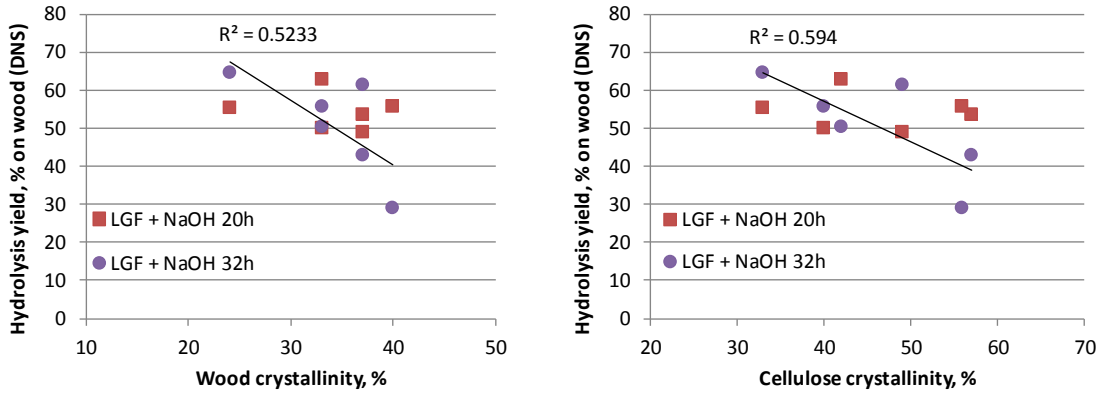


Figure 7. Correlation of wood (left) and wood cellulose (right) crystallinity with LGF pulp hydrolysability after 20 and 32 h LGF cooking followed by alkaline hydrolysis.

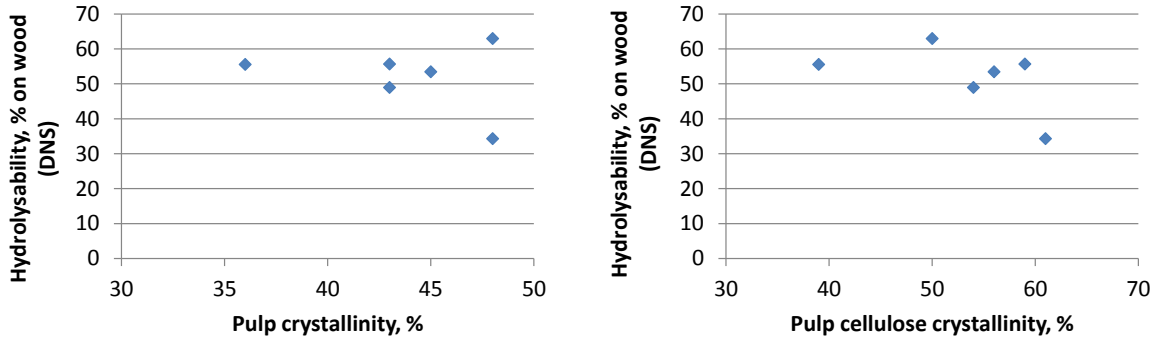


Figure 8. Correlation of LGF pulp (left) and pulp cellulose (right) crystallinity with the corresponding pulp hydrolysability after 20 h LGF cooking followed by alkaline hydrolysis.

Effect of alkaline cooking on cellulose crystallinity

Alkaline oxidation ($NaOH+O_2$) and soda-anthraquinone (Soda-AQ) treatments were studied as potential alkaline deconstruction methods for bioethanol production, and the effect of these alkaline treatments on cellulose crystallinity was also evaluated by solid state NMR spectroscopy. The alkaline treatments were performed in Suzano for elephant grass (*EG1*) and *G1xUGL* eucalyptus hybrid (kappa levels of 50, 30 and 15).

After alkaline deconstruction the cellulose crystallinity of elephant grass was clearly lower compared to eucalyptus pulps, as detected also with LGF pulps. With both raw materials, the **pulp crystallinity** determined from the ordinary CPMAS spectra (CrI_{CPMAS}) increased throughout cooking when more amorphous lignin and xylan were removed. There was increasing trend also in **cellulose crystallinity,** as detected with LGF pulps and reported also previously for kraft pulps (Liitiä 2002). No clear correlation between the crystallinity of alkaline pulps and their hydrolysability determined at WP4 could be detected, as was reported also for the LGF pulps.

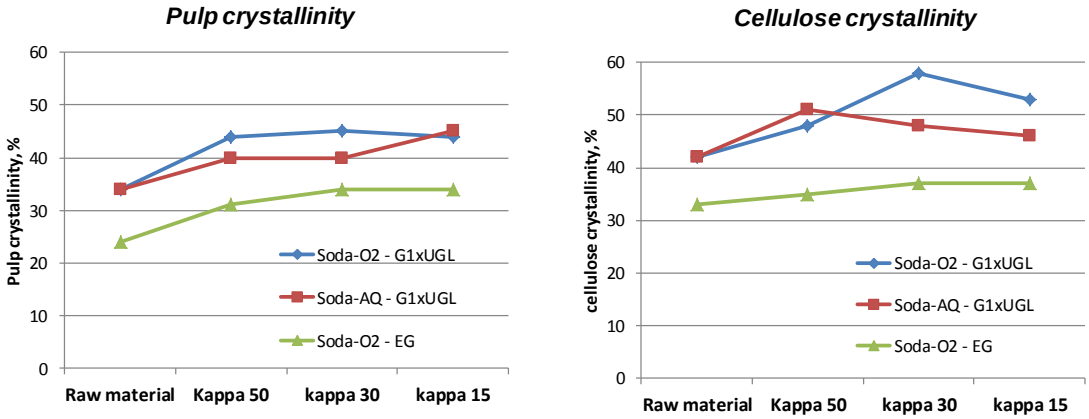


Figure 9. Pulp and cellulose crystallinities determined for alkaline pulps of elephant grass and G1xUGL clone.

Distribution of precipitated EG xylan on alkaline Eucalyptus pulp fibres

Immunolabelling microscopy was used for surface mapping of xylan on alkaline *Eucalyptus* pulp fibres after precipitation of elephant grass (EG) xylan. Two pulps with reprecipitated xylan loadings, and a corresponding reference was studied. The reprecipitated xylan was isolated using alkali charges of 400kg/t NaOH and 700kg/t NaOH. Labelling of the samples was carried out as previously reported and described (Lappalainen et al, 2004). A primary antibody was used, which recognises an epitope consisting of a short xylooligosaccharidic stretch carrying a 4-*O*-methyl-glucuronic acid residue (MeGlcA-Xyl_{2-3}). Previously, in the project the antibody was shown to recognise the xylan both on eucalyptus and elephant grass fibres.

Images on the labelled samples are shown in Figures 10-12. In general, xylan specific antibody was successfully used for labelling of xylan on *Eucalyptus* fibres. It is noteworthy that the method does not discriminate between the native and re-precipitated EG xylan and therefore in the image contribution of both components are merged.

On the untreated reference sample moderate labelling of fibres was observed (Fig. 10). Labelling appeared unevenly as faint and brighter patches on fibre surfaces. Especially, on damaged fibres, fibrillated fines and around pits more extensive labelling was observed. This is undoubtedly due to increased accessibility of xylan at kinks and dislocations and high surface area of fines for recognition by the rather large immunoglobulin molecules.

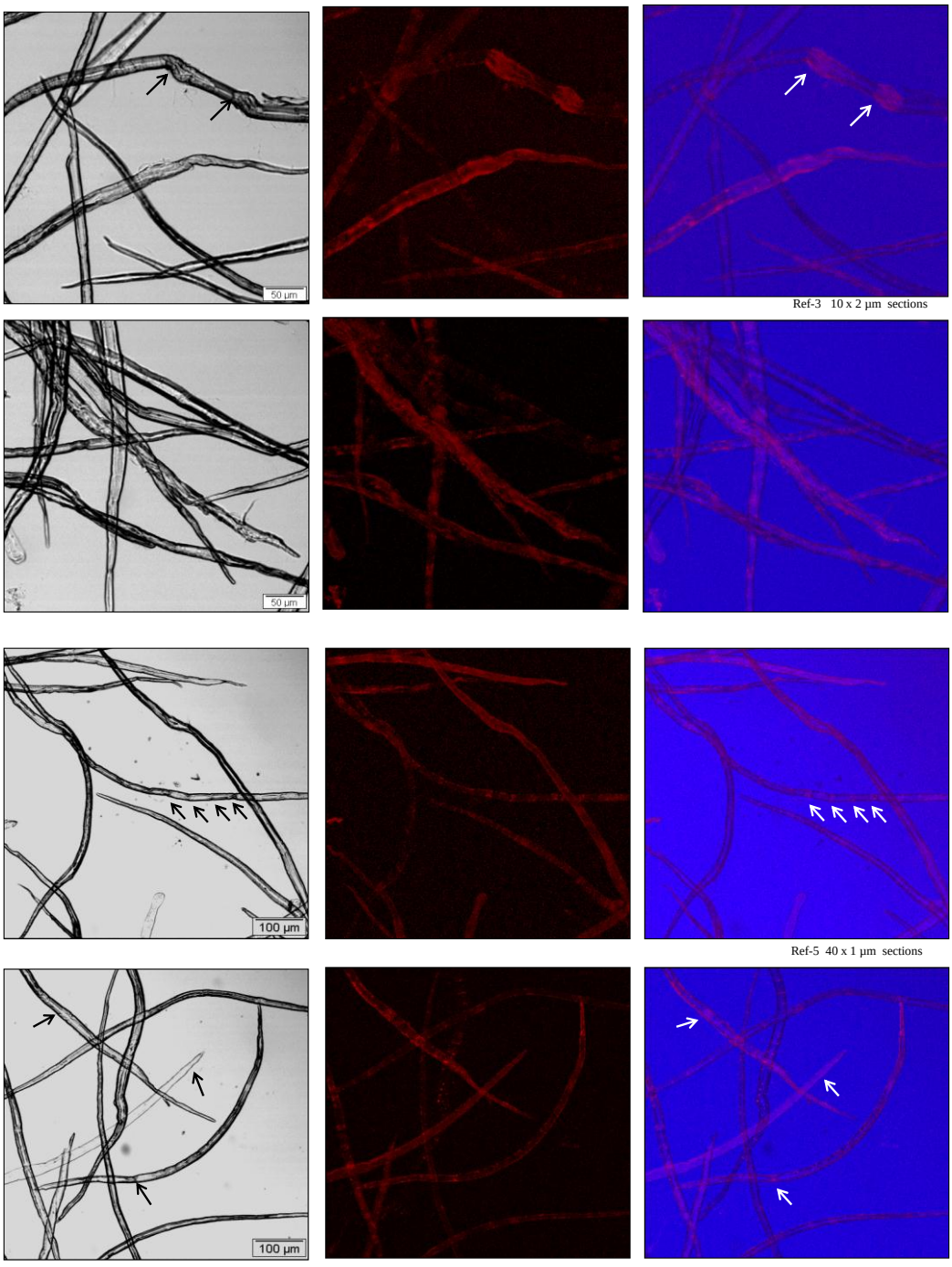


Fig 10. Immunolabelling of xylan on the untreated reference Eucalyptus fibres

Increased labelling of xylan was detected on the samples with precipitated EG xylan as compared with the reference (Figs. 11-12). Overall labelling of bulk fibres was increased and especially fine fibrils and damaged fibres were subjected to dense labelling. Some fibres were very evenly and heavily labelled. However, high background labeling of the 700kg/t NaOH sample indicated desorption of xylan (or antibodies) during preparation of the samples. No unspecific binding of the secondary antibody was detected with the labelling procedure used, *i.e.* the labelling was specific to xylan.

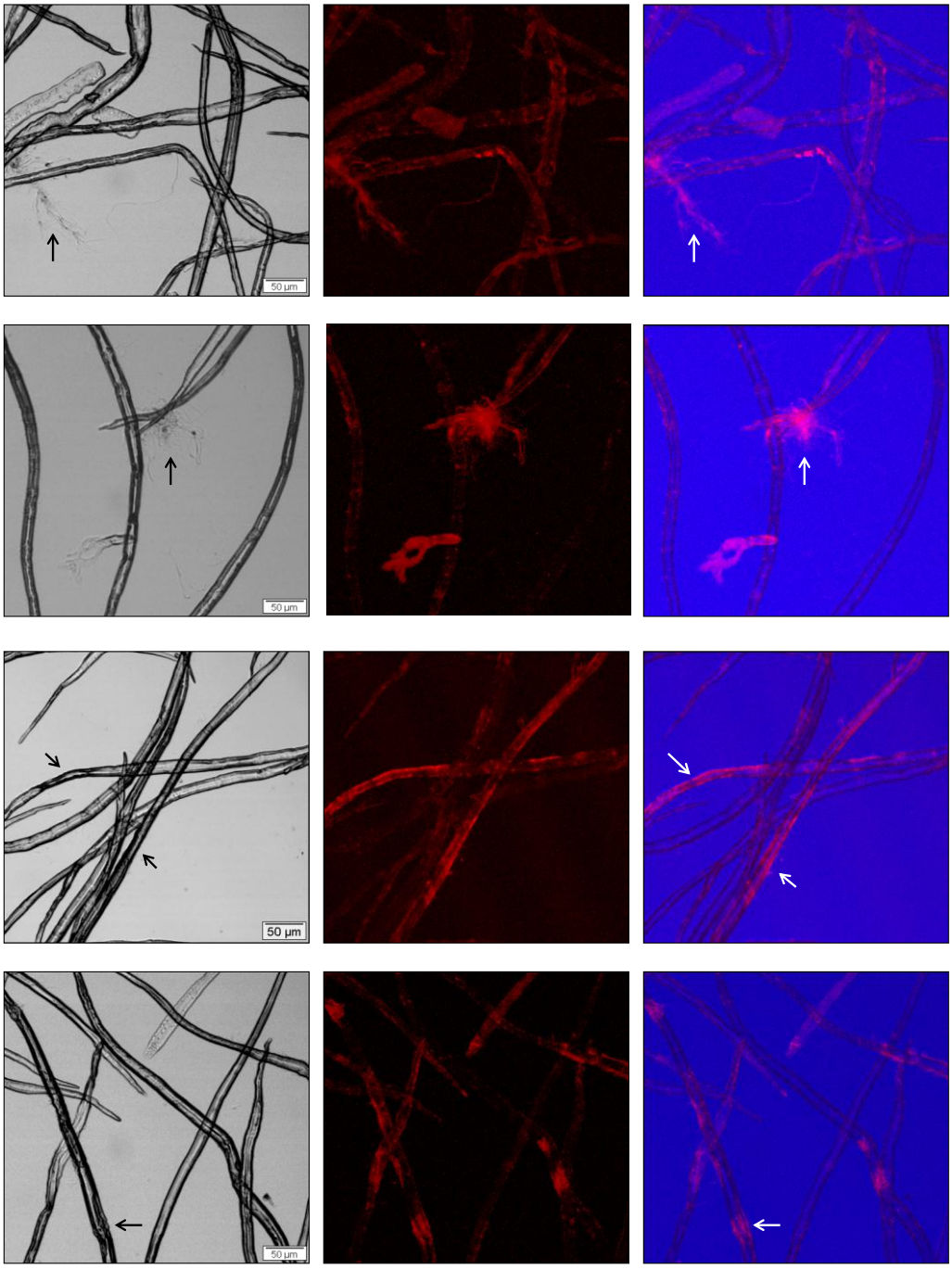


Fig 11. Immunolabelling of xylan on Eucalyptus fibres treated with 400 kg/t NaOH.

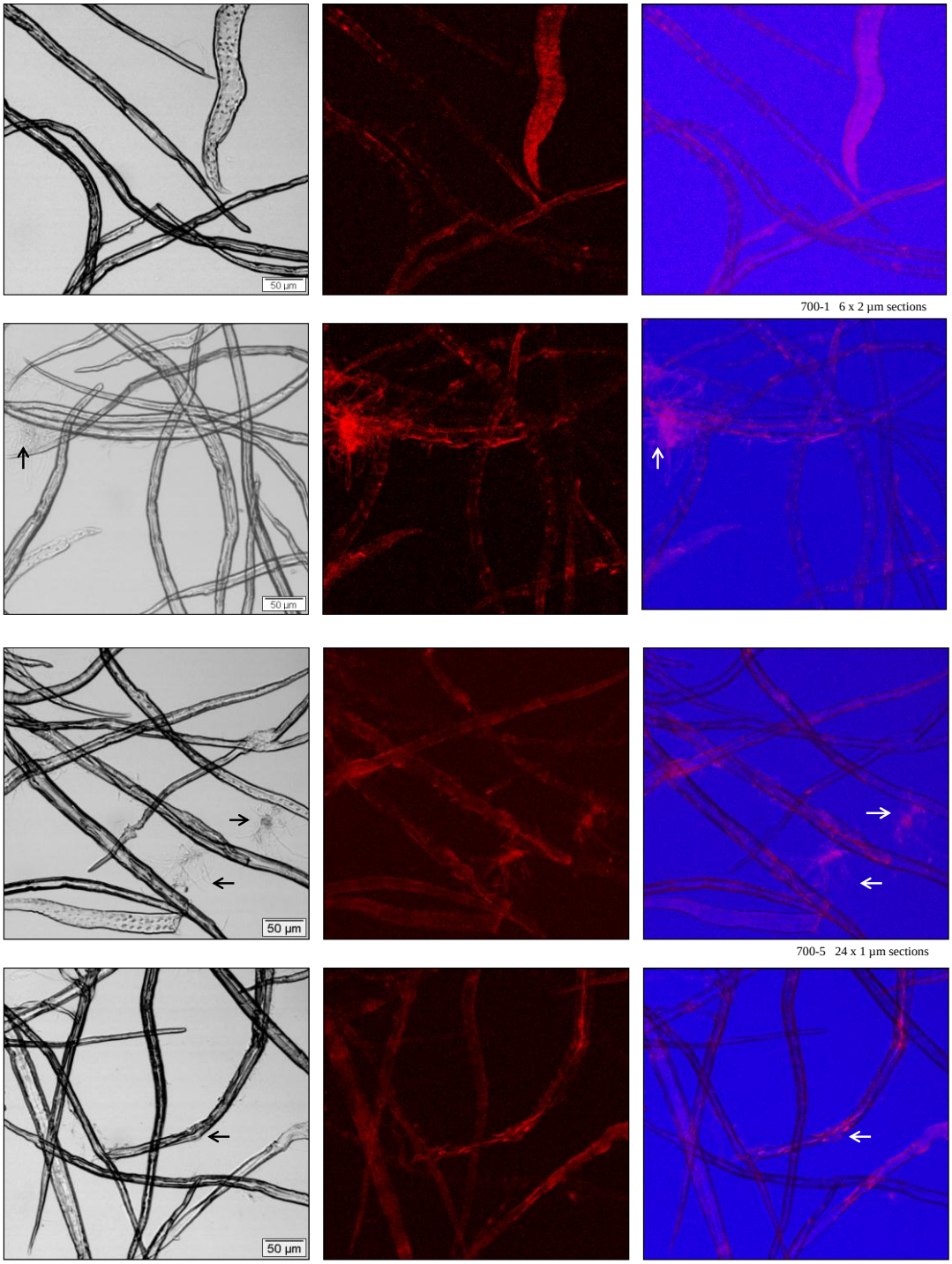


Fig 12. Immunolabelling of xylan on Eucalyptus fibres treated with 700 kg/t NaOH.

Task 3.3 Analysis of lignin and minor components

Effect of LGF cooking conditions on lignin structure

The spent liquor lignins (SLL) produced in LGF organosolv cooking experiments were characterized to better understand the delignification mechanisms, and also the utilization potential of the by-product lignins.

- Effect of phosphinic acid (PA) charge (0, 1.5, 3.5, 5, 15%) was evaluated with lignins originating from LGF cooking of G1xUGL clone (130°C, 15 % water content, 48h)
- Effect of cooking time (10, 16, 20, 32 h) was evaluated with lignins originating from LGF cooking of Suzano clone (3.5% PA, 130°C, 15 % water content)
- Effect of raw material was evaluated with lignins originating from LGF cooking of *EG, E.globulus, Suzano, G1xUGL, U1xU2, IP, and DGxU2* clones (3.5% PA, 130°C, 15 % water content, 20h)

Experimental

To minimize the scattering caused by impurities in analysis, the lignins were purified by dissolving 0.5g SLL into 35 ml 0.1M NaOH for 30 min at room temperature. After that the samples were filtered (Whatman no 4) to remove the insoluble impurities, and washed through the filters with 15 ml 0.1 M NaOH. The lignins were precipitated by acidification with 1M HCl to pH 2.4. The samples were centrifuged (25 min, 9000 rpm, 4°C) and washed with acidified milliQ water (pH 2.4) two times. Finally, the samples were freeze dryed and extractives removed by Soxhlet extraction (8h) using hexane.

The molar mass distributions and average molar masses (Mn, Mw) were determined by Size Exclusion Chromatography (SEC) which in this case is a relative method. The SEC measurements were performed by Waters HPLC in 0.1M NaOH eluent using PSS's MCX 1000 and 100 000 Å columns with UV detection at 280 nm. The average molar masses (Mn, Mw) were calculated relative to the polystyrene sulphonate (Na-PSS) standards using Waters Empower 2 software. For SEC analysis, lignins were dissolved overnight in 0.1M NaOH (1 mg/ml) and filtered with 0.45 μm PTFE membrane.

Lignin functionalities (phenolic and aliphatic hydroxyls and COOH groups) were analysed by ^{31}P NMR spectroscopy. For the ^{31}P NMR measurements, the phosphitylation of dry lignin samples was performed according to Granata and Argyropoulos (1995). The ^{31}P-NMR spectra were recorded on a Bruker Avance III 500 MHz spectrometer using a 90º pulse width and an inverse gated decoupling sequence. $CDCl_3$ was used as a locking solvent and 512 scans were accumulated with a delay time of 5 s between pulses. For processing data, the chemical shifts were calibrated to the sharp signal (132.2 ppm) of the reaction product between water and phosphitylation reagent. After establishing an optimal deconvolution parameters used for all samples, the deconvolutions of the syringylic - and condensed hydroxyl group signals were processed with a line broadening factor of 8 Hz. At the 144.2 – 141.ppm region, 12 and 20 peaks were used to obtain an exact deconvolution results (Bruker topspin software).

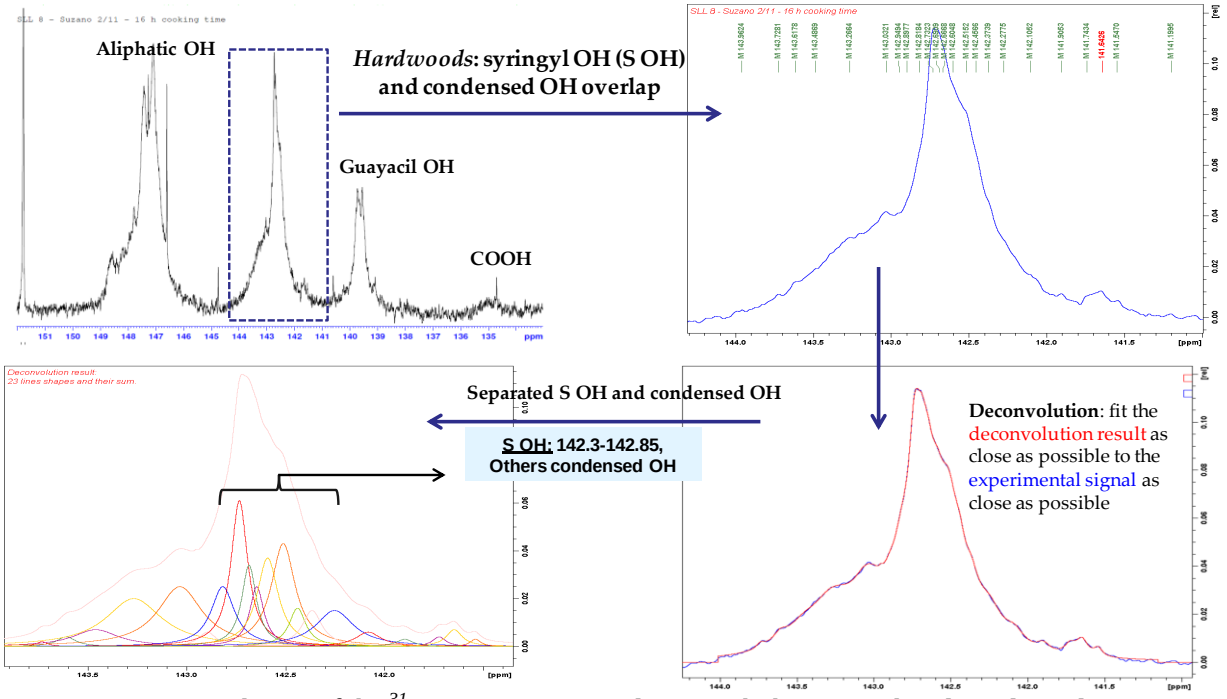


Figure 13. Deconvolution of the ^{31}P NMR spectra to distinguish the syringyl and condenced G units overlapping at 144.2 – 141.ppm region.

Structural features of LGF spent liquor lignins

Table 2. Average molar masses of LGF spent liquor lignins (SEC in 0.1M NaOH, UV-detection, Na-PSS standards).

Raw material	H_3PO_2, %	Cooking time, h	Mn, g/mol	Mw, g/mol	Polydispersity
G1*UGL	0	48	1900	2900	1,50
	1,5	48	2200	3400	1,54
	3,5	48	**2400**	**3700**	1,58
	5	48	2200	3600	1,62
	15	48	2000	3100	1,53
Suzano	3,5	10	2000	3300	1,53
	3,5	16	2100	3600	1,72
	3,5	20	2100	3500	1,67
	3,5	32	**2100**	**3400**	1,62
EG	3,5	20	**1400**	**2200**	1,55
E. Glob.	3,5	20	**2100**	**3500**	1,66
Suzano	3,5	20	2100	3500	1,67
G1*UGL	3,5	20	2200	3700	1,67
U1*U2	3,5	20	2200	3600	1,64
IP	3,5	20	2100	3300	1,63
DG*U2	3,5	20	2200	3700	1,65
Euc. Glob., pilot plant, not purified	3,5	20	**2000**	**3000**	1,52
Euc. Glob.	3,5	20	**2100**	**3200**	1,51

The molar mass results given in Table 2 show that small molecular easily soluble lignin fraction was dissolved in LGF cooking when no PA was used. The molar mass of dissolved lignin increased with increasing PA charge, reaching maximum with 3.5-5% PA charges. The same amount of PA was previously determined optimal for cooking efficiency in respect of pulp hydrolysability. With higher PA charges, the dissolved lignin was more extensively degraded. Cooking time or eucalyptus clone had no significant effect on lignin molar mass. Only for the EG lignin clearly lower Mw was detected. The

lower molar mass of pilot *E.globulus* lignin compared to the corresponding lab-scale lignin supports more efficient delignification process at pilot scale, as discussed in WP5.

As shown in Figures 14 and 15, the content of aliphatic hydroxyl groups reduced and proportion of phenolic hydroxyls increased as a function of cooking time and phosphinic acid charge. Without any added PA, more phenolic small molecular lignin was dissolved.
Also the S/G ratio of SSLs increased as cooking proceeded or higher PA charges were used. In both cases, the proportion of S-OH increased continously, whereas the content of G-OH was relatively stable. Interestingly, guaiacyl rich lignin was dissolved when no PA was used in LGF cooking. The proportion of condensed structures also increased with prolonged cooking times and increasing PA charge.

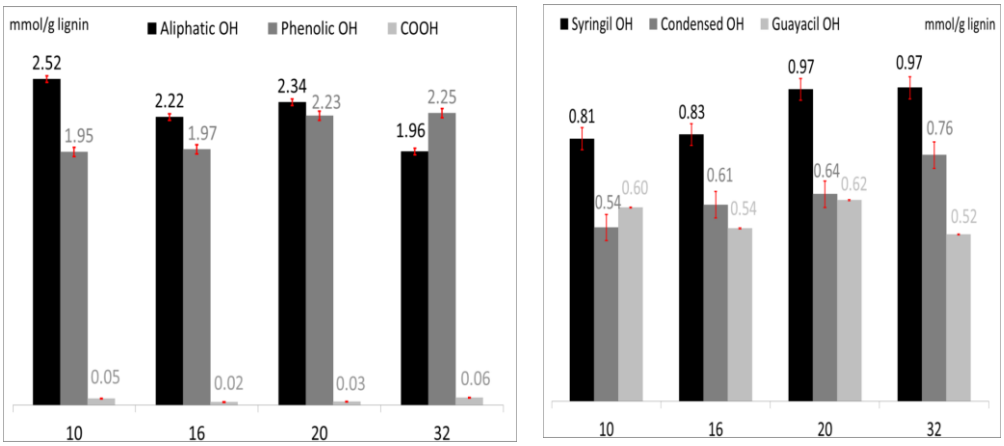


Figure 14. Effect of cooking time (h) on lignin functionalities (Suzano clone, 3.5% PA, 130°C, 15% water content).

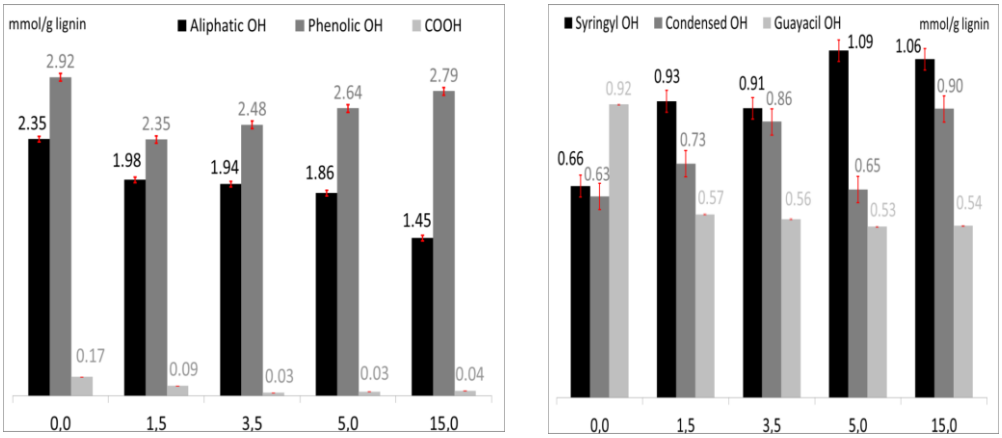


Figure 15. Effect of phosphinic acid charge (%) on lignin functionalities (G1xUGL clone, 130°C, 15% water content, 48h).

In comparison of raw materials, the SLL of *E. globulus* was most phenolic, whereas the *IP* and *EG* SLLs were least phenolic. Also the S/G ratio was highest in *E.globulus* SLL, whereas the proportion of condenced OH was lowest in *EG* and *IP*. This correlates well with the delignification efficiency.

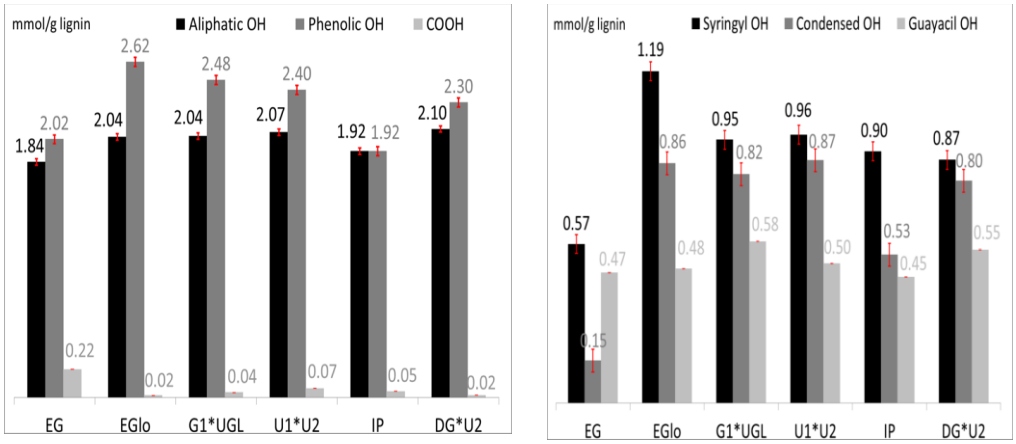


Figure 16. Comparison of lignin functionalities in SSLs of differenct raw materials (3.5% PA,130°C, 15% water content, 20h).

Interestingly, a previously unidentified lignin signals (A and B) were detected in most of the SLLs (Figure 17). The both signals most likely originate from the same structure, as their signal intensities correlated linearly. The identification of the structure is on-going.

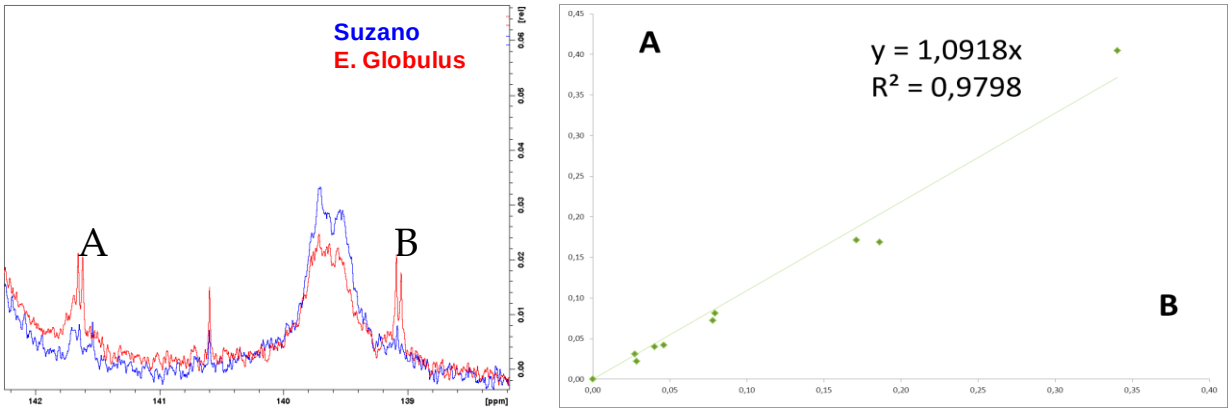


Figure 17. Previously unidentified lignin signals (A,B) and correlation of their signal intensities.

Effect of alkaline cooking on lignin structure

Pulp residual and spent liquor lignins of eucalyptus clone *G1xUGL* and elephant grass were isolated at IRNAS after alkaline cooking (kraft, Soda-AQ, Soda-O2) to kappa levels 50, 35, 20 and 15. The lignins were analysed at VTT during student exchange of Pepijn Prinsen/IRNAS.

The lignin functionalities were analysed by ^{31}P NMR spectroscopy as reported above. During alkaline cooking the content of aliphatic OH groups decreased, whereas the proportion of phenolic units increased mainly due to the cleavage of β-O-4 linkages. As expected, in spent liquor lignins the changes were higher compared to the corresponding pulp residual lignins. At high kappa levels, the decrease of aliphatic hydroxyls (Figure 18) and increase of phenolic units (Figure 19) was most efficient with Soda-O2 and lowest with kraft cooking. At kappa level 15, the content of aliphatic hydroxyl groups of residual lignins was quite same after all the studied alkaline cooking methods, whereas the phenolic content of Soda-O_2 residual lignin remains lower compared to the others. This is expected as the oxidation proceed via phenolic lignin units. This may affect the pulp reactivity in the following bleaching stages if paper pulp is produced, although the pretreatment was mainly tested for bioethanol production purposes. Also the Figure 20 shows, that the removal of aliphatic OH was higher than the formation of new phenolic units at lower kappa levels of Soda-O_2 cooking.

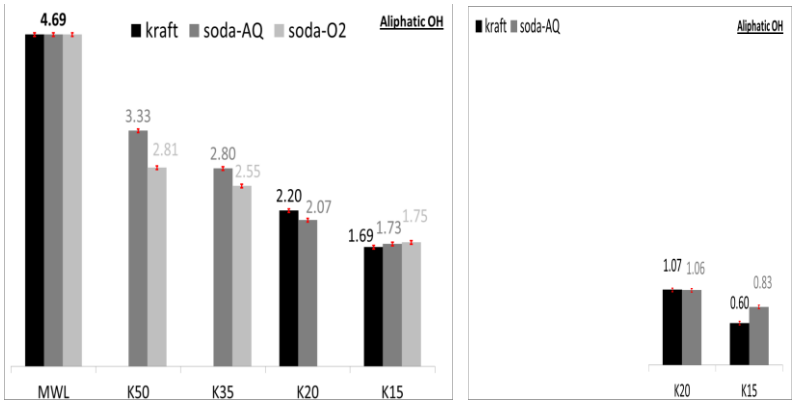


Figure 18. Effect of alkaline cooking on aliphatic hydroxyl content in pulp residual lignin (left) and spent liquor lignin (right).

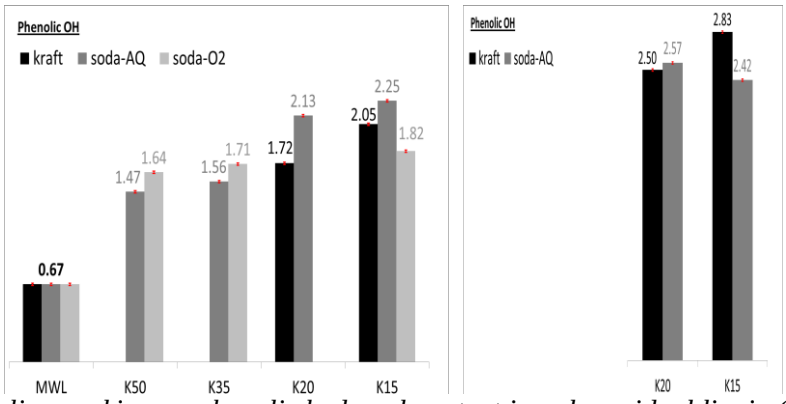


Figure 19. Effect of alkaline cooking on phenolic hydroxyl content in pulp residual lignin (left) and spent liquor lignin (right).

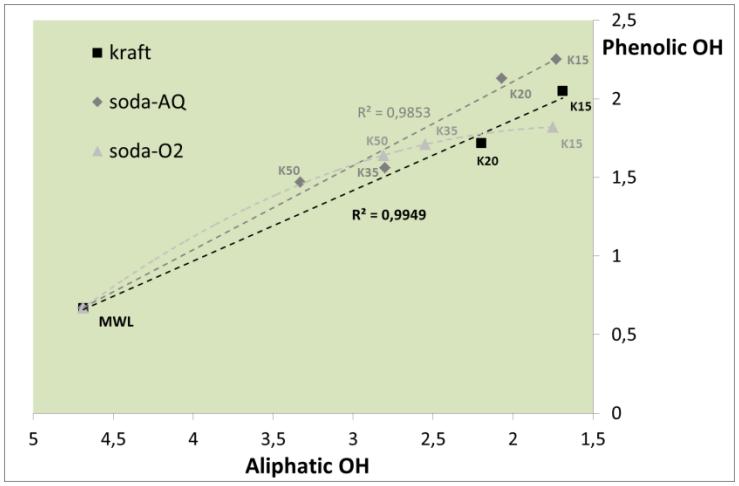


Figure 20. Correlation between phenolic hydroxyl groups formed and aliphatic hydroxyls removed.

The content of phenolic syringyl units increased in residual lignins during kraft and Soda-AQ cooks, but during Soda-O2 cooking remained rather constant at kappa levels 50-15 (Figure 21). The content of reactive phenolic syringyl units was higher in Soda-AQ pulp lignin compared to others. The changes in phenolic guaiacylic groups were lower, remaining relatively constant in pulp residual lignin throughout the alkaline cooks (Figure 21).

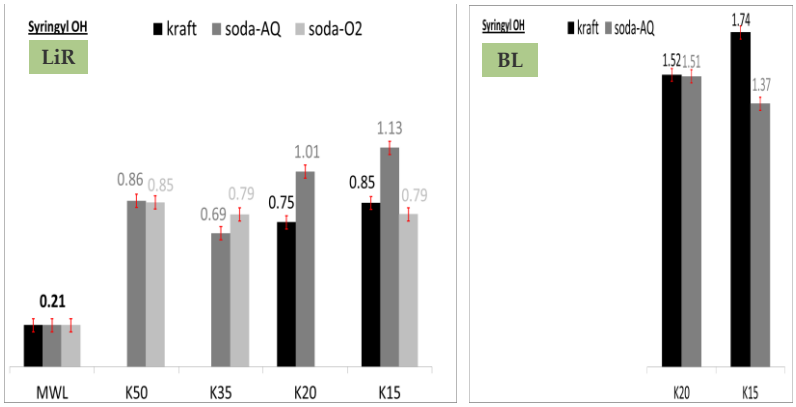


Figure 21. Effect of alkaline cooking on phenolic syringyl units in pulp residual lignin (left) and spent liquor lignin (right).

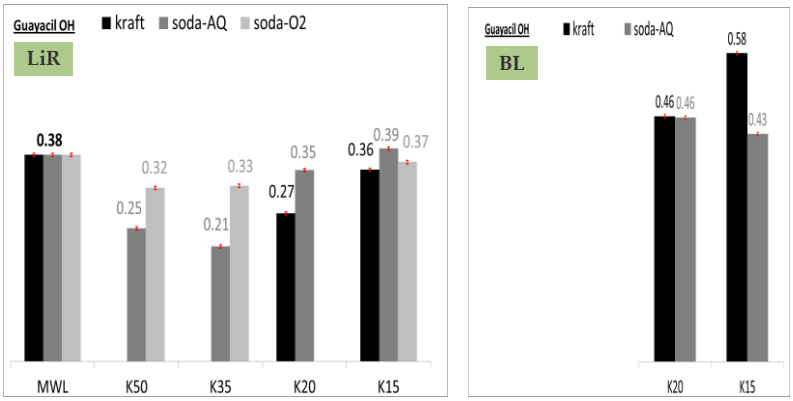


Figure 22. Effect of alkaline cooking on phenolic guaiacyl units in pulp residual lignin (left) and spent liquor lignin (right).

The structure of lignin became more condensed during all the alkaline cooks (Figure 23). In residual lignin, the condensation is most extensive at the end of kraft cooking (kappa 15). After the Soda-O2 cooking the residual lignin was least condensed, which may also be related to the lower phenol content in general. In spent liquor lignins, an opposite order was observed between the cooking methods. Also the content of carboxylic groups increased, being higher in black liquor lignins compared to the residual lignins (Figure 24).

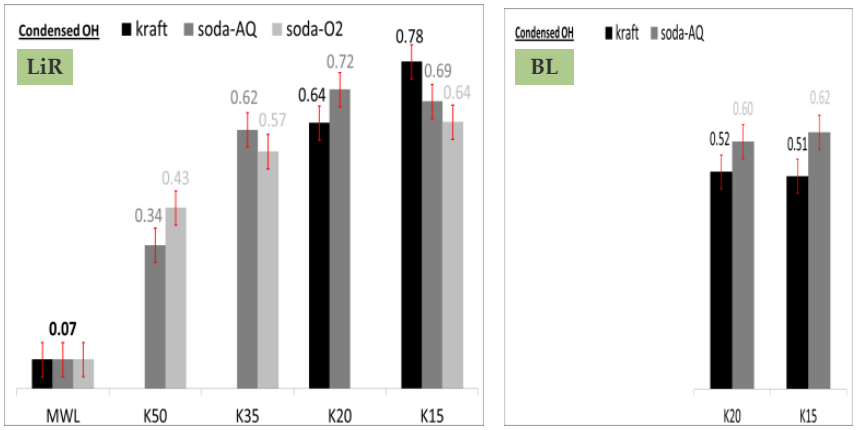


Figure 23. Effect of alkaline cooking on condensed phenolic units in pulp residual lignin (left) and spent liquor lignin (right).

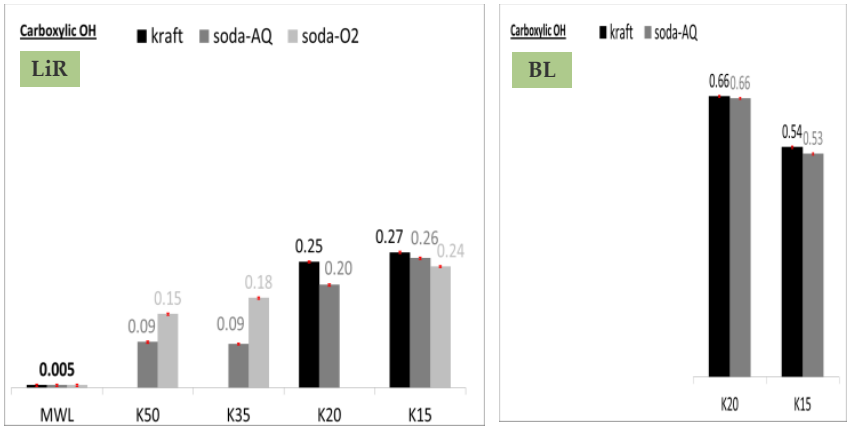


Figure 24. Effect of alkaline cooking on carboxylic acids in pulp residual lignin (left) and spent liquor lignin (right).

Unlike in MWL lignins, also in residual lignins and spent liquor lignins of the alkaline pretreatments, the A (141.65 - 141.61 ppm) and B (139.05 – 139.01 ppm) signals of a novel unidentified lignin structure were detected. Confirmation of the detailed structure is still on-going, but tentative identification suggests condensed catechol structure.

The molar mass of the alkaline lignins was determined by SEC as described above. The molar mass of residual lignin decreased with prolonged cooking, and as expected the molar mass of dissolved lignin was lower compared to the residual lignin remaining in pulp. The decreasing molar mass of Soda-AQ pulp residual lignin correlates well with the cleavage of β-O-4 linkages. Molar mass (Mw) of Soda-AQ pulp residual lignin was highest, whereas the Mw of lignin remaining in kraft pulps at the same kappa level was the lowest.

Table 3. The average molar mass(Mn, Mw) and polydispersity of alkaline lignins.

G1xUGL	**Mn (g/mol)**	**Mw (g/mol)**	**PD**
MWL	1900	4400	2.34
Residual lignins			
Kraft			
Kappa 20	2400	4300	1.78
Kappa 15	2400	3500	1.47
Soda-AQ			
Kappa 50	2500	10600	4.25
Kappa 35	2500	8300	3.30
Kappa 20	2700	4800	1.76
Kappa 15	2700	4200	1.54
Soda-O2			
Kappa 50	2400	9400	3.93
Kappa 35	2400	6100	2.58
Kappa 15	2300	3600	1.58
Black liquors			
Kraft			
Kappa 20	1700	2400	1.42
Kappa 15	1600	2300	1.39
Soda-AQ			
Kappa20	1700	2500	1.53
Kappa 15	1600	2300	1.48

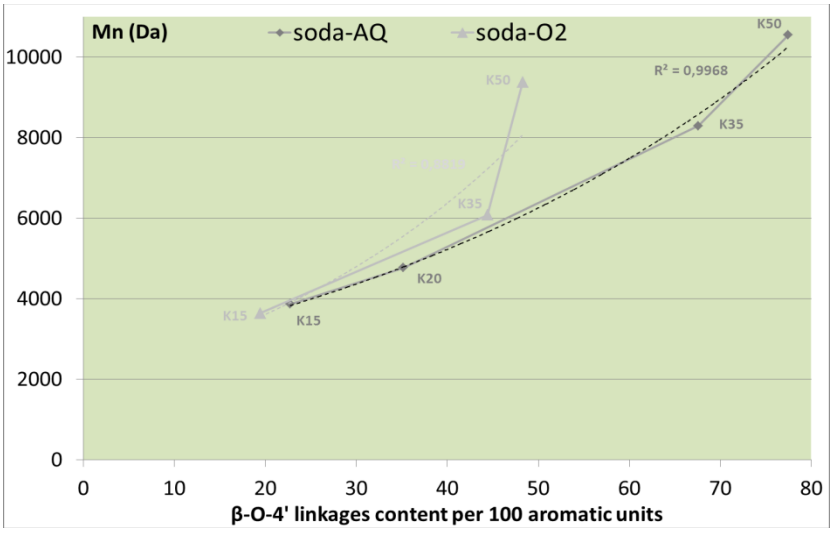


Figure 25. Correlation between residual lignin molar mass (Mw) and the content of β-O-4 linkages.

Conclusions of WP3

Cellulose crystallinity was not shown to correlate with hydrolysability of LGF and alkaline pulps. Other factors, *e.g.* lignin and xylan content seem to affect more. Structural changes in lignin revealed that also in LGF cooking the syringyl type units are more reactive, and condensation increases during cooking. PA is essential in these reactions. In alkaline cooks, the residual lignin of Soda-O2 pulp was less phenolic, which may restrict the reactivity in following bleaching stages if aimed at paper pulp. Also in this respect the method is more suitable for the bioethanol production. Most phenolic syringyl units were formed in Soda-AQ cooking, suggesting higher reactivity of the pulp lignin. In all the alkaline cooks, the condensation reactions were most extensive at the end of the cook.

The surface distribution of xylan was not even in the fibers of alkaline pulp, and higher xylan contents could be detected by labelling especially in damaged fibres, fibrillated fines and around pits. After reprecipitation of EG xylan, the overall labelling of bulk fibres was increased, and especially in fine fibrils and fibre defects. Some fibres were very evenly and heavily labelled.

References of WP3

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3.1.3 Progres on WP4. Tie between pre-treatment and industrial use of lignocellulosics

Summary on WP4

In WP4 VTT evaluated the suitability of the pre-treated woody and non-woody lignocellulosic materials produced in WP2 and WP5 as raw materials for bioethanol production. During the reporting period of 19-36 months

- The optimal LGF organosolv cooking time for bioethanol production was selected according to the enzymatic hydrolysability of LGF pulps.
- The bioethanol production potential of selected feedstocks after LGF cooking was evaluated based on enzymatic hydrolysability.
- Also the potential of other biomass pretreatment methods (alkaline deconstruction and enzymatic treatments) for bioethanol production was evaluated, and compared with LGF organosolv deconstruction.

Task 4.2 Biofuel potential of pre-treated materials and residues

Methods

In most cases, the enzymatic hydrolysability was evaluated according to sugar release (DNS). The washed pulp samples were suspended into 100 mM sodium citrate buffer, pH 5, 45ºC temperature at 1% consistency. Enzymatic hydrolysis was started by adding commercial Novozyme's cellulase mixture (Celluclast 1.5 FP) at the dosage of 10 FPU/g dry weight and β-glucosidase (Novozym 188) at the dosage of 500 nkat/g dry weight. The suspensions were incubated at 45ºC with magnetic stirring for 72 hours, and the content of released sugars was followed as a function of time. The 2,4-dinitrosalicylic acid (DNS) assay was used for the determination of reducing sugars. In some cases, the glucose, xylose, mannose and galactose concentrations were analysed from the supernatant by HPLC (without additional acid hydrolysis). The Aminex HPX-87H column (Bio Rad) was used with 0.6 ml/min flow of 5 mM H_2SO_4 as an eluent. To improve the separation of the monosaccharides, HPLC Fast Acid Analysis Column (Bio Rad) was used before Aminex HPX-87H column. In order to remove impurities from the samples, Cation-H Refill Cartridges (Bio Rad) was added as a pre-column.

Fermentations were carried out in erlenmeyer flasks (25 ml) in an incubator at 10% consistency. Same enzyme loading was used as in hydrolysis experiments (100 FPU/g Celluclast 1.5L and 500 nkat/g Novozyme 188). After 6h prehydrolysis at 45°C, the yeast (Red Star) was added with an OD_{600} of 3.5 ≈1g/l to the flasks to start the SSF and the temperature was lowered to 30°C with slow shaking (100rpm). Theoretical yield in fermentation is 0.51g ethanol/g glucose.

Selection of optimal LGF cooking time in respect of bioethanol production

As suggested on the basis of the chemical compositions (WP2), the shorter cooking times of 16-20h were sufficient for enzymatic hydrolysis of eucalyptus. Already during 16-20h cooking, the fiber structure was opened up and delignified enough for efficient hydrolysis (Figure 26), and highest proportion of the original feedstock could be released as sugars. The same was detected with both *G1xUGL* and *Suzano* clones. Cooking time of 20h was thus selected for the pilot demonstration performed in WP5.

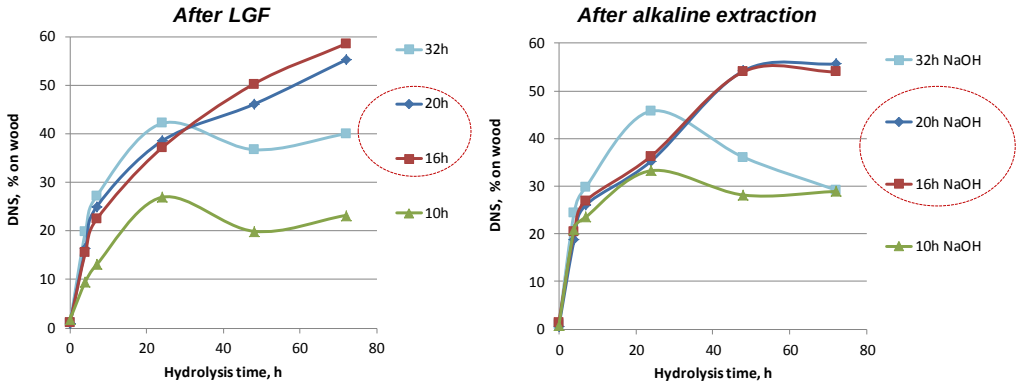


Figure 26. Effect of cooking time on sugar release in enzymatic hydrolysis of organosolv pulps (3.5% H_3PO_2, 130°C, 15% water content) for Suzano's eucalyptus clone. The results are corrected with pulp yields and shown as % on original wood raw material.

Bioethanol production potential of selected feedstocks after LGF cooking and alkaline extraction

As reported previously, the alkaline extraction improves LGF pulp hydrolysability because of enhanced lignin and xylan removal. In most cases, the hydrolysis time of 48h was required for the maximum sugar release. After LGF cooking and alkaline extraction, the *DGxU2* hybrid gave highest sugar release as could be expected based on highest cellulose yield reported in WP2. Despite the relatively good cellulose yield and better delignification efficiency compared to the other eucalyptus clones, the *E.globulus* unexpectedly gave lowest sugar release. As reported previously, the hydrolysis yield is to some extent dependent on cooking time, and optimal cooking time may vary between the clones. For *E.globulus* with lower initial lignin content the 20h cooking time may have been too long. No actual fermentation trials were performed for the LGF lab scale pulps.

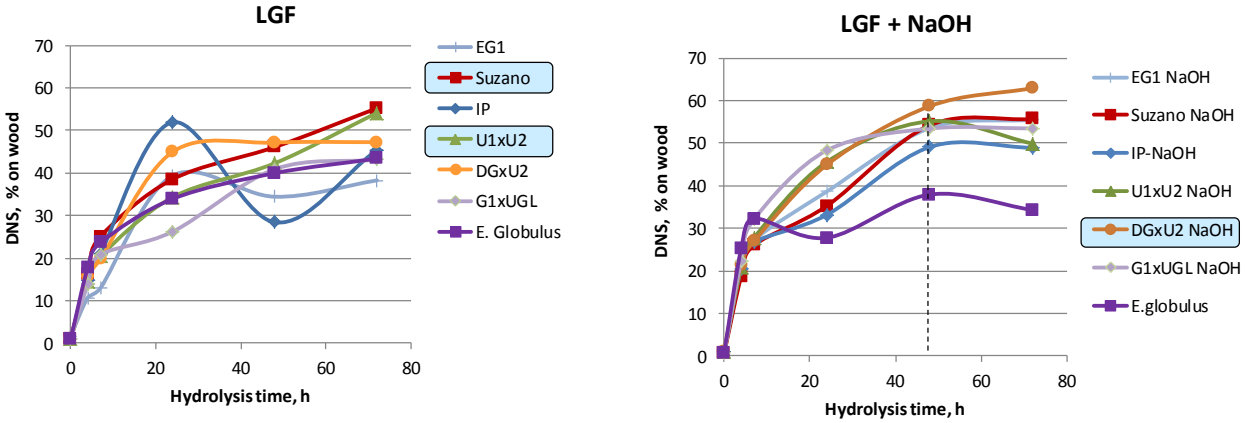


Figure 27. Effect of feedstock on sugar release in enzymatic hydrolysis of organosolv pulps (3.5% H_3PO_2, 130°C, 15% water content, 20h).The results are corrected with pulp yields and shown as % on original wood raw material .

Comparison of pretreatment methods

Effect of alkaline deconstruction on hydrolysis and fermentation efficiency

Alkaline oxidation (NaOH-O_2) and soda-anthraquinone (Soda-AQ) treatments were tested as potential alkaline deconstruction methods for bioethanol production. The alkaline treatments were performed in Suzano for elephant grass (EG1) and *G1xUGL* eucalyptus hybrid. In all cases, pulps at kappa levels 50, 30 and 15 were prepared.

In all cases, the *G1xUGL* eucalyptus provided higher ethanol yield than elephant grass. Cooking to low kappa level of 15 was necessary with both alkaline cooking methods (Soda-AQ, NaOH-O_2) to provide reasonable ethanol production with *EG*. With the eucalyptus, the NaOH-O_2 pretreatment was more suitable for bioethanol production than Soda-AQ. The Soda-AQ treatment required low kappa levels of

15, whereas after NaOH-O_2 treatment kappa levels of 35-50 provided well hydrolysable pulp for fermentation. The NaOH-O_2 treatment probably opens up the fiber ultrastructure better at the same lignin content, and even at high kappa levels the reject can be hydrolysed more efficiently.

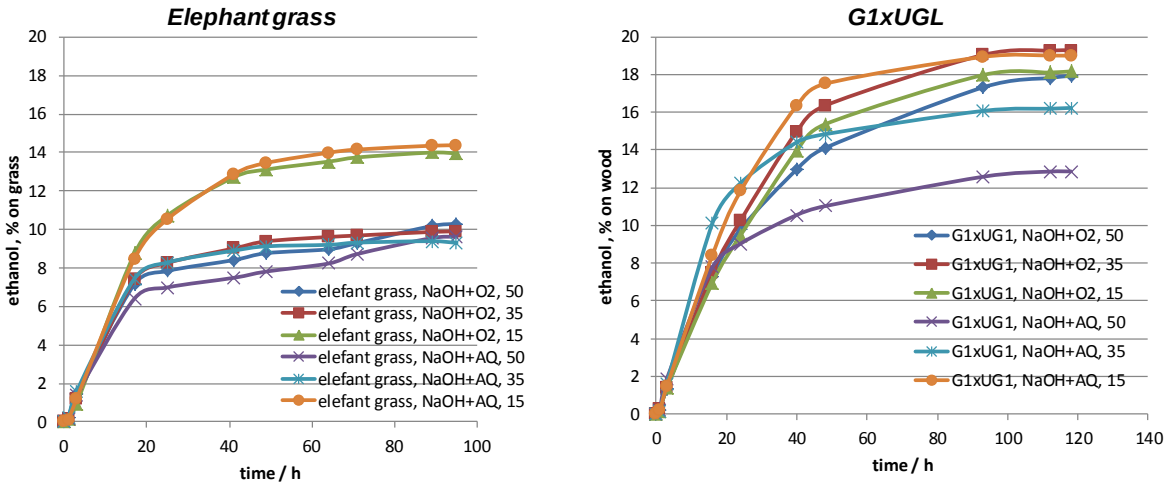


Figure 28. Effect of alkaline pretreatments (NaOH+O_2, Soda-AQ) on ethanol production from EG and G1xUGL pulps at kappa levels of 50, 30 and 15. The results are corrected with pulp yields and shown as % on original wood raw material.

Effect of pressafiner and hydrolase pretreatment on bioethanol production

Hydrolytic enzymes and laccase, were tested in CSIC and IRNAS for deconstruction of lignocellulosics for bioethanol production. After screening at lab scale, the elephant grass was treated in CTP by pressafiner (pilot scale) followed by hydrolytic enzyme treatment. After this pretreatment, only very limited ethanol production was detected unlike at lab scale. This is probably due to the insufficient biomass deconstruction and thereby hindered hydrolysability. As shown in Figure 30, the pressafiner had no significant mechanical crushing effect on EG. In previous lab scale experiments, the samples were ball milled, and higher ethanol yield was reported even for the reference without any enzymatic pretreatment (Fig. 31).

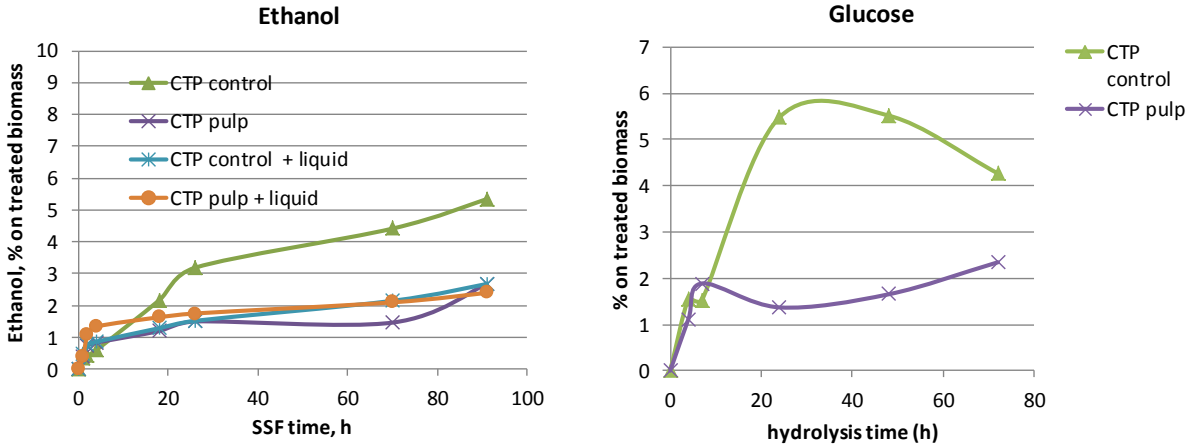


Figure 29. Effect of pressafiner and hydrolytic enzyme pretreatment on ethanol production from elephant grass.

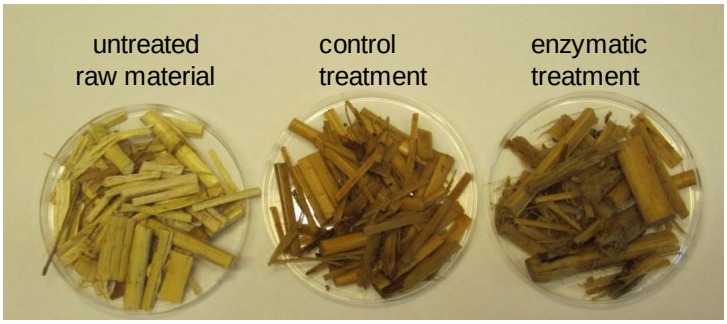


Figure 30. Effect of pressafiner (control) and following hydrolytic enzyme pretreatment on deconstruction of elephant grass.

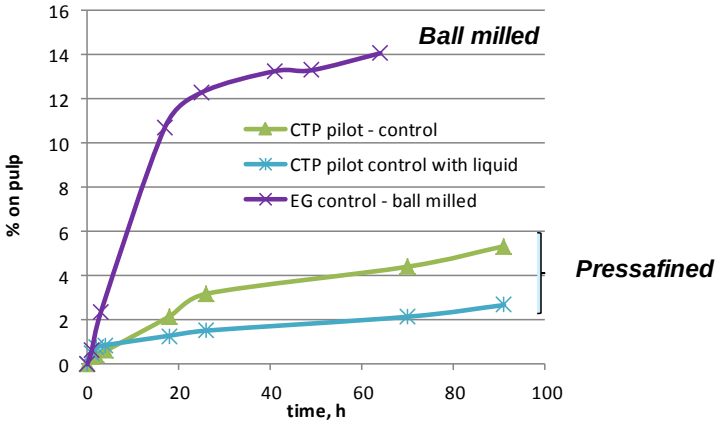
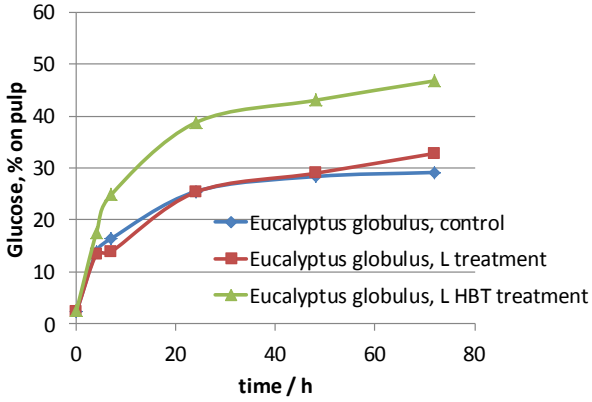


Figure 31. Effect of mechanical treatment on ethanol yield.

Effect of laccase treatment on hydrolysis and fermentation efficiency

The effect of laccase pretreatments performed at lab scale in IRNAS for *E. globulus* and elephant grass was investigated by enzymatic hydrolysis and fermentation experiments. As shown in Figure 32, the laccase treatment with HBT mediator (**L HBT)** clearly improved the enzymatic hydrolysability of elephant grass and *E. globulus*, and the effect was more pronounced for eucalyptus. The laccase treatment without mediator (**L)** had practically no effect, and hydrolysability was equal with the control sample. Probably due to the lower density and higher amorphicity, the elephant grass was much easier than *E. globulus* to hydrolyse even without any enzymatic pretreatment.

The actual bioethanol yields were well in line with the hydrolysability results. The elephant grass provided better ethanol yield compared to *E. globulus*, and laccase with HBT mediator was better than laccase without any mediator. The effect of HBT mediator was more pronounced with *E. globulus*. In all cases, the ethanol yield was relatively low even though the samples were ball milled before the enzymatic treatments.



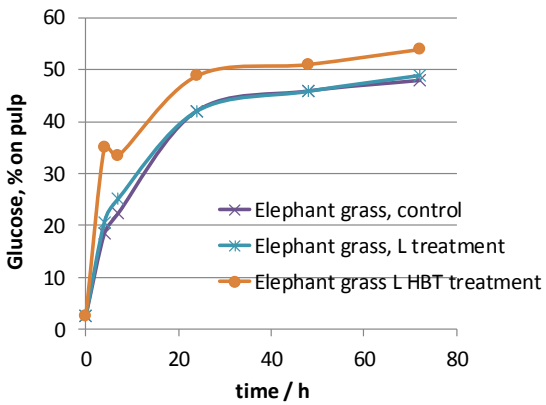


Figure 32. Effect of laccase treatment on glucose release in enzymatic hydrolysis. The results are not corrected with pulp yields.

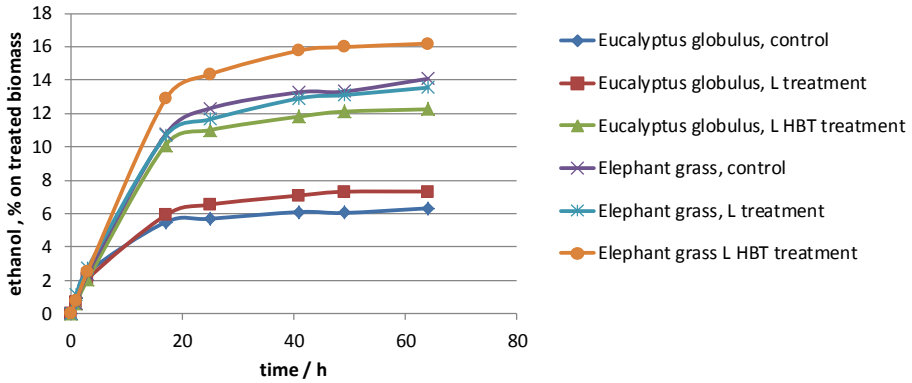


Figure 33. Effect of laccase treatment on ethanol yield.

At pilot scale, the same laccase treatments were performed for refined *E. globulus* at CTP. In this case, the ethanol production was very limited probably due to the insufficient biomass deconstruction and hydrolysis. The effect of ball milling on enzymatic hydrolysability was probably thus higher in previous lab scale experiments than the effect of laccase treatment.

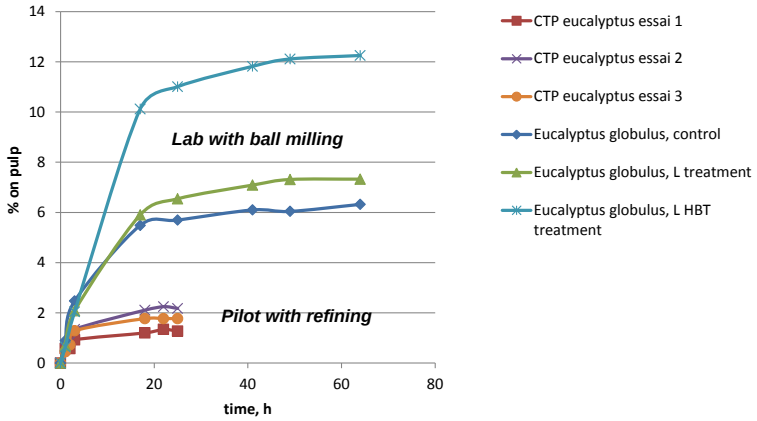


Figure 34. Effect of ball milling (lab scale) and refining (pilot scale) prior to the laccase treatment on bioethanol production yield.

Optimisation of enzymatic hydrolysis for the pilot pulps

To ensure reliable comparison, the enzymatic hydrolysis was optimised according to the raw material and pretreatment method. The optimisation was performed for the LGF pilot pulps (*E. globulus*) produced in WP5, and Soda-O2 pulp (*E. globulus*, kappa 35) showing the best bioethanol production potential.

Optimization of enzymatic hydrolysis was done using the Response Surface Methodology (RSM), which describes the relationship between several explanatory variables, i.e. different enzymes used in various loadings, and the response variable, i.e. the reducing sugars obtained, by a second degree polynomial. Additionally to the enzymes Celluclast 1.5 and Novozym 188, which were used in the screening experiments, purified xylanases with 3.2mg protein per ml (purified from Ecopulp, Ab Enzymes) was used. The dosages were chosen to be in the range of 5-15FPU/g_dm for Celluclast, 50-200nkat/g_dm for Novozym and 0.05-0.25mg protein/g_dm for the purified xylanases. In pretests, an addition of purified mannanases in the range of 0.05 -0.2mg protein/g·dm was found to have no statistically significant effect. Design of Experiment (DoE) of choise was a Central Composit Face Centered design (CCF) with the three enzymes as factors and reducing sugars as response variable. In the CCF design, each enzyme is used in three different dosages, the lowest, highest and mid point, in various combinations with the other enzymes.

The response surfaces obtained for all three pulps are shownin Figure 35. Xylanases were kept constant at 0.15mg protein per g·dm. Optimal reducing sugar concentration after 48h of hyrolysis were found to be 9.5 g/l at an enzyme loading of 13.9 FPU/g_dm and 130.3 nkat/gdm for LGF pulp, 11.3 g/l sugars at 11.3 FPU/g_dm and 160 nkat/gdm for LGF-OH pulp and 10.1g/l at 10.7 FPU/g_dm and 259.7 nkat/gdm for Suzano pulp. Compared to the constant charges used in sample comparisons (10FPU Celluclast, 500 nkat/g Novozym 188), the optimum Cellulcast dosage was slightly higher, but the Novozym charges could be reduced significantly.

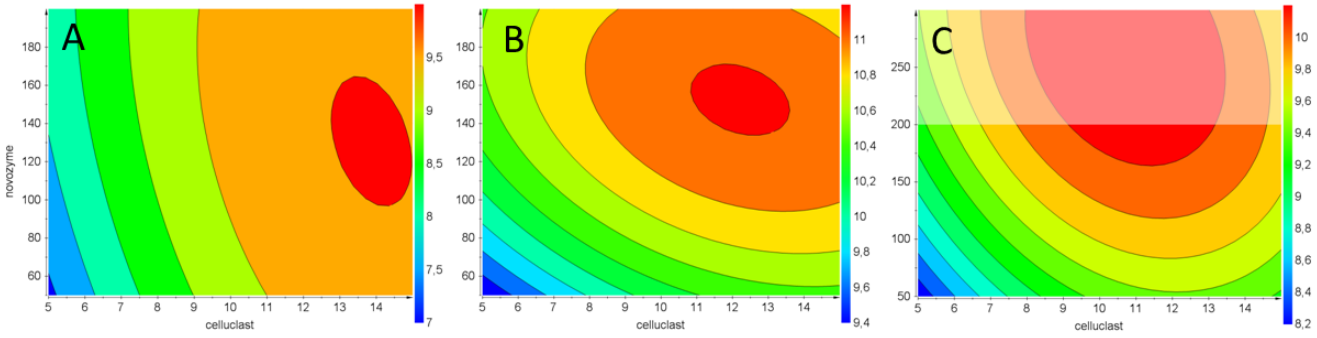


Figure 35. Response surfaces obtained from the optimization experiments. A: LGF; B: LGF-OH; C: Suzano.

As expected, the enzyme mixtures Celluclast and Novozym gave the highest variability in the response surface model. Xylanases only contributed minor variability (data not shown). The Celluclast preparation already contains traces of xylanases activity, and the pulps contain only small amounts of xylan (below 10%), therefore the addition of purified xylanases has only little effect on the resulting sugar concentrations.

Conclusions on WP4

The LGF organosolv cooking with ethanol solvent and phosphinic acid catalyst produced well hydrolysable pulp with high ethanol yield, and the hydrolysability was further improved by alkaline extraction of the LGF fibers. Cooking time between 16-20h was optimal at relatively low cooking temperature of 130˚C with 3.5% phosphinic acid and 15% water content. In comparison with other deconstruction methods, the oxidative alkaline pretreatment ($NaOH+O_2$) provided also well hydrolysable pulp at high kappa levels of 35-50, being another potential pretreatment method for bioethanol production from eucalyptus and elephant grass. Enzymatic laccase and hydrolytic pretreatments alone were not sufficient to open up fiber ultrastructure for efficient enzymatic hydrolysis, and thus ethanol fermentation.

WP5. Pilot demonstration

Summary of WP5

The feasibility of LGF organosolv as potential pretreatment method for bioethanol production was demonstrated at pilot scale. Another aim of the pilot cook was to produce sufficient amount of pulp for optimization of enzymatic hydrolysis dosages, as reported in WP4. The bioethanol production potential of other pilot pulps pretreated by alkaline cooking (Suzano) and enzymatic pretreatments (CTP, CIB) were evaluated at VTT, as described in WP4.

T.5.1. VTT organosolv demonstrations

The upscaling of LGF organosolv process was performed using *E. globulus*. Although the *E. globulus* did not show best bioethanol production potential in lab scale comparison of feedstocks, it was selected for the pilot demo as it best represents the Eucalyptus species available also in Europe. The same raw material was also used in pilot demonstration of CTP, allowing direct comparison of the pretreatments.

The LGF cooking was performed for 20 kg (b.d.) batch of screened and dried *E. globulus* chips using a forced circulation reactor of 250 l at temperature of 130°C with 3.5% H_3PO_2 and 15% water for 20 h. During cooking, the organosolv liquor was circulated through the chips continuously. After cooking, the pulp was washed with hot ethanol:water (85:15) mixture, followed by washing with water. After LGF cooking, the alkaline extraction of the pulp was performed with 1M NaOH at 2.5% consistency over night at room temperature.

Pilot scale cooking efficiency compared to lab results

The pilot demonstration resulted in LGF pulp with 56 % yield, and 51% total yield after alkaline extraction. This was somewhat lower than at lab scale, but also the delignification efficiency was better. This is probably due to the circulation of the liquor through chip pad throughout the cooking, and more efficient washing with hot liquors. Cellulose yield was comparable to the laboratory pulp.

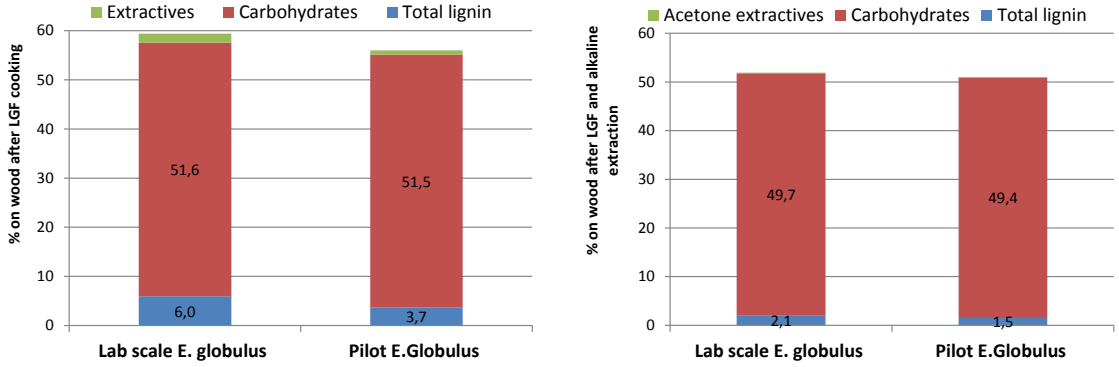


Figure 36. Chemical composition of E. globulus lab and pilot pulps after LGF cooking (left) and the following alkaline extraction (right). All the results are normalised to pulp yield.

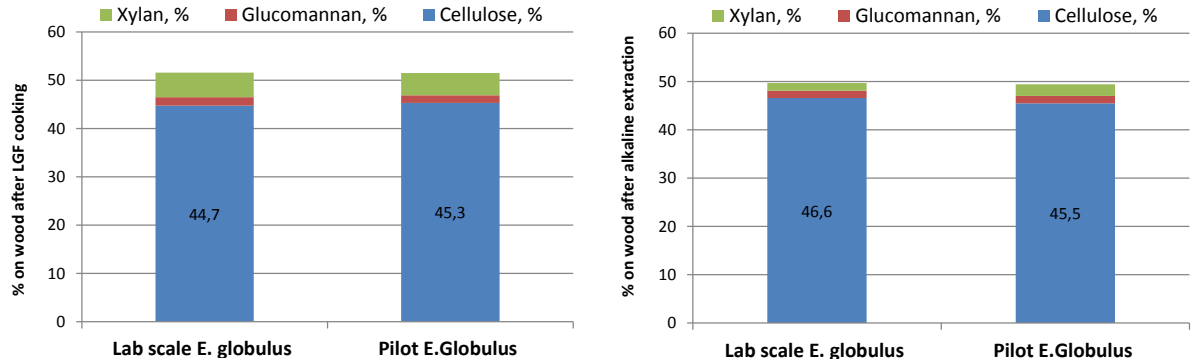


Figure 37. Carbohydrate composition of E. globulus lab and pilot pulps after LGF cooking (left) and the following alkaline extraction (right). All the results are normalised to pulp yield.

Bioethanol production potential

Despite the similar cellulose content, the hydrolysability of pilot pulp was significantly better compared to the corresponding lab pulp, or any of the LGF pulps produced at lab scale (*Suzano/DGxU2*). This is probably due to the more efficient delignification and also lower xylan content. After alkaline extraction, the maximum hydrolysis yield was reached already after 24h hydrolysis.

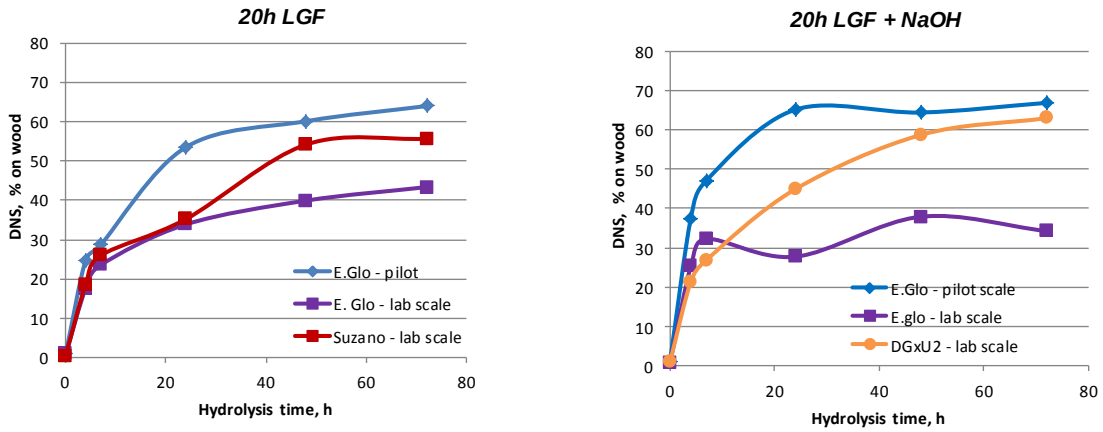


Figure 38. Enzymatic hydrolysability of the E. globulus pilot and lab pulps compared to the best pulps prepared in similar conditions at lab scale.

In comparison with the alkaline oxidation pretreatment, the LGF organosolv cooking at pilot scale produced slightly better bioethanol yield. After alkaline extraction of LGF pulp, clearly higher bioethanol production was shown compared to the Soda-O2 pulp. The better bioethanol yield of LGF pulp was in line with higher cellulose and lower xylan content compared to Soda-O_2 pulp.

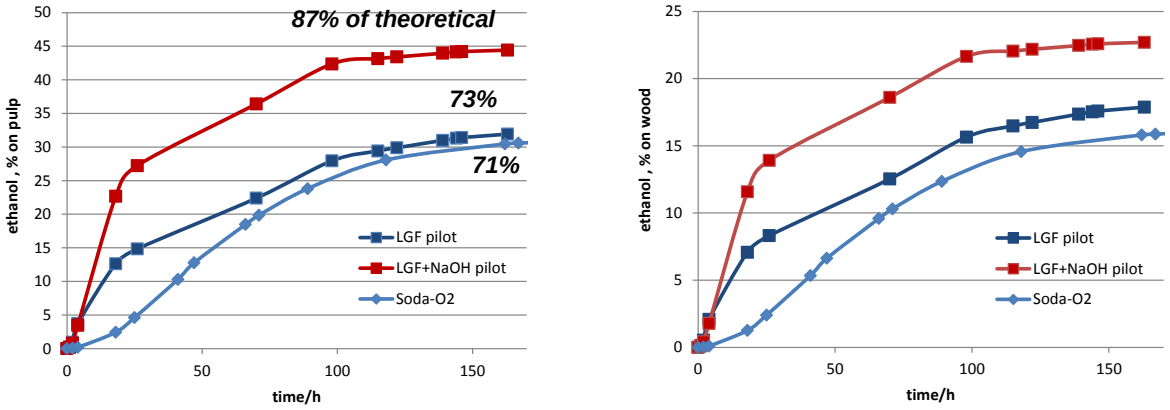


Figure 39. The bioethanol yield of LGF pilot pulps compared to the pulp after alkaline oxidation pretreatment.

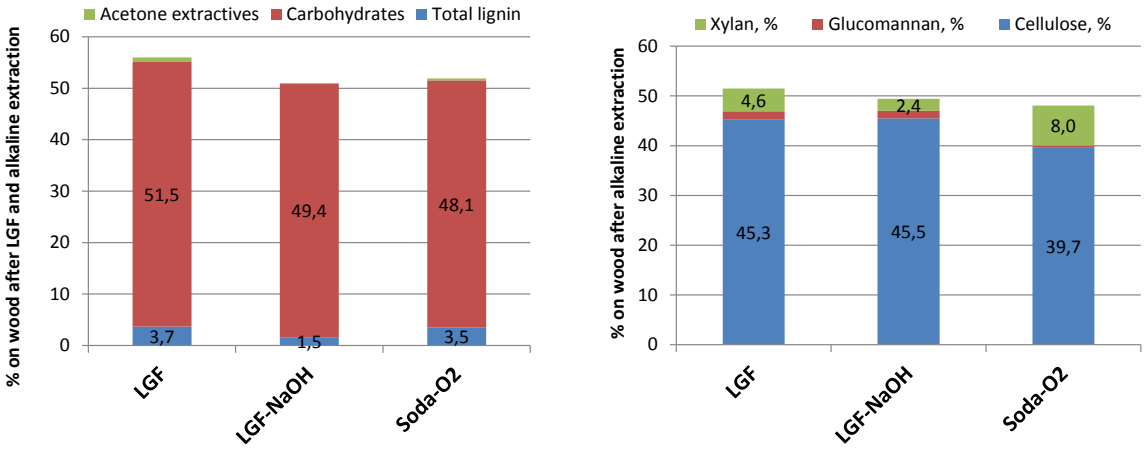


Figure 40. The chemical compositions of LGF pilot pulps compared to the pulp after alkaline oxidation pretreatment.

Conclusions of WP5

The LGF organosolv cooking was successfully demonstrated also at pilot scale, producing well hydrolysable biomass with high ethanol yield. Nearly 90% of the theoretical ethanol yield was reached after the alkaline extraction of LGF pulp. This was higher compared to the alkaline oxidation, which also produced well hydrolysable pulp for bioethanol production. As reported in WP4, the bioethanol yield after enzymatic treatments at pilot scale was very limited.

3.2. Deviations from the Work Plan

Project progress was according to the work plan. Only the optimisation of the LGF cooking conditions took longer time than expected (**WP1**). Therefore, the pilot demonstration in **WP5** was postponed with the acceptance of the coordinator and EU officer, and was carried out when the fully optimised cooking conditions with shortest possible cooking time were defined. The pilot demonstration was performed according to the new dead line (month 24) as planned, but had to be repeated later on due to the damaged raw material.

4. PLANS FOR THE DISSEMINATION AFTER THE 36th months

The results of LGF pulping as a potential pretreatment method for bioethanol production will be presented in the 17th International Symposium on Wood, Fibre and Pulping Chemistry, Vancouver, Canada, June 2013.

- Tamminen, T., Barth, D., Colodette, J., Liitiä, T. Organosolv pulping as pretreatment for bioethanol production from Eucalyptus and Elephant grass

Two joint papers together with IRNAS are under preparation on characterisation of residual and spent liquor lignins of alkaline and LGF cooking.

5. DELIVERABLES AND MILESTONES

Deliverables during the 19-36 months reporting period were achieved, as follows:

DL3.4. & DL 3.7 Characterisation of pretreated non-woody materials using advanced analytical tools - 1 & 2 (month 18 & 32, R, CO)

- Surface xylan of elephant grass LGF pulps was detected, and found to be enriched especially in parenchyma cells. Also the removal of surface xylan by alkaline extraction was not as efficient as with eucalyptus
- Distribution of reprecipitated EG xylan on eucalyptus pulp was detected by immunolabeling, showing accumulation in fine fibrils and damaged fibers, but also evenly and heavily labelled fibers were dedtected.
- Crystallinity of EG increased during LGF and alkaline pulping, but remained lower compared to the eucalyptus pulps.
- Molar mass and content of phenolic units was lower in elephant grass LGF lignin compared to eucalyptus.

LD 3.5 & DL 3.8 Characterisation of pretreated woody materials by advanced characterisation methods – 1 & 2 (month 24 and 32, R, CO)

- Surface xylan of G1xUGL clone after 48h LGF cooking and alkaline extraction was detected by immunolabeling, and removal of surface xylan after alkaline extraction was evident.
- Moderate and uneven labeling of surface xylan was detected in alkaline paper pulp fibers, showing more dense labelling in damaged fibers, fibrillated fines, pits and dislocations. Also the content of reprecipitated xylan was higher in fines and more open fiber defects.

- Effect of LGF cooking and the following alkaline extraction on cellulose crystallinity was evaluated using solid-state NMR spectroscopic means.
  - Eucalyptus crystallinity increased during LGF cooking and alkaline extraction. In LGF cooking conditions, the cellulose crystallinity increased first, but reduced with long cooking times. With prolonged cooking times, the phosphinic acid charge had no effect on cellulose crystallinity.
  - Increase of crystallinity was detected also during all alkaline cooks (Soda AQ, Soda-O2, kappa level).
  - This is due to removal of amorphous cellulose, but also enhanced aggregation when amorphous lignin and hemicelluloses are removed between cellulose fibrils.
  - No correlation between pulp crystallinity and hydrolysability was detected

DL 3.6. Characterisation of black liquors and other side streams (month 24, R, CO)

- The residual lignins and the spent liquor lignins of alkaline cooks and the LGF spent liquor lignins were characterised by SEC to follow changes in molar mass, and by ^{31}P NMR for functionalities (aliphatic OH, type of phenolic OH, and COOH).

DL 5.1. Pilot scale solvent deconstruction trials (month 24, D, CO)

- LGF cooking and alkaline extraction was demonstrated in pilot scale for E. globulus, providing LGF pulp with good glucose release and ethanol yield.

DL 4.2 Evaluation of pretreated materials and residues for bioethanol production (month 36)

- Enzymatic hydrolysability of LFG pulps (effect of cooking conditions, raw materials) was evaluated.
  - Optimised LGF conditions were identified based on hydrolysability (3.5% PA, 130C, 15% water, 20h).
  - Resuls on hydrolysability of different feedstocks were dependent on LGF cooking time (20h, 32h) and whether alkaline extraction was performed or not.
- Soda-O_2 provided better hydrolysable pulp at higher kappa levels than Soda-AQ.
- LGF pilot treatment especially after alkaline extraction provided higher bioethanol production than Soda-O2 treatment for E.globulus.
- Very limited bioethanol production after enzymatic treatments was detected at pilot scale due to insufficient biomass deconstruction.

6. NEW CONTACT PERSONS

No changes in the contact persons.

APPENDIX D

NOVOZYMES

Grant agreement no: KBBE-2009-3-244362
Optimized pre-treatment of fast growing woody and nonwoody Brazilian crops by detailed characterization of chemical changes produced in the lignin-carbohydrate matrix

6th Periodic Report

Date: 15 January 2012
Partner P4 (Novozymes)

Grant agreement no: **KBBE-2009-3-244362**

Project acronym: LIGNODECO

Project title: Optimized pre-treatment of fast growing woody and nonwoody Brazilian crops by detailed characterization of chemical changes produced in the lignin-carbohydrate matrix

Funding Scheme: Collaborative project (small or medium-scale focused research project)

Thematic Priority: KBBE-2009-3

Period covered: From 1 July 2012 to 31 December 2012

Date of preparation: 15 January 2012

Start date of project: 1 January 2010 Duration: 36 months

Partner name: **Novozymes**
Author 1 *Kasper Klausen (Novozymes)*
Author 2 *Pedro Loureiro (Novozymes)*

1. SUMMARY OF THE WORK

The establishment and validation of the previous described technique enabled us to identify a novel xylanase-based product, NS51115, among more than 30 enzymes and enzyme blends, both commercial and experimental, for the pre-treatment of eucalyptus wood prior to mechanical deconstruction. This candidate repeatedly showed to have a destabilizing effect on the cell wall structure and was chosen for the optimization trials which generated a prediction model based on 120 data points revealing the optimum conditions.

Several enzymatic fiber modification trials have been conducted with the unconventionally deconstructed materials. Most of the deconstructed materials were readily modified by the cellulase treatments and the prepared handsheets showed improvement in tensile strength. Specifically the Cel45A and Cel7b endoglucanase families demonstrated the ability to improve resultant handsheet physical strength properties, the latter enzyme treatment without sacrificing tear strength. Also the Soda-AQ*E. globulus* fiber (both kappa 15 and 20) showed to be susceptible to enzymatic strengthening, resulting in tensile strengths equal to the otherwise stronger kraft fiber, thus enabling a completely sulphur-free pulping process. Refining trials carried out on the selected Soda-AQ*E. Globulus* pulps did not reveal any significant difference in the physical properties between the kappa 15 and 20 pulps, except in tear strength where the kappa 20 pulp was superior. The enzymatic treatments of these pulps with both Cel45A and Cel7b revealed the need for further process optimization in order to obtain the expected benefits, although large increases in freeness developments were identified as well as increases around 10% in tensile strength after 1000 PFI rev.

Furthermore these unconventional pulps were investigated with regards to their susceptibility enzymatic delignification (i.e. bleaching) by the application of oxidoreductases both alone and in combination with mediators. These trials revealed that the high redox-potential laccase from *Polyporus pinsitus* showed synergistic behaviour when combined with violuric acid, reaching a brightness increase of 16 units for the kappa 20 pulp. Also interesting was it that the same absolute brightness values were obtained when kappa 15 and kappa 20 pulps were subjected to the enzymatic bleaching, which can be translated into increased pulp yield and cost savings on chemicals. Data is presented throughout the report which continuously favours the use of this unconventional sulphur-free pulp, especially at kappa 20, with regards to enzymatic strengthening and enzymatic bleaching. The enzymatic delignification system was further optimised with the *Coprinus cinereus* peroxidase combined with violuric acid and it was shown that this system increased brightness by 7 units at a very low dosage of 0,3 mM of violuric acid and 0,5 mM of hydrogen peroxide.

It was also shown that conventional bleached kraft pulp with little effort could be converted into dissolving pulp of a proper grad via enzymatic routes. The application of xylanases and cellulase had positive influence on the solubilities (S10 and S18) and the intrinsic viscosities of the investigated pulps, thus enabeling a regular kraft mill to obtain dissolving pulp grades without the need for a pre-hydrolysis step.

The investigations of the biogas potential of the various substrates used in LignoDeco revealed that the raw feedstock's themselves were rather recalcitrant and produced very little biogas, except for elephant grass which showed the highest yields. With regards to both the conventional and unconventional deconstructed materials, all showed to be good substrates for biogas production, except the elephant grass which was inferior to the other tested substrates. However it is not advised to produce biogas from these perfectly nice pulped fibres, rather to used waste streams containing these and rejects for biogas production.

2. PROJECT OBJECTIVES FOR THE PERIOD

In accordance with work package 4, "*Pulp characteristics and papermaking evaluation"* (T4.1.), which includes the assessment of the various deconstruction procedures on the sensitivities of the deconstructed materials to enzymatic application has been conducted. In this regard the main objectives has been on the beatability of the selected *E. globulus* pulps and the application of enzymes in this regard to reduce the need for refining, i.e. development of the fiber by enzymatic means to obtain freeness and strength properties.

In T4.1 Novozymes is also tasked with the evaluation of enzymatic delignification technologies based on oxidative enzymes including laccase-mediator systems. Work has been done within this task including set-up of a medium-throughput small scale bleaching assay which enables the investigation of several oxidoreductases and mediator systems on the deconstructed materials.

T4.1 also included exploratory work done within the field of dissolving pulps, where enzymatic benefits were shows which enables a conventional kraft mill to produced dissolving pulps without the need for a pre-hydrolysis step, and thus we have identified a promising substrate for the further processing into a high-value specialty grade pulp.

In T4.2 Novozymes is tasked with the development of an assay for the determination of biogas potential from different feedstocks and the actual biogas determination of both deconstructed and raw materials.

In accordance with work package 2, specifically task 2.3, Novozymes' primary effort over the duration of the first half of the LignoDeco project was the development and execution of screens of the commercial and experimental enzyme portfolios to identify suitable enzyme candidates and ideal application conditions for subsequent use in lignocellulose deconstruction pilot trials. The task specifically stipulates the identification of at least one novel candidate, with a significant impact on the mechanical integrity of at least one feedstock (i.e. eucalyptus) for evaluation within pilot trials (DL2.1).

In parallel with our internal enzymatic deconstruction assays, several experimental and commercial enzymes would be provided to relevant LignoDeco partners for use in mechanistic investigations defining the interaction between enzymes and model substrates (DL 2.5) and for the demonstration activities described in DL 5.2.

3. WORK PROGRESS AND ACHIEVEMENTS DURING THE PERIOD

3.1 Work Progress

3.1.1 WP2- Optimized pre-treatments for woody and non woody materials

In WP2, Novozymes is tasked with the identification of suitable commercial and/or experimental enzymes to be used as lignocellulose pre-treatments prior to deconstruction (Task 2.3). Development and validation of a suitable procedure to screen the extensive commercial and experimental enzyme portfolios of Novozymes has evolved from simple post-incubation liquor analyses to bench-top simulations of mechanical deconstruction.

Several techniques to gauge the impact of mechanical deconstruction and thereby enable the isolation and quantification of the impact of specific enzyme applications have been explored and a promising

xylanase-based product (NS51115) has been identified to have a positive effect on the mechanical deconstruction of eucalyptus wood. This enzyme blend was selected among more than 30 tested enzymes and enzyme blends, both commercial and experiential, representing a wide variety of enzyme classes such as oxidoreductases, xylanases, cellulases, mannanases and pectinases.

Task 2.3 - Enzymatic deconstruction using hydrolases and oxidoreductases

Optimization of NS51115 pre-treatment based on a bench-top simulation of mechanical deconstruction

Previous experiments identified a novel xylanase-based product, NS51115, to repeatedly have a significant impact on the integrity of eucalyptus wood when used for pre-treatment prior to mechanical deconstruction. The past 6 months has been devoted to further optimization of the enzymatic deconstruction using this xylanase of the LignoDeco raw materials. The previously used bench-top screening procedure, comprising an enzymatic incubation with the eucalyptus wood followed by a mechanical deconstruction and, ultimately, an estimation of surface area, has been further developed to include an enzyme "impregnation" step in order to simulate an impressafiner. This operation was included in the bench-top simulation of a mechanical deconstruction in order to introduce the enzymes to the interior of the lignocellulosic material and was used for the optimization trials with varying pH and temperature.

Suzano wood chips were comminuted by passing through a Wiley knife mill without a screen installed. These "mini-chips" were further separated into various size fractions by sieving before use in the procedure. Two oven dry grams of wood chip were allowed to pre-soak in 15 ml of 0,2M Britton & Robinson buffer (varying pH) for 30 min. 1 ml of enzyme preparation was added and mixed and the sample was transferred to custom impregnation cups, see Figure 1, immediately prior to compression. The temperature of the press was set according to the temperature used during the incubation and the wood chips were compressed for 5 min at a pressure of 45 kg/cm^2.

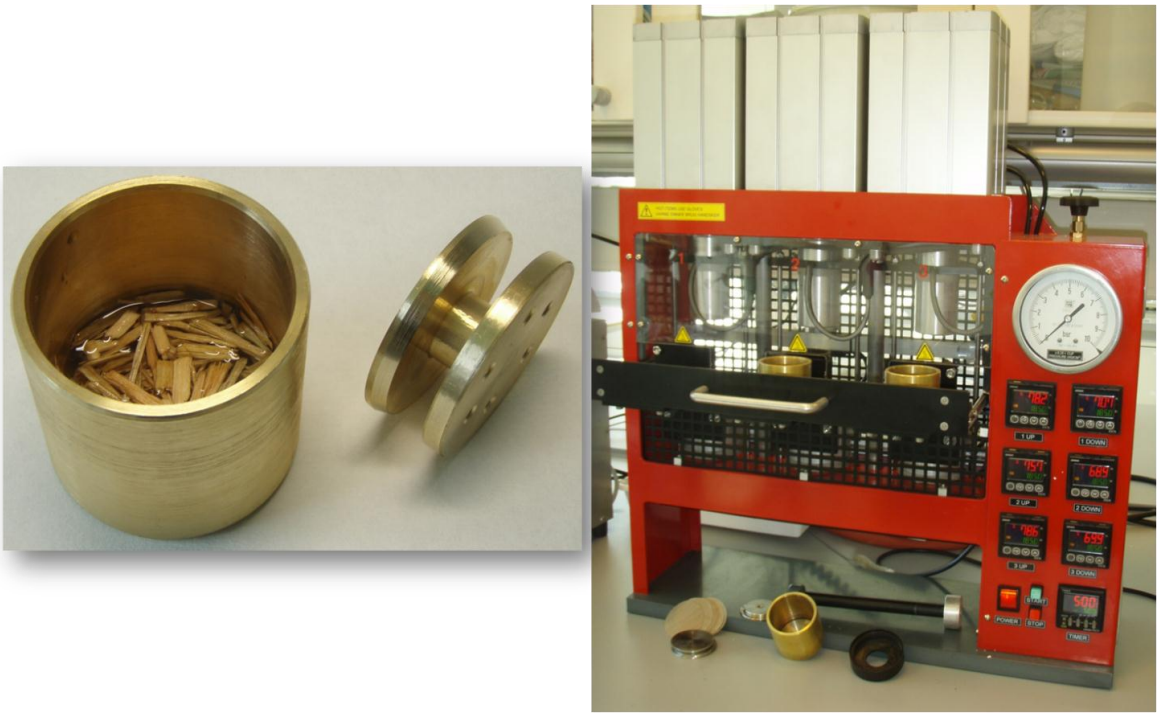


Figure 1 On the left the cup and insertion platen used for the impregnation of eucalyptus wood chips. The perforated platen, allows rapid penetration of the impregnation liquor, containing enzyme, upon pressure relief. On right is shown the mini-impregnator which is used for the actual compression of the wood chips within the cups.

After pressure relief, the samples were transferred to 50 ml Falcon tubes and submerged within preheated water in large 1L Lab-O-Mat beakers. These were incubated for 4 hours with rotary mixing at 20 rpm. After incubation the samples were placed in a 90°C water bath in order to inactivate the enzymes. The contents of the tubes (i.e. chips and liquor) were transferred directly from the 90°C bath to a Waring blender in which the blades were reversed (by reversing the blades to reveal the blunt edges, less "cutting" of the wood chips during mechanical action was observed). The sample was then "refined" within the Waring blender, operating at the low speed setting, for 5 minutes. After recovery from the blender, the samples were centrifuged for 10 minutes at 4000 rpm. After decanting the supernatant, the samples were diluted with 100 ml of fresh MilliQ water and once again concentrated by centrifugation. The supernatant was discarded and the samples were washed once more and 45ml 0,1M sodium acetate buffer pH 5,5 was added and allowed to equilibrate overnight. The samples were centrifuged again and the supernatant was discarded and the samples were suspended to a target volume in MilliQ water. A pre-determined excessive dose of 0,003 N polyDADMAC was introduced and allowed to absorb to accessible surfaces within the sample during 2 hours of continuous stirring at room temperature. Afterwards, an aliquot of the liquor from each sample was filtered via 0,2 µm syringe filters and 10 ml of each filtered liquor titrated to zero charge within the Mütek PCD-04 using 0,001 N PesNa to determine the overall cationic demand (1) of the original mechanically disrupted and washed sample.

Ideally, a difference in cationic demand between two samples of the same initial substrate is a (partial) function of a difference in surface area between the two samples. Increased cationic demand is presumed an indirect indicator of increased surface area after enzymatic pre-treatment and mechanical disruption. This method was further validated with the more time-consuming Simons Stain method, which shows good correlation to the cationic demand measurements (data not shown refer to LignoDeco 18 months report for details).

The optimization trials were conducted under these conditions and SAS JMP statistical software (version 8.0.1) was used to design the varying experiential conditions allowing for a surface plot to be generated in order to identify the optimal conditions for the enzymatic pre-treatment. A total of 120 data points was generated by the above assay for the NS51115 pre-treatment with pH ranging from 3-8 and temperatures from 30-90°C. These data were used to generate a model by the Standard Least Squares method describing the development of cationic demand by enzymatic pre-treatment as a function of pH and temperature.

The prediction surface plot can be viewed in Figure 2 and identifies the optimal pH for the enzymatic pre-treatment with NS51115 to be between pH 4,5 and 5,5 at temperatures ranging from 40-60°C using a predicted 20% increase in cationic demand as the cut-off value. At pH 5 and 55°C the actual increase in cationic demand is 45% and shows good repeatability with the screening trials conducted at similar conditions which further substantiate the destabilizing effect of the xylanase on the cell wall structure. It is therefore recommended that the enzymatic pre-treatment of eucalyptus wood for the mechanical deconstruction pilot scale trials is to be conducted at a pH of 5 and a temperature of 55°C.

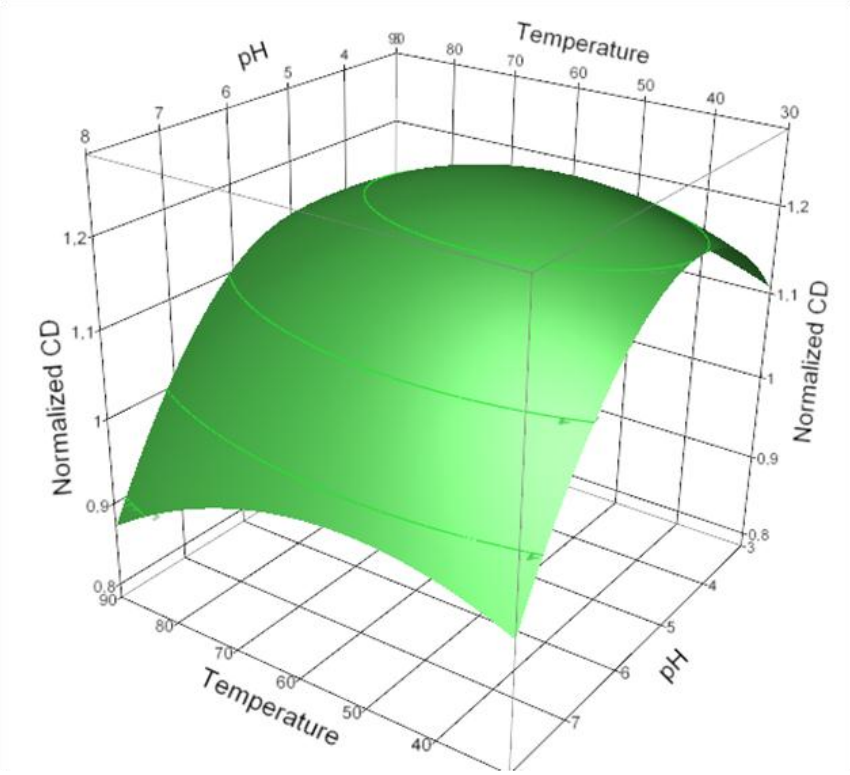


Figure 2 Prediction surface plot describing the development of cationic demand as a function of pH and temperature based on 120 data points using the Standard Least Squares method.

Conclusions

The establishment and validation of the previous described technique enabled us to identify a novel xylanase-based product, NS51115, among more than 30 enzymes and enzyme blends, both commercial and experimental, for the pre-treatment of eucalyptus wood prior to mechanical deconstruction. This candidate repeatedly showed to have a destabilizing effect on the cell wall structure and was chosen for the optimization trials which generated a prediction model based on 120 data points revealing the optimum conditions.

References

(1) **SCAN-W 12:04, Cationic demand; Polyelectrolyte titration with a streaming current detector. 2004**

3.1.2 WP4 - Tie between pre-treatment and industrial use of lignocellulosics

Task 4.1 – Pulp characteristics and papermaking evaluation

Enzymatic modification of unconventional eucalyptus kraft and soda + AQ

A general premise of LignoDeco is the use of unconventional means to produce papermaking fiber from eucalyptus wood. Although favorable from the environmental and/or sustainability standpoint, unconventional "deconstruction" of lignocellulose generally produces inferior papermaking fiber. To enable unconventional deconstruction, enzymatic treatments of the resultant fiber may recover desirable papermaking qualities. Moreover, the unconventionally deconstructed fiber may exhibit altered sensitivity/amenability to enzymatic modification relative to the standard fiber.

14 sets of deconstructed materials were provided for quantitative evaluation of sensitivity towards enzymatic treatments by applying both proven fiber modifying cellulases as well as experimental

enzymes. The enzymatic treatments were carried out at pH 7 using a 40 mM Britton & Robinson buffer at 50°C for 2 hours at 5% consistency, followed by an inactivation at 80°C for 30 min. All handsheets were prepared and tested according to Tappi Standard procedure. The results from the physical testing of the handsheets was normalized against their respective controls and can be viewed in Figure 3-Figure 5 and the freeness values for the enzymatically treated pulps can be viewed in Figure 6.

Most of the deconstructed materials were readily modified by the cellulase treatments. The prepared handsheets showed improvement in tensile strength in practically every case. Of all eucalyptus species and deconstruction techniques analyzed in these trials, the triple hybrid G1 x UGL, pulped to kappa 15, appeared to be most susceptible to cellulase enhanced tensile strengthening and showed increases from 25-35%. A reduction in the strengthening effect of the cellulases is observed on the handsheets prepared from the kappa 20 pulps which may be caused by a reduced margin for improvements as these pulps had higher initial tensile strength. There is a clear difference between the Cel45A (Fibercare R and U) and Cel7b (NS16081) endoglucanase families: while the increase in tensile strength came at the expense of tear strength for the Cel45A treatments, the Cel7b treatment surprisingly increased both tear and tensile strength of the handsheets.

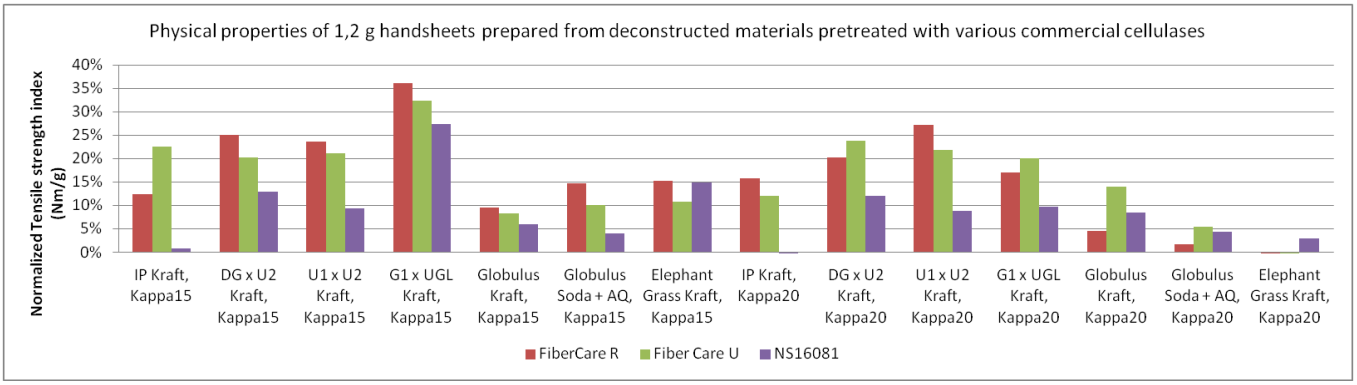


Figure 3 Normalized tensile strength data obtained from Tappi standard handsheets prepared from several different types of materials (eucalyptus spp and elephant grass), deconstructed via several different techniques to static Kappa levels of 15 and 20. All data has been normalized against the untreated control for each separate material.

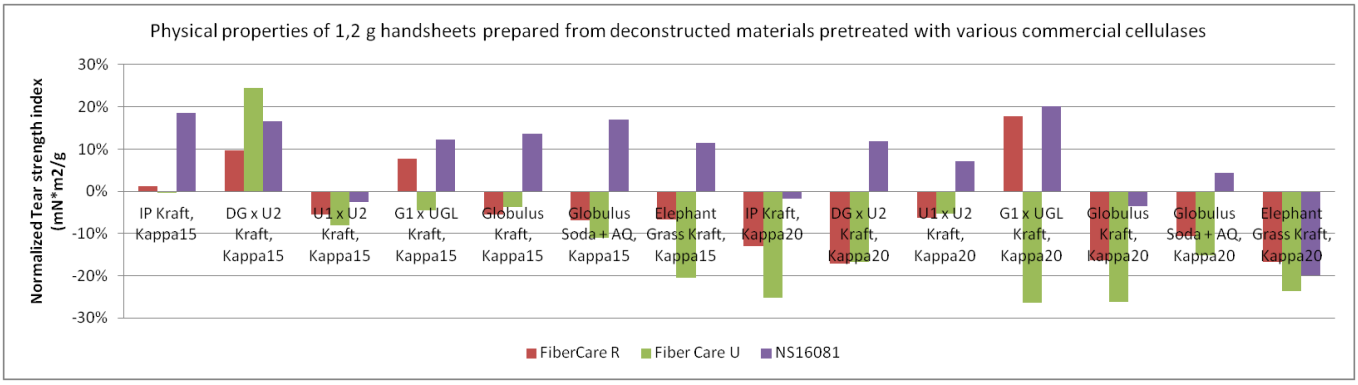


Figure 4 Normalized tear strength data obtained from Tappi standard handsheets prepared from several different types of materials (eucalyptus spp and elephant grass), deconstructed via several different techniques to static Kappa levels of 15 and 20. All data has been normalized against the untreated control for each separate material.

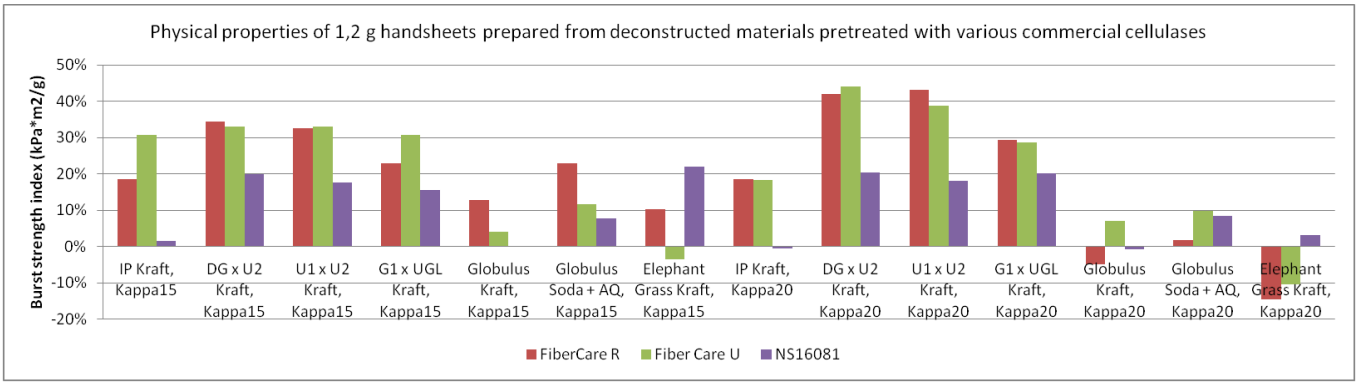


Figure 5 Normalized burst strength data obtained from Tappi standard handsheets prepared from several different types of materials (eucalyptus spp and elephant grass), deconstructed via several different techniques to static Kappa levels of 15 and 20. All data has been normalized against the untreated control for each separate material.

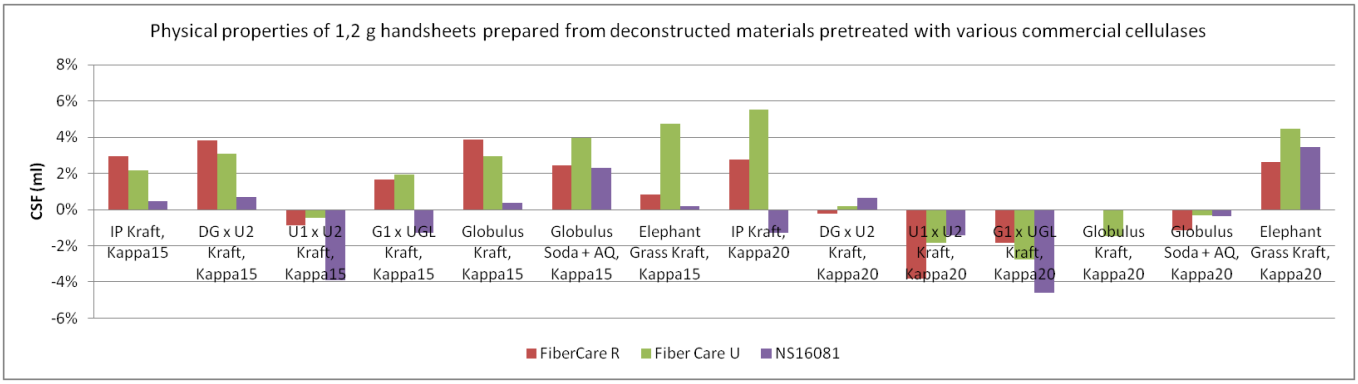


Figure 6 Normalized freeness data obtained from several different types of materials (eucalyptus spp and elephant grass), deconstructed via several different techniques to static Kappa levels of 15 and 20 after incubation with commercial cellulases. All data has been normalized against the untreated control for each separate material.

When comparing the different deconstruction techniques (i.e. the kraft and the Soda-AQprocess), it becomes evident that the physical properties of the kraft fiber are superior to those from the Soda-AQprocess with regard to the *E. globulus* (**Figure 7**). However the enzymatic treatment with Cel45A recovers sufficient strength from the Soda-AQprocess fiber to compete equally with the otherwise stronger kraft fiber.

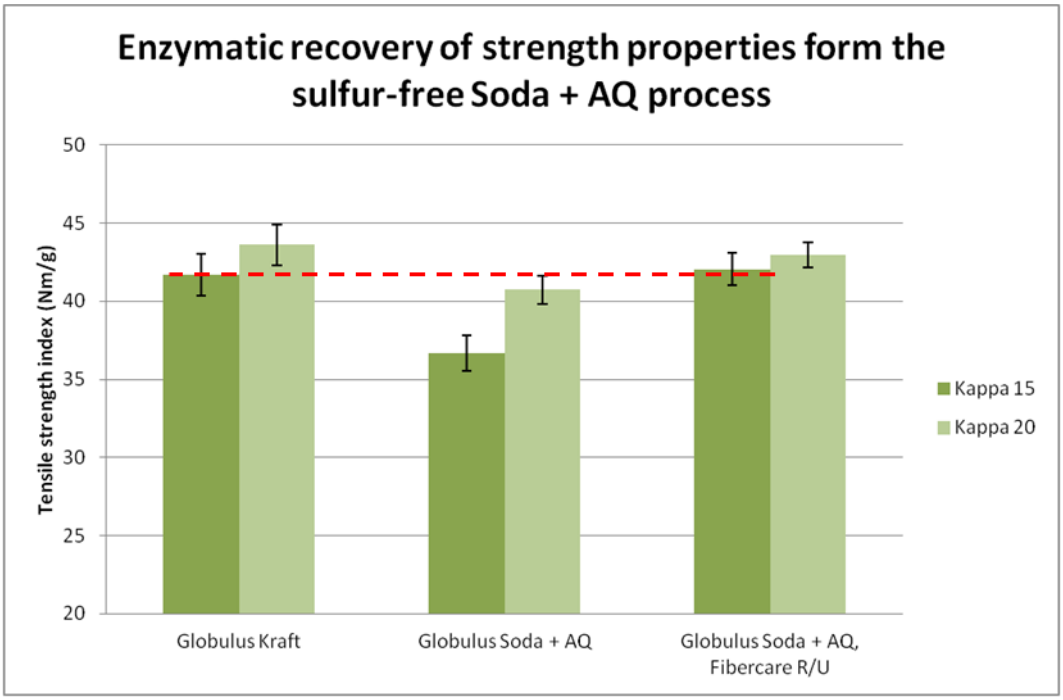


Figure 7 Comparison of tensile strength for the two different pulping processes and the enzymatic recovery of strength properties.

The following describes the evaluation of beatability and enzymatic strengthening during refining for the selected *E. globulus* Soda-AQkappa 20 pulp, where the kappa 15 pulp of same species was also included in order to evaluate which degree of delignification would be most favourable in this context.

The investigated pulps were diluted to 5% consistency (30 odg pulp total) in 40 mM Britton-Robinson buffer pH 7 and added to 1000 ml Lab-O-Mat beakers. Enzyme additions were made and the beaker were sealed and incubated for 120 min at 50°C while rotating in a Lab-O-Mat. After the incubation the enzymes were inactivated at 80°C for 30 min, followed by dilution to 2L and disintegrated for 10.000 revolutions. The pulp was filtrated and diluted to 10% consistency (assuming 30 odg transfer between steps) and distributed equally in PFI mill. Besides the non-refined controls, the pulps were refined for 1000 rev, 2000 rev or 3000 rev. After refining the samples were further diluted to 0,5% consistency and disintegrated for 10.000 revolutions and 60 g/m^2 handsheets were prepared according to Standard Tappi methods according to T-205 sp-95. Freeness measurements were carried out on a Mütek DFR-05 and all physical testing were carried out according to Standard Tappi methods, with a minor deviation in the prescribed climate conditioning of the samples.

The refining curve for the two pulps can be viewed in Figure 8 and shows the °SR freeness as a function of PFI revolutions. From this it is clear the two pulps behave rather similar with respect to increasing refining except at 3000 PFI revolutions.

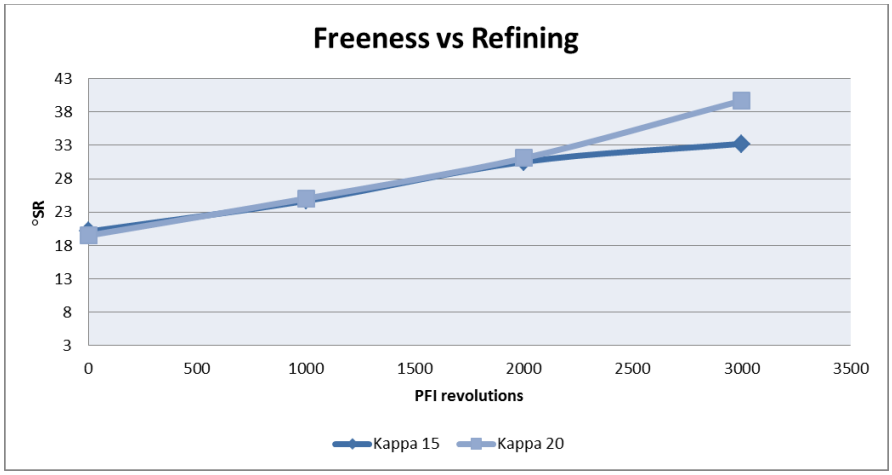


Figure 8 °SR freeness as a function of refining for *E. globulus* Soda-AQpulps at kappa 15 and 20.

The treatment of the pulps with a Cel45A (Fibercare U) gave rise to an improvement in pulp behaviour during the refining, where the pulp drainability is developed rather fast compared to the control. As can be seen in Figure 9 the °SR of the enzymatically treated pulp after 1000 rev corresponds to roughly 2200 rev for the control pulp, and would lead to decreased refining time/energy giving the other pulp properties follows. The major factor influencing the drainability of the pulp is external fibre fibrillation and is caused by the cellulase action on the surface of the fibre, which cuts the cellulose chains without liberating them and hence gives rise to increased fibrillation upon refining for the enzymatically treated pulps. The same behaviour can be seen when the kappa 15 pulp was subjected to refining as can be seen in Figure 10 where an increase of 95% in °SR can be seen after 3000 PFI rev.

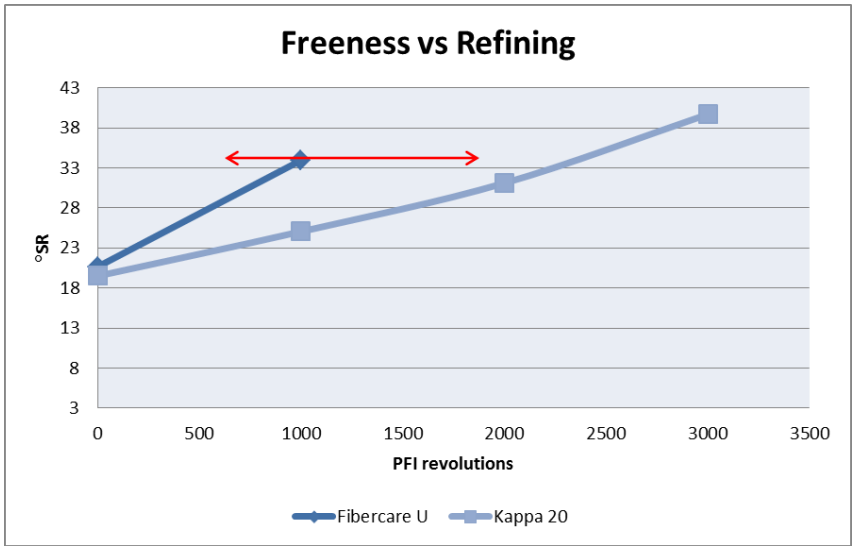


Figure 9 °SR as a function of refining for *E. globulus* Soda-AQpulp at kappa 20 compared to enzymatically treated pulp.

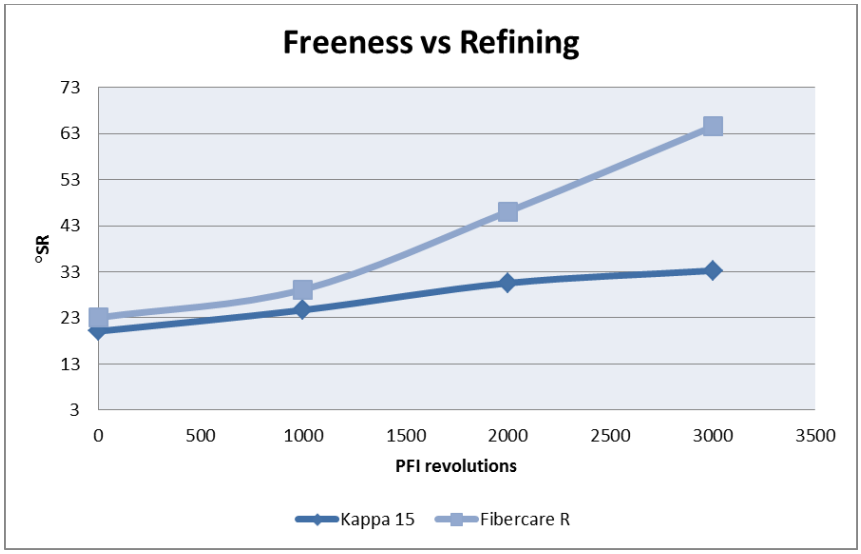


Figure 10 °SR as a function of refining for *E. globulus* Soda-AQpulp at kappa 15 compared to enzymatically treated pulp.

With regards to the physical properties of the refined samples, there does not seem to be any significant difference between kappa 15 and kappa 20 as can be viewed in Figure 11, although the kappa 20 pulp can be refined to obtain a slightly higher tensile strength after 300 PFI rev.

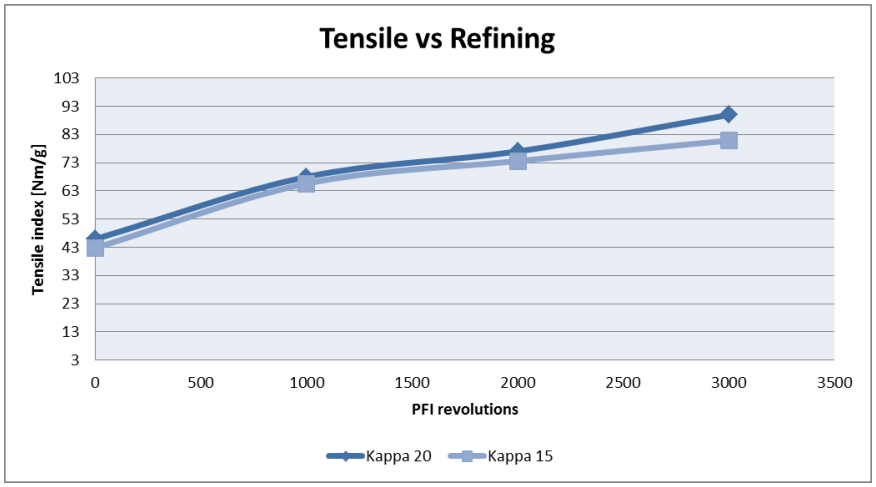


Figure 11 Tensile strength as a function of PFI revolutions for kappa 15 and 20 pulp.

The enzymatic treatment of the kappa 20 pulp revealed slight improvements with regards to tensile strengths of the prepared handsheets as can be seen in Figure 12, both before and after refining at 1000 PFI rev. This increase again indicates increase surface fibrillation which leads to increased fiber-fiber bonding and thus an increase in tensile strength is observed.

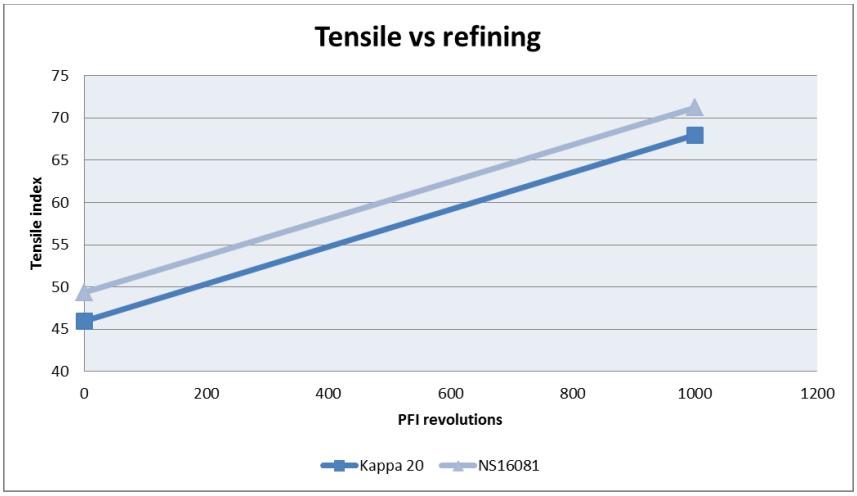


Figure 12 Tensile strength improvements as a result of enzymatic treatment with NS16081

The tear strength of the two pulps can be seen in Figure 13 as a function of pulp freeness. When comparing the two pulps there is a clear difference in the behaviour of the pulp during the refining, where the kappa 20 pulp exhibits the highest tear strength of the two at the same freeness level. One would expect to see the curve to drop, giving lower tear strength after a relatively low amount of refining, however this does not seem to be the case here, but may be a result of insufficient refining.

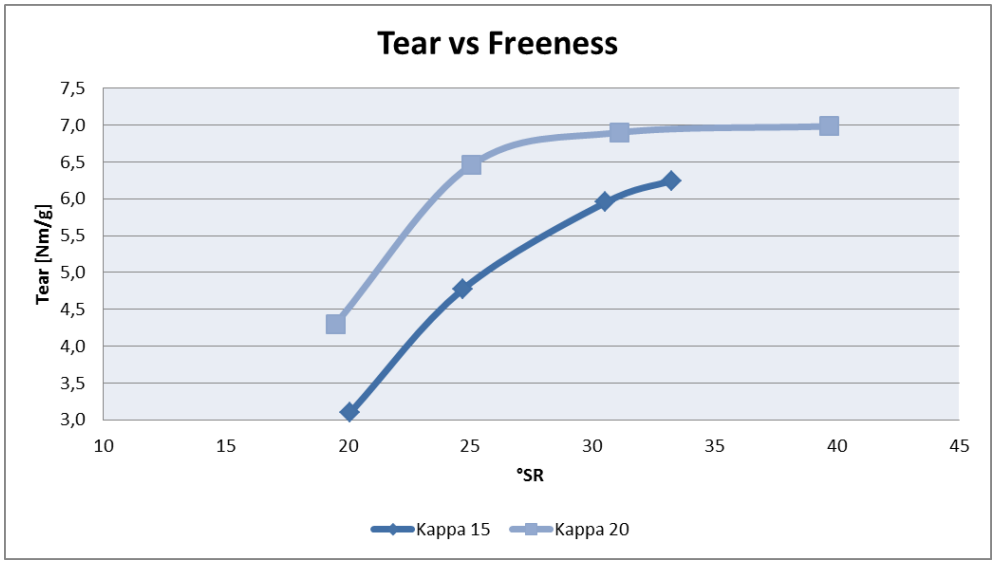


Figure 13 Tear strength as a function of freeness for kappa 15 and kappa 20 pulps.

Normally the enzymatic strengthening effect obtained by cellulase treatments of the pulps on the tensile strength of the prepared handsheets comes at the expense of lower tear strength similar to what increased refining would have. However this does not seem to be case when treating the kappa 20 pulp with NS16081 (Cel7b), where an increase in both tensile strength and tear strength is obtained without refining of the pulp as can be seen in Figure 14. However the tear strength seems to be impaired by the refining at 1000 rev giving lower tear strength for the enzymatically treated pulp compared to the control.

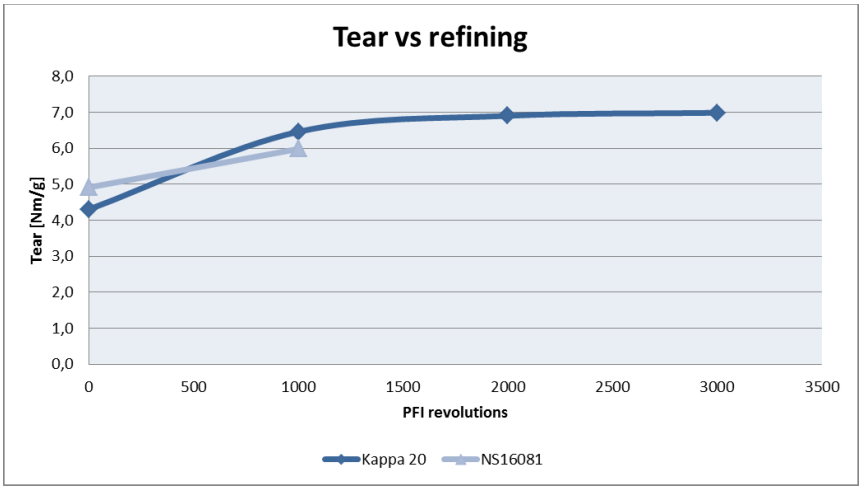


Figure 14 Tear as a function of refining for the kappa 20 pulp and the enzymatically treated pulp.

The above results generally does not indicate any benefits from pulping down to kappa 15, rather the opposite when looking at the higher tear strength of the kappa 20 pulp compared to kappa 15. The data also shows that this unconventional Soda-AQpulp is susceptible to enzymatic modifications which are most evident when looking at the development of freeness during the refining, where the enzymatically treated pulp (kappa 20) shows the same freeness after 1000 rev as the corresponding control at approximately 2200 rev. With regards to the strengthening effect both the tensile and tear strength are improved by the enzymatic treatment, however the effect is lower than would be expected when comparing to conventional *Eucalyptus* kraft pulps, but this is likely due to lack of optimization of the enzymatically treatment itself, and should be further investigated in order to obtain the maximum benefit from the treatments. It is believed that the enzymatic treatment has been too severe on the fibers especially when being refined. Also the development of the tear strength throughout the refining suggests that the revolutions should be further investigated in order to substantiate the results.

Enzymatic delignification using oxidative enzymes (laccase mediator systems)

In T4.1 Novozymes is also tasked with the evaluation of enzymatic delignification technologies based on oxidative enzymes including laccase-mediator systems. Preliminary work has been done within this task including set-up of a medium-throughput small scale bleaching assay which enables the investigation of several oxidoreductases and mediator systems on the deconstructed materials.

Investigations have been conducted in order to evaluate the unconventional Soda-AQpulp to enzymatic delignification with regards to brightness increase. Two different laccases has been investigated with various mediators. A low redox-potential laccase from *Myceliophthora thermophila* (MtL 0,1 g/L) and a high redox potential laccase from *Polyporus pinsitus* (PpL 0,1 g/L) were incubated with and without the methyl syringate (MES, 1mM) (for MTL) and violuric acid (2 mM) and 1-hydroxybenzotriazole (1 mM) (for PpL) followed by an alkaline/peroxide extraction in order to evaluate the bleaching effect of these laccase-mediator systems.

As can be seen in Figure 15 the MtL alone is able to increase the brightness with 3 units, as does the MES itself, and a total of 6 units increase is obtained using the combined treatments, so there does not seem to be any synergistic effect under these conditions. However there is a clear synergistic effect when combining the high-redox laccase with violuric acid, which by themselves does not seem to improve the brightness, but together there is a drastic increase in brightness of 16 units. Keeping in mind that these are preliminary investigations and no optimization has been conducted with regard to concentrations, temperature or time, this is a very promising enzymatic treatment, and shows that the *E. globulus* kappa 20 pulp is very much susceptible to enzymatic delignification by the PpL-violuric acid system and also outperformed the PpL-1-hydroxybenzotriazole treatment (data not shown).

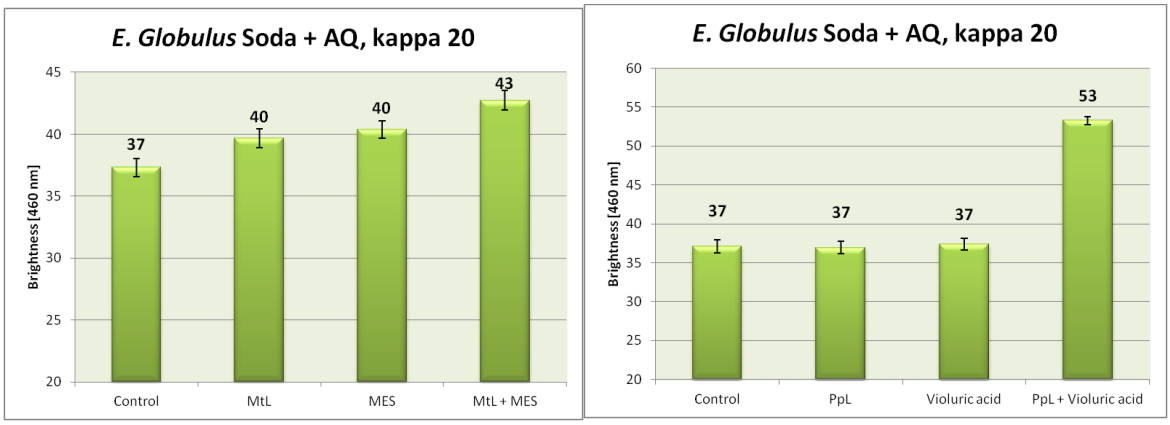


Figure 15 Brightness of the prepared mini-pads after enzymatic bleaching with MtL-Mes and PpL-violuric acid on *E. globulus* kappa 20 pulp. The pulps (60 mg dw in 4 ml) were incubated at pH 6 (50mM phosphate buffer) (MtL) and pH 4,5 (50 mM acetate buffer) (PpL) for 60 min at 50°C with O_2 added via tube, followed by thorough centrifugation and washing. All samples were extracted with 1,1 g/L NaOH and 0,9 g/L H_2O_2. Brightness is measured at 460nm using a Macbeth Color-Eye 7000 Remissions spec.

Another interesting result was made when kappa 15 and kappa 20 pulps were bleached by the PpL-violuric acid system . The enzymatic bleaching system responds equally well on both the kappa 15 and kappa 20 pulps, meaning the absolute values after bleaching and extraction are the same for the two pulps. This validates the further use of the high kappa 20 pulp versus the kappa 15, where one would obtain an increase in pulp yield and cost savings on chemicals as a result.

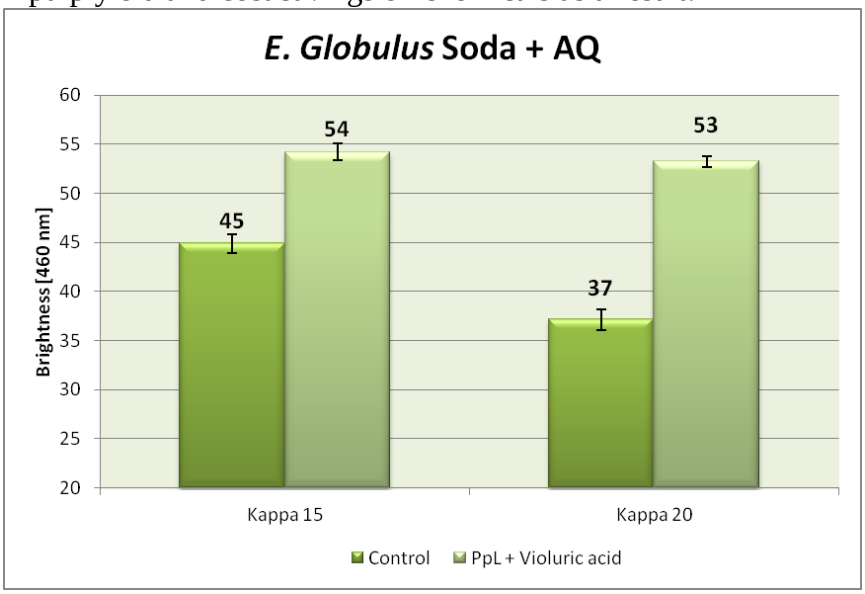


Figure 16 Comparison of brightness for the kappa 15 and kappa 20 pulps after enzymatic bleaching.

The above data clearly shows that the unconventional Soda-AQpulps are very much susceptible to enzymatic delignification giving rise to rather large brightness increases. Also the fact that the laccase-mediator system results in the same brightness for the kappa 15 and kappa 20 pulps makes this substrate and the pulping process very interesting in terms of enzymatic bleaching.

The dosages used in the before mentioned experiments carried out with the PpL + violuric acid system were however rather high for this system to be commercially relevant, however interesting from an academic point of view. A dosage/response profile was therefore created in order to evaluate the commercial validity of this system. **Figure 17** shows the brightness increase as a function of violuric acid concentration during the enzymatic delignification, and it can be seen that the optimal dosage lies between 3,5 mM and 5 mM violuric acid, which unfortunately is still rather high for an commercial application of this system.

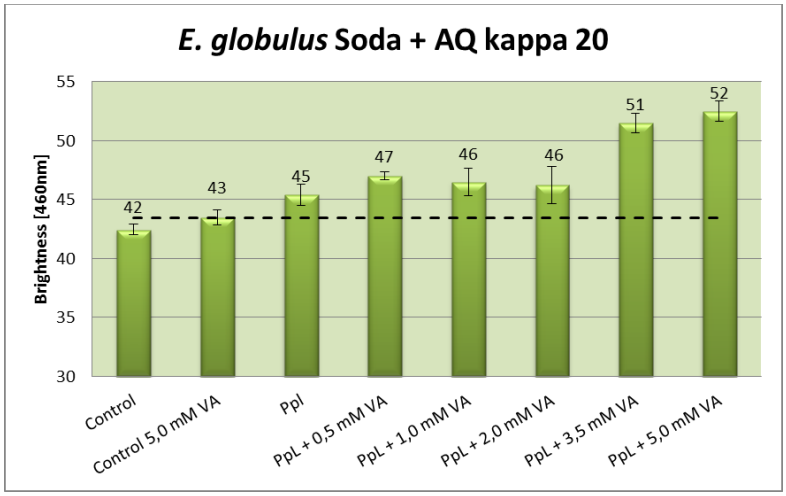


Figure 17 Dose response profile for the PpL + violuric acid system. Error bars represent standard deviations. The black dotted line depicts the high violuric acid control.

Another oxidoreductase + mediator system was investigated which hopefully could perform equally well at lower concentrations. A peroxidase (isolated from *Coprinus cinereus(CiP)*) was chosen for this investigation due to the fact that it had shown promising results in other applications, where it performed very well at low concentrations of both hydrogen peroxide and violuric acid. The results of these investigations can be seen in **Figure 18** and compared to the PpL + violuric acid system clearly shows a good performance at very low dosages of both mediator and peroxide. A 6 unit brightness increase is obtained at 0,3 mM violuric acid and 0,5 mM H_2O_2 and an enzyme loading at 170 mg ep/kg dm.

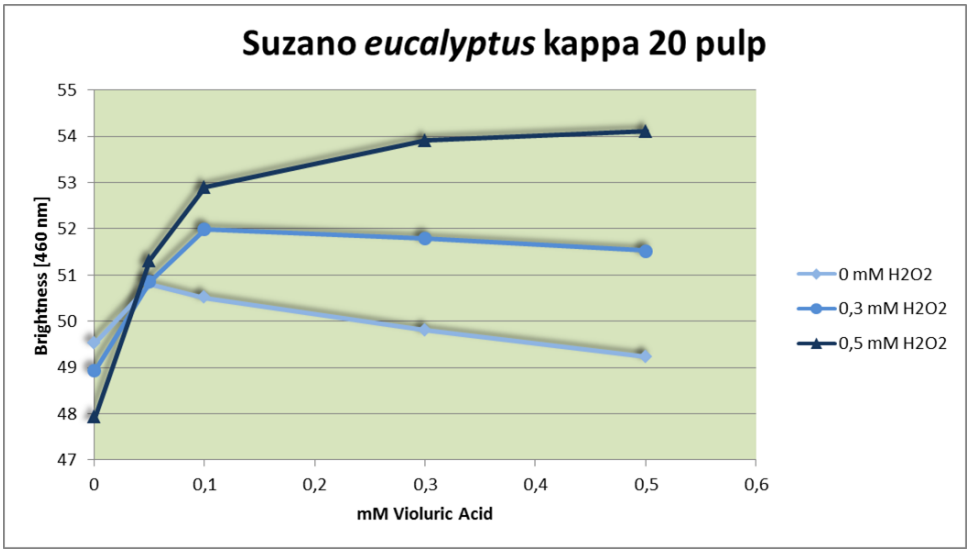


Figure 18 Dose/response of *Coprinus cinereus* peroxidase combined with violuric acid at varying concentrations and at various hydrogen peroxide levels. It should be noted that this experiment is carried out on conventional kraft pulp, and not the soda + AQ.

Similarly the performance of this CiP + violuric acid system was evaluated on the unconventional Soda-AQpulp. Again here the system performed very well at low concentrations as can be seen in **Figure 19**, where a brightness increase of 7 units is obtained at 0,3 mM violuric acid and 0,5 mM H_2O_2 and an enzyme loading at 170 mg ep/kg dm.

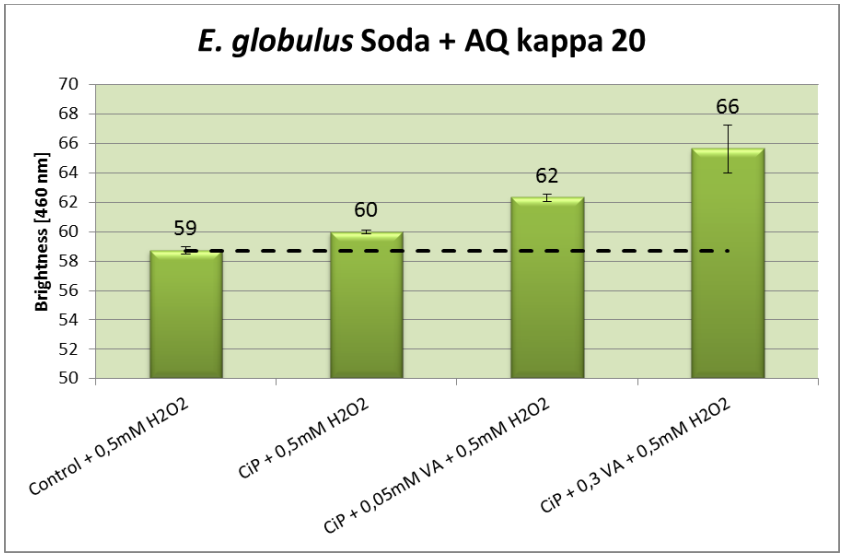


Figure 19 Performance of the peroxidase + violuric acid system on Soda-AQkappa 20 pulp. Error bars represent standard deviations.

Specialty grade pulps; enzymatic benefits in dissolving pulp

Dissolving-grade pulps have specific quality requirements such as high cellulose content, low hemicellulose content and only trace amounts of residual lignin, extractives and inorganics.

The increased market demand for dissolving pulp in recent years has result in much interest from many paper-grade pulp manufacturers into the conversion of their production sites into dissolving pulp.

As most paper-grade pulp manufacturers use kraft pulping technology, the pre-hydrolysis step is needed for the production of dissolving pulps. However, opportunities to convert conventional bleached kraft pulp with little effort have emerged via enzymatic treatment of the pulp. Taken into account the research conducted within LignoDeco on the bleachability of the selected eucalypt pulp, we have identified it as a promising substrate for the further processing into a high-value specialty grade pulp.

The paper-grade bleached eucalypt kraft pulp (BEKP) from Suzano was treated with xylanase Pulpzyme HC® (X-stage), also combined with cellulase FiberCare R® (FCR), and followed by either a cold caustic extraction (CCE-stage) or hot-caustic extraction (HCE-stage).

As determinant market properties of dissolving pulps, their alkali solubilities at 18% (S18) and 10% (S10) NaOH as well as their intrinsic viscosities were measured according to TAPPI T 235 and ISO 5351 standard procedures, respectivelly.

The results presented in Figure 20show that the xylanase treatment (BEKP) increased the pulp S18 value (ca. 9% increase). This can be explained higher alkaline solubility of the residual xylan left in the pulp after the xylanase treatment. This residual xylan after the xylanase treatment likely became more degraded (lower molecular weight) and more vulnerable to alkali solubilization thus increasing S18 (1).

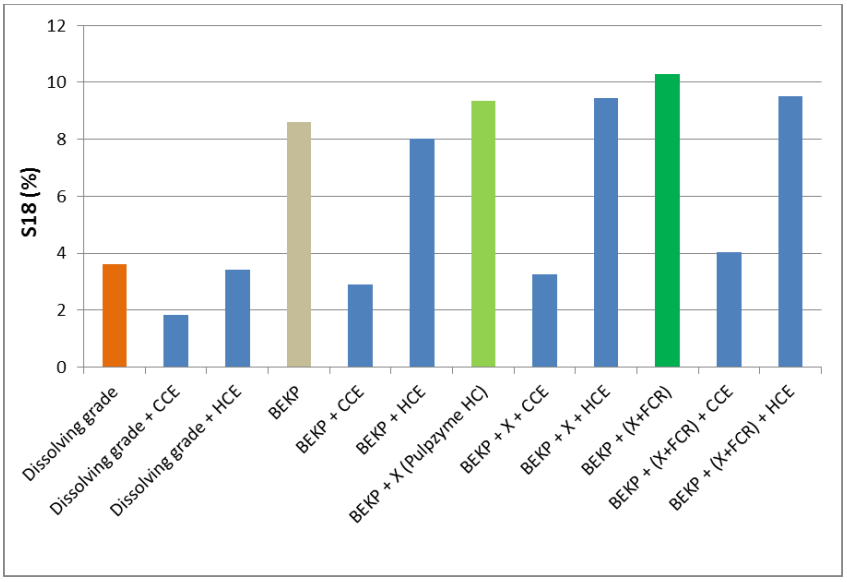


Figure 20 Alkali solubility of pulp at 18% NaOH (S18). X-stage: 0.10% odp Pulpzyme HC (0.05% odp FiberCare R); 60 ºC; pH 7; 120 min. CCE-stage: 70 g/L; 30 ºC; 30 min. HCE-stage: 4 g/L; 90 ºC; 60 min.Trials run at 10% consistency in sealed polyethylene bags imersed in a water bath.

Only after the introduction of a CCE-stage it is possible to reach the same S18-level of a standard dissolving grade pulp being the values slightly higher after the X-stage. However, if more xylan is dissolved there is the potential benefit on pulp reactivity and filterability of the dope in the viscose production process.

As regards the S10-values presented in Figure 21, the same trend is obtained with higher solubilities obtained when a xylanase treatment is included, being the pulp more soluble when combined with a cellulase treatment. The S10 determination comprises the dissolution of hemicelluloses and degraded cellulose being the S10-S18 usually an estimate of low molecular weight (LMW) cellulose (2). The solubility difference reveals that while the cellulase treated pulp have the highest amount of LMW cellulose the xylanase (monocomponent) treated pulps have the lowest after alkaline extraction (X-CCE).

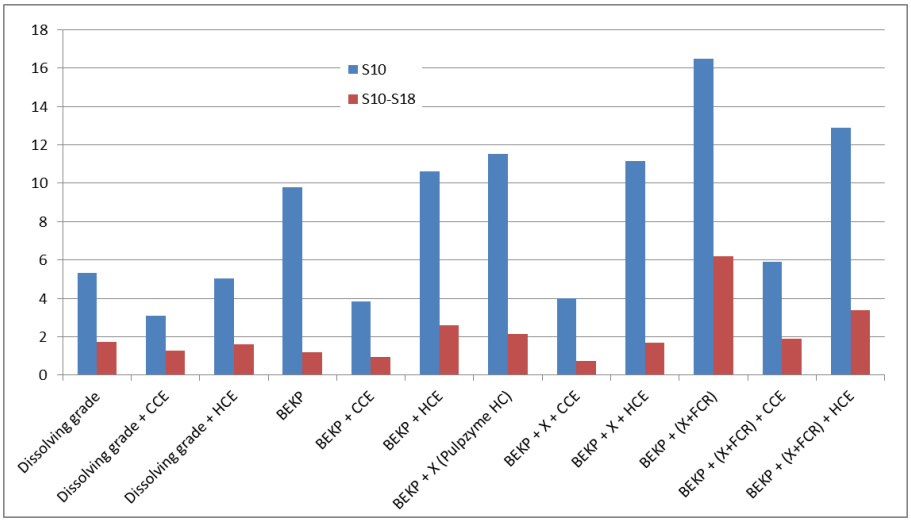


Figure 21 Alkali solubility of pulp at 10% NaOH (S10) and S10-S18 values. Conditions presented in Figure 20.

Using a higher dosage of xylanase the same increased alkali solubility (S18) is obtained before and after the CCE-stage with different NaOH dosages (20-70 g/L), as depicted in Figure 22. On the other hand,

the intrinsic viscosity values of the xylanase treated pulps are always somewhat higher than the controls without enzyme. Therefore, the xylanase treatment up-shifted the average degree of polymerization. This indicates that the X-CCE pulps have a lower amount of xylan and that this degraded remainning amount is more soluble at 18% NaOH (higher S18).

To convert a paper-grade pulp into dissolving grade pulp, the intrinsic viscosity shall be reduced. The use of monocomponent endoglucanase preparations have shown in previous studies to be able to adjust the final intrinsic viscosity and at the same time increasing the reactivity of the dissolving pulp for viscose application (1,3,4).

Three different monocomponent cellulases (endoglucanases) products were tested regarding the control of pulp intrinsic viscosity. The results shown in Figure 23reveal that FiberCare R and FiberCare U decreased the intrinisc viscosity to a similar degree while Novozym 613 hardly had any effect even using a higher dosage.

A higher dosage of enzyme leads to higher decrease of pulp viscosity. This decrease is more pronounced with the more pure dissolving pulp: ca. - 200 vs. - 300 mL/kg.

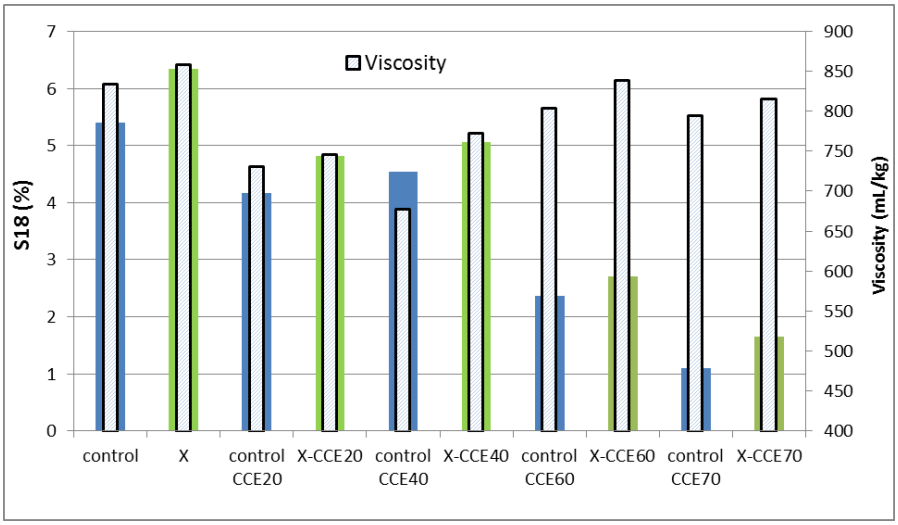


Figure 22 Intrinsic viscosity and alkali solubility of pulp at 18% NaOH (S18). X-stage: 0.30% odp Pulpzyme HC (0.05% odp FiberCare R); 60 ºC; pH 7; 120 min. CCE-stage: 20-70 g/L; 30 ºC; 30 min. Trials run at 10% consistency in sealed polyethylene bags imsersed in a water bath.

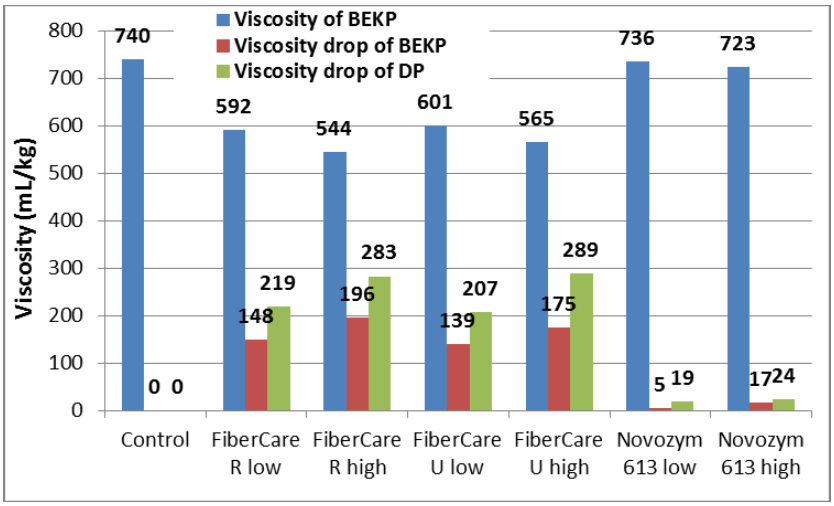


Figure 23 Intrinsic viscosity of pulps. Cellulase treatments at low and high dosage of enzyme (0.02-0.20% odp); pH 7; 60 ºC; 120 min; Trials run at 10% consistency in sealed polyethylene bags imersed in a water bath.

1.1 Conclusion

Several enzymatic fiber modification trials have been conducted with the unconventionally deconstructed materials. Most of the deconstructed materials were readily modified by the cellulase treatments and the prepared handsheets showed improvement in tensile strength. Specifically the Cel45A and Cel7b endoglucanase families demonstrated the ability to improve resultant handsheet physical strength properties, the latter enzyme treatment without sacrificing tear strength. Also the Soda-AQ*E. globulus* fiber (both kappa 15 and 20) showed to be susceptible to enzymatic strengthening, resulting in tensile strengths equal to the otherwise stronger kraft fiber, thus enabling a completely sulphur-free pulping process. Refining trials carried out on the selected Soda-AQ*E. Globulus* pulps did not reveal any significant difference in the physical properties between the kappa 15 and 20 pulps, except in tear strength where the kappa 20 pulp was superior. The enzymatic treatments of these pulps with both Cel45A and Cel7b revealed the need for further process optimization in order to obtain the expected benefits, although large increases in freeness developments were identified as well as increases around 10% in tensile strength after 100 PFI rev.

Furthermore these unconventional pulps were investigated with regards to their susceptibility enzymatic delignification (i.e. bleaching) by the application of oxidoreductases both alone and in combination with mediators. These trials revealed that the high redox-potential laccase from *Polyporus pinsitus* showed synergistic behaviour when combined with violuric acid, reaching a brightness increase of 16 units for the kappa 20 pulp. Also interesting was it that the same absolute brightness values were obtained when kappa 15 and kappa 20 pulps were subjected to the enzymatic bleaching, which can be translated into increased pulp yield and cost savings on chemicals. Data is presented throughout the report which continuously favours the use of this unconventional sulphur-free pulp, especially at kappa 20, with regards to enzymatic strengthening and enzymatic bleaching. The enzymatic delignification system was further optimised with the *Coprinus cinereus* peroxidase combined with violuric acid and it was shown that this system increased brightness by 7 units at a very low dosage of 0,3 mM of violuric acid and 0,5 mM of hydrogen peroxide.

It was also shown that conventional bleached kraft pulp with little effort could be converted into dissolving pulp of a proper grad via enzymatic routes. The application of xylanases and cellulase had positive influence on the solubilities (S10 and S18) and the intrinsic viscosities of the investigated pulps, thus enabeling a regular kraft mill to obtain dissolving pulp grades without the need for a pre-hydrolysis step.

1.2 References

(1) Gehmayr, V.; Schild, G.; Sixta, H. A precise study on the feasibility of enzyme treatments of a kraft pulp for viscose application. Cellulose (2011) 18:479-491.
(2) Sixta, H. Handbook of Pulp. Volume 1. Weinheim: WILEY-VCH Verlag GmbH &Co. KGaA; 2006.
(3) Kvarnlöf, N.; Germgård, U.; Jönsson, L. J.; Söderlund, C.-A. Enzymatic treatment to increase the reactivity of a dissolving pulp for viscose preparation. Appita J (2006) 59:242-246.
(4) Henriksson, G.; Christiernin, M.; Agnemo, R. Monocomponent endoglucanase treatment increases the reactivity of softwood sulphite dissolving pulp. J Ind Microbiol Biotechnol (2005) 32:211-214.

Task 4.2 – Biofuel potential of pre-treated materials and residues

A bench-top procedure was developed to gauge the biogas potential of several raw feedstocks and deconstructed materials from the LignoDeco partners.

4 different raw eucalyptus species and the elephant grass were milled to < 0,5 mm with a Wiley knife mill. An amount of wood powder or elephant grass, equivalent to 0,9 grams of cellulose (based on the

amount of volatile solids, VS), was added to three 1 L laboratory digesters (see Figure 24) for each sample. The deconstructed materials i.e. various kraft eucalyptus clone pulps (kappa 20) and Soda-AQeucalyptus globulus pulp (kappa 20) were homogenized in a food-processor for 15 min, and an equivalent amount based on VS were added to the bioreactors. As a positive control, 0,9 grams of pure crystalline cellulose (Avicel) was added to 3 separate digesters. A third set of three digesters was reserved for the seeding sludge only, in order to determine the background biogas produced by the inoculum itself.

Seeding sludge was provided by Snertinge, Denmark, which was allowed to degas for 3 days at 49°C in order to minimize the background biogas production. 100 g of inoculum was added to the digesters. This results in a ratio of 0,7/0,3 for the inoculum and substrate/cellulose, respectively, based on the amount of volatile substrate. Each digester was then flushed with N_2 for 1 min, and sealed with OxiTop Control AN 6/AN 12 (WTW) and incubated anaerobically for 14 days at 49 °C. The biogas produced results in increased pressure within the digester which is automatically measured by the OxiTop.

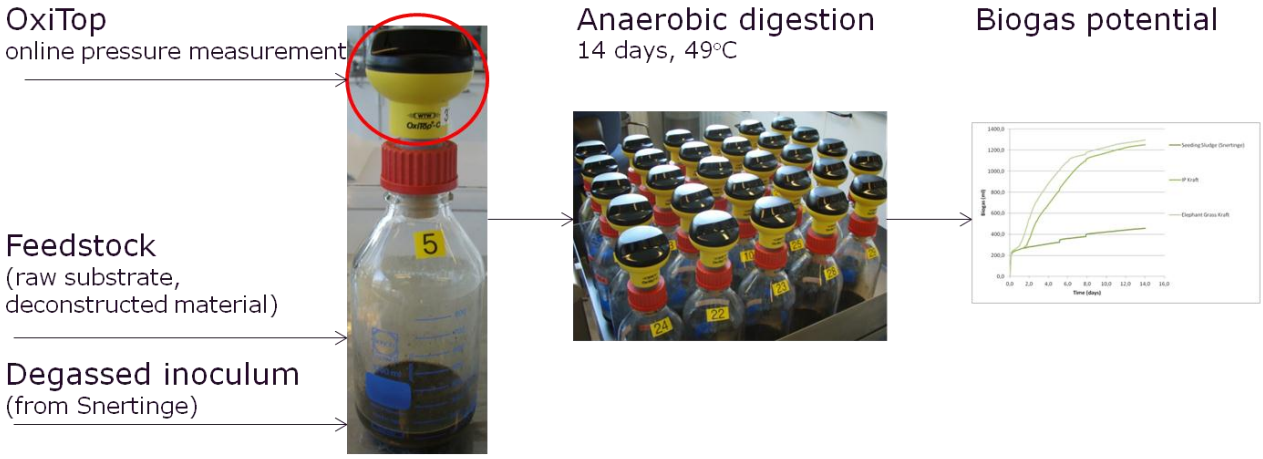


Figure 24 Outline of the procedure developed to gauge the biogas potential of the raw feedstocks and deconstructed materials.

The biogas development over the 14 days of anaerobic digestion at 49°C can be viewed in Figure 25. As is immediately obvious, raw eucalyptus wood presents a rather recalcitrant feedstock for anaerobic digestion. The absolute biogas potential after the 14 days of incubation corresponds to a theoretical carbohydrate conversion between 7-9% for the eucalyptus species, when using the compositional data from the 6th months technical report for these species. This low conversion may be caused by either the presence inhibitory compounds from the lignin fraction of the wood which hinders the microbial degradation of the substrate and/or more probable that the carbohydrate fraction is simply inaccessible to the degradative mechanisms of the microbes. The raw elephant grass did however show to be a suitable substrate for biogas production where the theoretical carbohydrate conversion is 71% and thus appears to be decidedly more bioavaible than the raw eucalyptus wood.

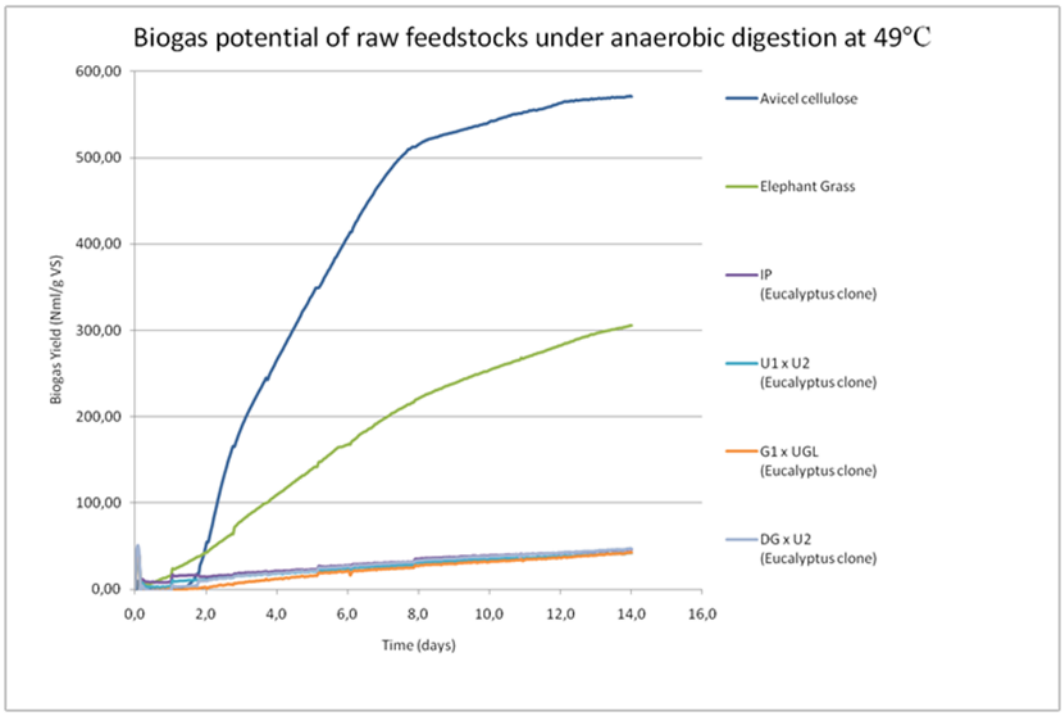


Figure 25 Biogas development over 14 days of anaerobic digestion at 49°C for the raw feedstocks including the positive cellulose control.

The total biogas yield after 14 days of anaerobic digestion at 49°C for the deconstructed materials can be viewed in Figure 26. The pulps investigated were deconstructed to kappa 20 and were mainly kraft pulps. However, *E. glubulus* Soda-AQ(kappa 20) pulp was included in order to identify possible differences between in biogas yield between the two pulping processes. Generally, when comparing the biogas yield of the kraft pulps to the raw feedstocks, it is clear that the deconstruction has had a positive impact on the digestibility. This may be a result of several factors including making the carbohydrate fraction much more accessible for the microbes. Moreover the removal of a large part of the lignin fraction may also have a positive impact on the digestibility of the substrate. Giving that compositional data for these deconstructed materials has yet to be provided, a theoretical carbohydrate conversion cannot be calculated at this point. However when comparing the absolute values for the biogas yield of these deconstructed materials (580-690 Nml/g VS for the eucalyptus species) to the positive cellulose control (570 Nml/g VS) it is clear that the substrates are easily digested. However the higher biogas yield for the pulps may be a result of other volatile gasses (sulfur compounds) released from the pulps and may not represent methane and carbon dioxide arising from the anaerobic digestion of carbohydrates.

A minor contribution from the digestion of protein and fats in the feedstock is also included in the total biogas yield measured, but is considered to be negligible in the conversion calculation due to the small amounts present in the samples compared to the carbohydrate content. The theoretical biogas yield arising from carbohydrate conversion is 750 Nml/g VS and results in 50% methane and 50% carbon dioxide v/v.

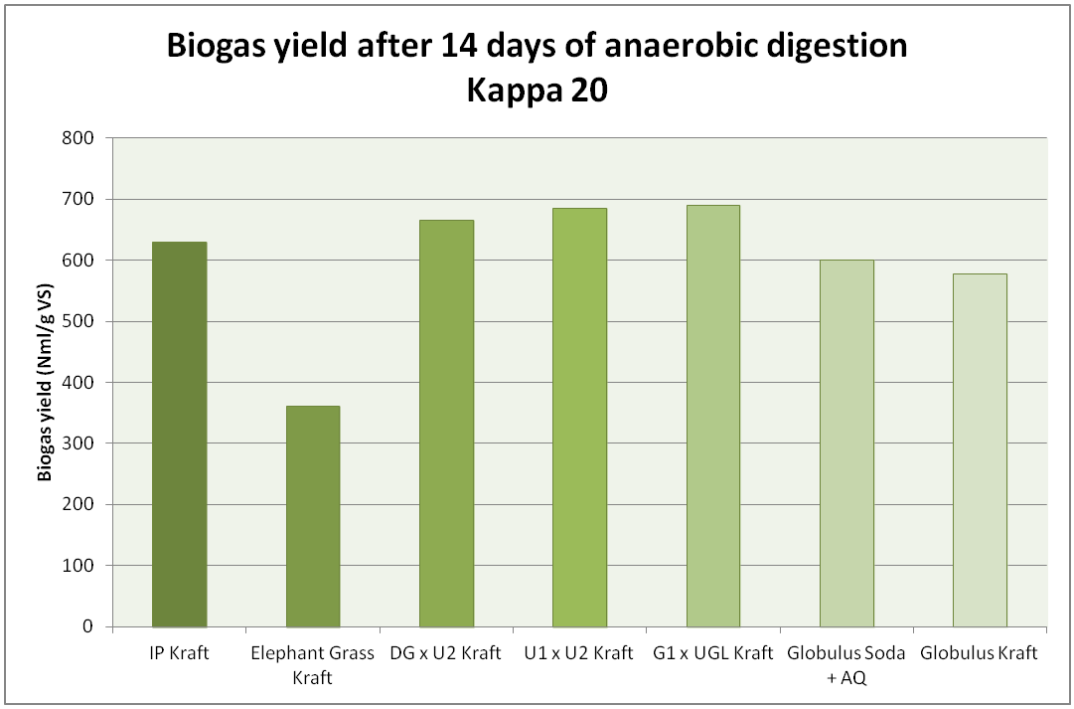


Figure 26 Biogas yield for the deconstructed materials after 14 days of anaerobic digestion at 49°C. All investigated pulps were kraft kappa 20 pulps but also the Soda-AQ*E. globules* kappa 20 pulp were included.

The biogas potential of mill sludge effluent was also investigated. However due to delays of samples (see 3.2. Deviations from the Work Plan) these investigations have not come to an end yet due to the long incubation times associated with these experiments (incubation alone is 14 days). Initial results have however been obtained after two days of incubation, which is clearly not enough to get a proper estimation of the total biogas potential of the mill sludge. Some of the biogas reactors containing the mill sludge and inoculum were also treated with a multicomponent cellulase mixture, a xylanase multicomponent mixture and a protease (and their respective inactivated mixtures). The idea with these treatments is to get a faster release of fermentable sugars from the substrate (and the inoculum itself) to increase the rate of biogas production. The data presented in **Figure 27** shows that in this instance there is no effect of the enzymes on the initial biogas yield. This is can be caused by presence of enzyme inhibitors in the effluent or simply that these are not the optimal blend of enzymes for this specific mill sludge substrate. Another possibility could be to treat the mill sludge separately with for example an laccase to remove aromatic substances which may be inhibitory for the inoculum itself and thereby boost the biogas production.

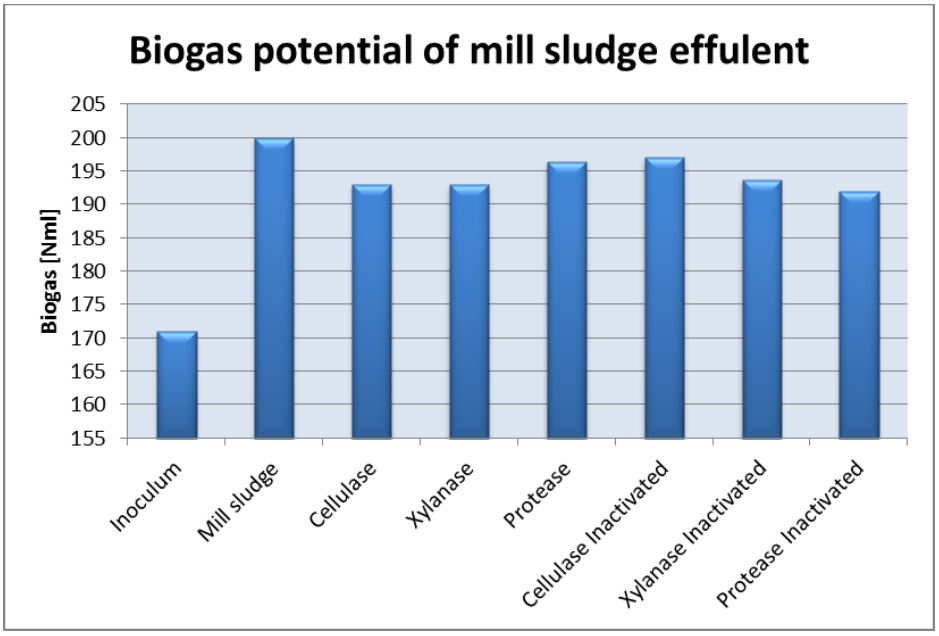


Figure 27 Biogas potential after two days of anaerobic incubation at 49°C. Mill sludge samples were treated with a multicomponent cellulase product, multicomponent xylanase product, a protease and their inactivated versions (1hour incubation at 90°C)

1.3 Conclusion

The investigations of the biogas potential of the various substrates used in LignoDeco revealed that the raw feedstock's themselves were rather recalcitrant and produced very little biogas, except for elephant grass which showed the highest yields. With regards to both the conventional and unconventional deconstructed materials, all showed to be good substrates for biogas production, except the elephant grass which was inferior to the other tested substrates. However it is not advised to produce biogas from these perfectly nice pulped fibres, rather to used waste streams containing these and rejects for biogas production. The investigations of the mill sludge effluent are still inconclusive giving the very short incubation time, which hardly gives off any right evaluation of the potential biogas potential.

3.2. Deviations from the Work Plan

WP4: *Task 4.2, Biofuel potential of pre-treated materials and residues.* All of the different tasks in this WP have been performed as expected with one exception being the investigation of biogas potential of mill sludge effluent. The laboratory investigations concerning this specific task is currently ongoing (initial findings described in this report) and will come to an end the 28th of January (14 days incubation of materials). The reason for this delay is lack of mill sludge effluent from Suzano needed to conduct the trials, which was received the 3rd of January 2013. It appeared that the shipment had both been delayed in the Brazilian and Danish customs which resulted in an unusual delay in delivery.

4. PLANS FOR THE DISSEMINATION AFTER THE 36th months

5. DELIVERABLES AND MILESTONES

DL 2.7 Pre-treatment conditions using selected enzymes
Achieved

DL 4.1 Pulp and papermaking evaluation after optimized pre-treatment *Achieved*

DL 4.3 Setup of application method for anaerobic digestion and evaluation of pre-treated materials for biogas production. *Achieved*

6. NEW CONTACT PERSONS

In case that any of the responsible persons of any of the beneficiaries is replaced for a particular reason, please explain and indicate the name and contact details of the new contact person.

APPENDIX E

SUZANO

Grant agreement no: KBBE-2009-3-244362

Optimized pre-treatment of fast growing woody and nonwoody Brazilian crops by detailed characterization of chemical changes produced in the lignin-carbohydrate matrix

36th Month Periodic Report

Date: 28 February 2012
Partner P5 (Suzano)

Partner name: SUZANO PAPEL E CELULOSE

Author 1 *Juliana Andreotti (SUZANO)*
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Author 3 *Jonatas Carlos Pereira de Araújo (SUZANO)*
Author 4 *Dimas Effgen (SUZANO)*
Author 5 *Fabiano Rosso (SUZANO)*

*Use the font **Times New Roman**. Font size in text is **12 points**. Page numbering is header - right, no numbering on the front page. Line spacing single, 0 point before and after paragraph.*

For those who have been active in research, the appropriate length would be 5–10 pages. For the institutes/companies that have not worked, please report only the appropriate items e.g. plans.

1. SUMMARY OF THE WORK

In this Second Periodic Report Suzano is presented all the work and conclusions of the last 18 months of the Lignodeco project.
The task **T2.1: Chemical deconstruction by alkaline processing** was fully completed in the First Period of the Lignodeco Project: seven materials were analyzed in four different processes, at different conditions. The group selected the best alternatives for each case. For the Second Period of the project, it was necessary to produce more samples to some partners, and Suzano was responsible for that.
Also some new material were used, and new delignification curve was done with it. The new *Eucalyptus globulus* were cooked in two different processes, Kraft and Soda-AQ, at several kappa numbers, in a range from 15 to 70, to develop the delignification curve.
For each cooking trial we analyze kappa number, the alkaline charge, screened yield, rejects content, and analysis to complete the characterization of the cooking, as viscosity, pH at the end of the cooking, residual active alkali, brightness of the pulp, solids content and organic and inorganic content of the liquor at the end of each cooking. A brief comparison between the two *Eucalyptus globulus*, the first material used and this second one, was also performed.
Were also presented the cooking trials done at the $NaOH+O_2$ process, which was produced pulp for the partners at kappa 70. The comparison between the previous results and the new trials are also explained.
After that, were developed the Task **T3.4 Analysis of black liquors and other side streams**, which was assumed for Suzano after the impossibility of developing the task **T5.3 Suzano pulp beatability pilot trials**, as explained before, due to the amount of chips available to do the trial (the pilot plant at Suzano mill is too big for the amount of chips available).
In this new task for Suzano, T3.4, were analyzed the main liquor samples of the processes developed and chosen during the Lignodeco project. After this characterization, applications uses were proposed for each case, considering its main characteristic, contaminant content, economic value in the market and potential use. In this case we considered our experience as a pulp producer, and also our knowledge in the extraction of kraft lignin and development of its market.

2. PROJECTS OBJECTIVE FOR THE PERIOD

The objective of Suzano work during this 18 month period was to evaluate the new *Eucalyptus globulus* material, develop the delignification curve. This is part of the task **T2.1: Chemical deconstruction by alkaline processing**, of the **WP2.: Optimised pre-treatments for woody and nonwoody materials**.
Also was produced sample of the $NaOH+O_2$ process for the partners. In this case was analyzed the most important variables of the process in order to have a complete characterization of the trial done and the sample produced.
The last objective was to analyze the main side streams of each process elected during the Lignodeco project, according to the task **T3.4 Analysis of black liquors and other side streams**. With a complete characterization was proposed the preferable utilization and commercial use of each side stream.

3. WORK PROGRESS AND ACHIEVEMENTS DURING THE PERIOD

3.1. Work Progress

Provide a concise overview of the progress of your work (results and discussion). Report according to WPs and Tasks
- Highlight clearly significant results.
- Explain the reasons for possible deviations from Annex1 and their impact
- as well as corrective actions taken / suggested

Task 2.1 foi WP2: Chemical deconstruction by alkaline processing

Due to preservation problems with the *Eucalyptus globulus*, it was necessary to get more wood chips to proceed with the research at the Lignodeco. UFV have arranged a new sample material for us to do the trials.
Wood chips samples of *Eucalyptus globulus* were evaluated on different types of cooking processes: Kraft and Soda-AQ (soda anthraquinone). The wood chips were delivered at Suzano Mucuri mill by the Pulp and Paper Laboratory of Universidade Federal de Viçosa (UFV). New delignification curves were done in order to generate data to the partners, so they can continue with the research. As all the work were done before, we focus on the development of new delignification curves for the already chosen processes, Kraft and Soda-AQ, also in the range defined as appropriated in each case. No need to redo all the study in all processes.
A brief comparison for the two *Eucalyptus globulus* material was done also, the main characteristics and the pros and cons of each wood sample.
The preparation of chips for cooking was performed identically for all types of cooking simulated. Chips were classified according to standard SCAN-CM 40: 94 [1]. The chip quantity classified between 4 to 6 mm thicknesses was packed in bags made of polyethylene (that prevents moisture exchange with the environment) in the amount established for each cooking and then stored.
Cooking trials were held in a CRS digester (CPS 5010 Recycle Digester System), with 2 individual reactors of 10 liters each, equipped with a forced liquor circulation system and electrically heated with temperature and pressure control. The digester is coupled with a cooling system (Coil System with residual liquor, involved with water at room temperature), to ensure the cooling of the liquor after the cooking simulation.
Several cooking experiments were performed for each sample, using different active alkaline charge expressed as NaOH, to establish the delignification curves for each sample. At this case, as we didn't have enough samples to perform the trials and all the repetitions required for the development of the mathematical model, we based on the previous results with the *Eucalyptus globulus*, and then developed a new delignification curve with these samples. This strategy was proven to be a good one at the end, when the results were obtained and analyzed.
Alkaline content, yield and reject content were estimated for each wood using the previous equations for retrieval of kappa numbers 15, 20, 35, 50 and 70. For the kraft process, we just perform the trials for the kappa numbers 15 and 20, which were elected the most important for this process, as we are analyzing the kraft process with high yield, to achieve a good quality pulp for paper production.
The conditions of the Soda-AQ cooking are: Anthraquinone (AQ): 0,05% wood based, Liquor/Wood Ratio = 4/1, Temperature of the cooking = maximum of 170°C, Time at the temperature = 90 minutes, Time at the temperature = 50 minutes. For the Kraft Cooking, the fixed conditions were Sulfidity (S): 25%, Liquor/Wood Ratio = 4/1, Temperature of the cooking = maximum of 170°C, Time at the temperature = 90 minutes, Time at the temperature = 50 minutes.

Table 1: Main results of the Soda-AQ cooking trials. Kappa number between 15 and 70.

	Alkali Charge	kappa	Screened Yield	Rejects Content	Viscosity	pH	Residual Active Alkali	Brightness	Solids Content	Organic	Inorganic
	%		%	%	dm³/kg		g/L	ISO	%	%	%
NaOH+AQ	25,0	15,0	50,2	0,1	962	13,7	18,0	30,6	10,7	50,3	49,7
	19,0	21,2	51,8	1,7	1083	12,7	11,2	29,8	10,5	56,2	43,8
	15,0	29,9	49,8	7,1	1105	12,4	7,1	22,7	10,4	58,5	41,5
	14,5	36,5	46,4	12,4	1130	12,4	6,8	20,9	10,1	59,0	41,0
	13,5	44,7	44,0	16,5	1148	12,1	5,9	18,1	9,3	60,1	39,9
	13,0	57,5	33,4	29,9	1170	11,7	4,8	16,4	9,2	62,4	37,6
	12,0	73,1	26,9	42,0	1228	11,2	3,1	13,1	8,0	61,0	39,0

The major advantage of using the non-sulfur processes is the simplification of gas handling and black liquor recovery in a mill operating as a biorrefinery. The results of the pulping are good. The beneficial effect of AQ (protection against peeling reactions) was quite significant at the lowest kappa target.

At this time of the project was not possible to generate a good mathematical model for this cooking trial. With few points in the curve, the model was not reliable enough. To do such analysis would be necessary to have more wood sample to generate data. Anyway, it was enough material to give some guidance to the partners to proceed with the research, as planned. The main objective was accomplished.

Kraft Process: Also were performed with the new *Eucalyptus globulus* wood chips cooking trials for the kraft process. For those we prefer to focus on the lower kappa number, between kappa numbers 15 and 20. The main reason for that is that the kraft process is the best choice to produce pulp to paper purposes, so, the lower kappa number will gives us a higher screened yield.

Table 2: Main results of the Soda-AQ cooking trials. Kappa number objective were between 15 and 20.

	Alkali Charge	kappa	Screened Yield	Rejects Content	Viscosity	pH	Residual Active Alkali	Brightness	Solids Content	Organic	Inorganic
	%		%	%	dm³/kg		g/L	ISO	%	%	%
Kraft	22,0	14,9	53,1	0,2	1077,0	13,1	15,1	31,4	12,4	50,5	49,5
	18,0	17,3	53,2	1,1	1083,0	12,8	13,0	26,7	12,1	57,5	42,5
	16,0	20,2	52,5	3,5	1112,0	11,5	7,4	26,3	10,4	59,2	40,8

Comparing the Soda-AQ process with the kraft process for the same wood material, we can observe that the kraft pulping delivers a higher screened yield, and a lower alkali charge is necessary to reach the same kappa level. The Soda-AQ process gives also a good screened yield, with similar results to the kraft.

Figure 1 and 2. Cooking trial for both Globulus at the Soda-AQ process, comparing screened yield results and reject content for each kappa number.

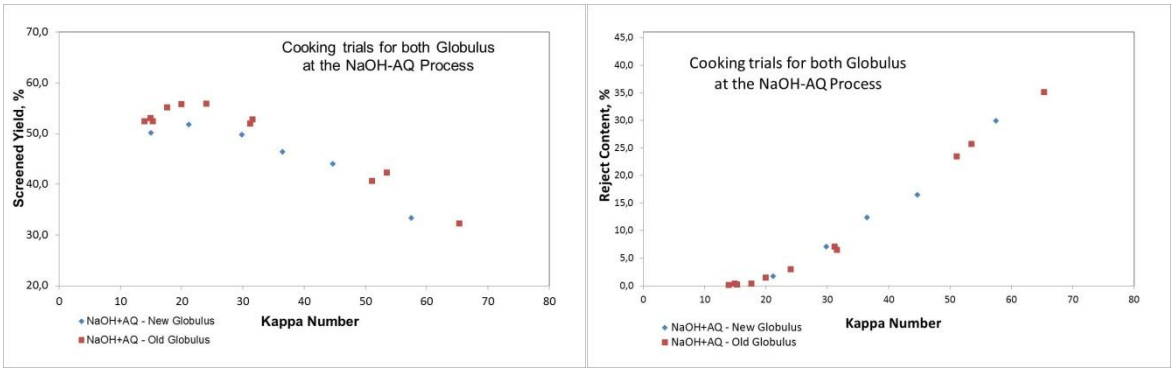
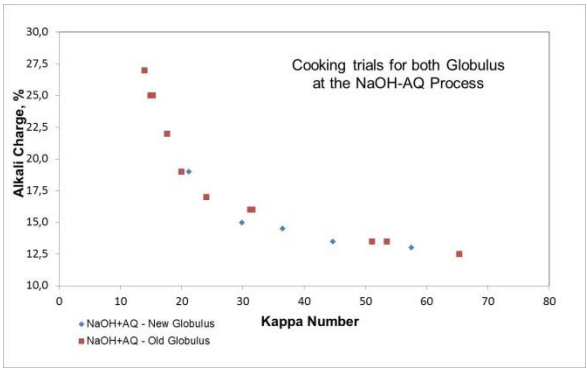


Figure 3. Cooking trial for both Globulus at the Soda-AQ process, comparing alkali charge for each kappa number.



The results for the previous wood material (Figures 1, 2 and 3), can be observed that the new material delivers a slightly lower screened yield, and a higher alkali charge is necessary to reach the same kappa level. The results of rejects content are similar. In general, both materials are good, the differences are small, and with more chips available maybe the optimization of these results could be done to reach the same results of the previous material.

Production of NaOH + O_2 samples for the partners; Analysis of the pulp produced and its characterization

Pulp was cooked in the NaOH+O_2 process, at kappa number 35. It was utilized 7,0% of O_2 in each cooking trial, added in 3 different times, to guarantee excess of oxygen. The time of addiction was after 50 minutes, after 70 minutes and after 110 minutes. The temperature curve applied was: 90 min to reach the temperature of 170°C and more 50 min at the temperature of 170°C. Pulp was sent to the partner without screening, as done before, and as agreed. The CRS equipment was used to perform this trial. The capacity of the digester is 10 liters each. The digester has forced liquor circulation system and electrically heated with temperature and pressure control. A cooling system completes the equipment.

Table 3. Results for the trials of the NaOH+O_2 process

New globulus	Alkaline Charge	Kappa Number	Yield, %	pH	Residual Active Alkali, g/L
NaOH+O_2	30,0	36,1	51,1	12,1	10,1
	30,0	37,4	51,9	12,0	9,1
	30,0	33,9	52,4	12,5	12,0

This new wood material, which is a *globulus* from Brazil, contains more lignin and extractives. This explains that this wood material requires more alkaline charge to reach the same kappa number.

Task T3.4. Analysis of black liquors and other side streams

The original plan for Suzano was to develop the task T5.3. Suzano pulp beatability pilot trials. Between all the partners was agreed to do changes in the plan, and the main reason is related to the wood quantity available to proceed with this trials. As we, at Suzano, have a big pilot plant, would be necessary a lot of pulp sample to complete this task, and this would be difficult to send all the samples from France to Brazil, as the pulp would be produced at CTP facilities. We estimate that the pulp required would be around 500 kg for all trials that we plan, as each batch requires around 80 kg of pulp. There is also no enough wood available.
So, together with the partners, in the last meeting was decided the following: CTP was be responsible do the task T5.3 in its facilities, and Suzano will give assistance in the analysis of the results, as we have experience in the subject.
Another change in the original plan is that Suzano assumed the task *T3.4. Analysis of black liquors and other side streams*. The main idea is to evaluate all the main side streams of the developing process proposed in the Lignodeco project, characterize them, understand their value and propose solutions for their use. Suzano is already producing lignin, in your bench plant, and is well qualified to analyze this side streams and propose destination to it.

Analysis done for this task: kraft and Soda-AQ process black liquors. This characterization was done for each material at each process: Elementary analysis, lower heating value, solids content, organic and inorganic content, Cl, K, Na, Mg, P, SiO_2, residual alkali and pH. The main kappa numbers were chosen for each process, according to the chosen process and material in each case.
For the alternative process developed in the project, also the relevant side streams were analyzed. The same analysis was performed (Elementary analysis, lower heating value, solids content, organic and inorganic content, Cl, K, Na, Mg, P, SiO_2, residual alkali and pH), and some additional analysis was included to complete the entire characterization of each stream (viscosity, S/G ratio, for instance). In each case it was defined after receive the sample for each partner.

The organic composition includes alkali lignin, hydroxy acids extractives, acetic acid, formic acid, methanol, etc. The elemental analyses was carried out using elemental analyzer, atomic absorption spectrometer and ion chromatograph. The organic compounds were measured by GC and py-GC-MS. A number of standard procedures for paper pulp and residual liquor analysis were used including the ones listed below:

Table 4: List of methodologies utilized in the side streams analysis

Analysis	**Methodology**
Black liquor chloride content	Tappi T699 om-87
Black liquor residual alkali	Tappi T625 cm-85
Black liquor sodium content	Tappi T25 cm-85 (adaptation)
Black liquor sulphur content	Tappi 625 cm-85
Black liquor chloride contente	Tappi T699 om-87
Black liquor residual álcali	Tappi T625 cm-85
Ash	Tappi T211
SiO2	Tappi T 244
Metals	SCAN CM 38:96
Wood/black liquor potassium content	Atomic Absorption Spectroscopy
Wood/black liquor high heating value	Tappi T684 om-97
Wood/black liquor elemental analysis	Direct measurement in CHNO equipment - dry samples

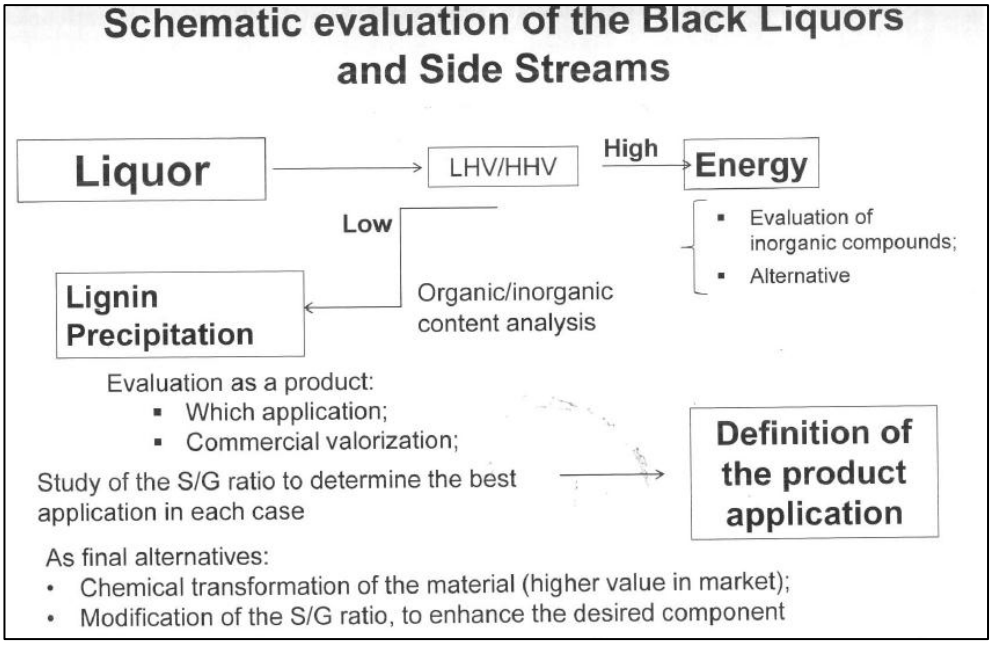
Based on the selected process, we defined which side streams would be important to be analyzed. The criterion utilized was the amount of the side stream produced and relevance in the process. Also, as a comparison, a characterization of the Suzano kraft liquor and kraft lignin was done. The main reason for

this choice was to have a good reference of a consolidated process. So was chosen a side stream that is well known and very much utilized.

Selected material:

- ✓ Liquor NaOH+O_2, *globulus*, kappa 35
- ✓ Soda-AQ Liquor, Elephant Grass, kappa 20
- ✓ Kraft Liquor, Elephant Grass, kappa 20
- ✓ Organosolv Lignin
- ✓ Suzano kraft Lignin, *eucalyptus*, kappa 17
- ✓ Suzano Kraft Liquor, *eucalyptus*, kappa 17

Figure 4: Schematic evaluation of the Side streams: the strategy utilized in the project.



The figure 4 summarizes the way analyzed to define the best use and application for each stream. The division for each use was defined based on the heating value of each sample: if the sample provides a high heating value, it could be a good option for energy purposes. A more complete analysis was done to evaluate the inorganic content. This is important to understand any possible impact in the boiler operation, for instance, as chloride and potassium, which were known for its bad effect at boilers. Otherwise, if the high heating value is not high enough, a detailed analysis of the organic compounds was done. With that, could be understood more its characteristics and the value of the side stream as a product.

To have a full evaluation of the processes, a complete analysis of the compounds of each side stream was done either way: even if the liquor or lignin is good for energy, would be important to evaluate all the main elements and understand if any could cause a bad impact in a boiler, for instance,.

A deeper analysis is the study of the S/G ratio of the lignin, and with that could be defined the product application. As Suzano experience, the S/G ratio could determine the possible application and the value of the final product.

The understanding of the main characteristics of each side stream, and it main destination, as a product development or for energy purpose already fulfill the requirements of the Lignodeco project and complete the task and the deliverables related to this subject. It was also understood that the complete understand of the process was done, and all impacts were considered. With all the information provided

by the Lignodeco study, would be possible to recommend the best process available for each purpose without doubt.

Table 5: High Heating Value of the selected Liquor samples. Lignin content in this sample

Sample Code		**High Heating Value, MJ/kg**	**Lignin, %**
Suzano liquor – kraft process	A	14,7	37,5
	B	14,5	38,1
Average		**14,6**	**37,8**
Black Liquor EG Kraft Kappa 20	A	14,8	38,9
	B	14,9	38,4
Average		**14,9**	**38,7**
Black Liquor EG Soda-AQ Kappa 20	A	16,8	42,1
	B	17,1	41,8
Average		**16,9**	**42,0**
Black Liquor NaOH + O_2 kappa 35	A	11,6	30,8
	B	11,6	30,2
Average		**11,6**	**30,5**

The best liquor for energy purpose, considering the high heating value, is the EG Soda-AQ. This could be explained for the high lignin content, which provides a better HHV. The black liquor from the kraft process, for both materials, Elephant grass and *eucalyptus* from Suzano, results in almost the same HHV. Also the lignin content is very similar, with a slightly higher lignin content for the EG.
The kraft liquor is a consolidated product to generate energy. The use of EG for this purpose in not a reality already, but it is shown that is a good possibility.

Table 6: Solid, Organic and Inorganic content of Liquor samples.

Liquor Samples	Solid Content, %	Organic Content, %	Inorganic Content, %
NaOH+O_2, *globulus*	11,7	45,8	54,2
Suzano Kraft	14,5	51,2	48,8

The lower solid content and higher inorganic content of the NaOH+O_2 explains its lower high heating value, in MJ/kg. This also would impact in higher recovery and recausticizing areas in a mill running in a NaOH+O_2 process.

Kraft Liquor with low kappa number is more suitable to be concentrated and burned then liquors from NaOH + O_2 process. This also confirms the high heating values from the table 5. So at this point also the Kraft Liquor is more suitable for energy purposes than NaOH+O_2 process.

Table 7: High and Low heating value of lignin samples.

Lignin Samples	LHV, MJ/kg	HHV, MJ/kg
Organosolv	23,3	24,8
Suzano Kraft	22,3	23,3

Results for already concentrated lignins. The Organosolv lignin presents a higher HHV than the kraft lignin. Comparing to the concentrated kraft liquor, which HHV is 14,5 MJ/kg, the lignin HHV is very good, with 60% higher number. We should not forget that 14,5 MJ/kg is already considered a good and viable number, as the Kraft process is a reality in large scale.
Considering only the comparison between numbers, this Organosolv lignin is better than the kraft one for energy purposes. Considering also the production cost of the lignin, the Organosolv process is more expensive than the kraft process, so this one is more suitable to be burnt.

Table 8: Elemental Analysis

Sample code	**Elemental Analysis, % BLS**						
	C	**H**	**N**	**O**	**S**		
					Total	**Unbound Sulfur**	**Bound Sulfur**
Black Liquor EG Kraft Kappa 20	37,1	3,7	0,7	36,0	2,9	1,95	0,95
	37,3	3,9	0,7	36,2	3,0	1,91	1,09
Average	**37,2**	**3,8**	**0,7**	**36,1**	**3,0**	**1,93**	**1,02**
Black Liquor EG Soda-AQ Kappa 20	39,0	3,9	0,4	37,5	1,0	0,52	0,48
	39,0	4,0	0,3	37,4	1,1	0,55	0,55
Average	**39,0**	**4,0**	**0,4**	**37,5**	**1,1**	**0,53**	**0,52**
Organosolv Lignin	67,9	5,6	0,2	25,2	0,1	0,03	0,07
	67,7	5,6	0,2	25,4	0,1	0,04	0,06
Average	**67,8**	**5,6**	**0,2**	**25,3**	**0,1**	**0,03**	**0,07**

Organosolv process gives a lignin with low sulfur content. If we compare with kraft process, it would have more sulfur, as sulfur is one of the raw materials applied in the digester.

Considering the elemental analysis of the black liquor, they are very similar for the kraft and soda-AQ process. The main difference is the sulfur content, which obviously is present in the kraft black liquor in a higher amount.

The comparison between black liquor and lignin is unfair: a concentrated material would present a HHV than a diluted one.

Table 9: SG ratio of the lignin samples

Lignin Samples	SG ratio
Organosolv	2,54
Suzano Kraft	2,55

Considering only the comparison between numbers, this Organosolv lignin is better than the kraft one for energy purposes. Considering also the production cost of the lignin, the Organosolv process is more expensive than the kraft process, so this one is more suitable to be burnt.

More results were done, but none in particular were shown to be a problem in the process of the side streams. The complete results are presented in the Appendix.

Conclusions

The new material was evaluated in the kraft and Soda-AQ process. The results were good in both processes. For the kraft process, the *Eucalyptus globulus* presented a good screened yield, but a higher amount of alkali was necessary, comparing with the previous globulus analyzed. Also for the previous material the screened yield was significantly higher.
For the Soda-AQ process, also a higher alkali charge was required to reach the same kappa level, comparing with the previous wood analyzed. The screened yield was lower than expected, with a difference of 4% in some cases, comparing the same kappa number for both wood.
With the results obtained, we conclude that this *Eucalyptus globulus* are good to continue the research and finalize the Lignodeco project, even though the previous material performed in a better way. As additional comment, we highlight that we didn't have as much sample as necessary to complete the optimization of these processes. So maybe producing pulp with this *Eucalyptus globulus* in batch process could be optimized and better results will appear.
It was possible to conclude the task, developing the delignification curve for this new material, to generate data and pulp sample to the partners to continue the study. This was not planned at the beginning, and it was decided to do more trials due to problems with preservation of the wood chips.
The side streams were analyzed, giving special attention to the lignin produced. All the materials could be considered for energy purposes, even though some of them present better high heating value, giving a higher energy production.
The characterization of the organic and inorganic content of each side stream didn't present any material that should be a concern or could hinder the use and application. Only when we think in a high scale process in continuous looping and closed system the inorganic content could hinder the production and jeopardize the investment.
Lignin could be used in several applications, and its sustainable source is only one of the good aspects of it as a product. Also its properties provide several applications and can have a good value as a commercial product.
A further study would be the chemical transformation of the material. For instance, the modification of the S/G ratio, in order to enhance the desired component and customize the side stream as a desired product. An approach like this was not done, but stay as a suggestion for further developments.

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3.2. Deviations from the Work Plan

No deviation from the original plan.

5. DELIVERABLES AND MILESTONES

List the deliverables and milestones you are responsible due during the period covered by the report indicating whether they have been achieved.

Deliverables accomplished:

T3.4 Analysis of black liquors and other side streams

6. NEW CONTACT PERSONS

In case that any of the responsible persons of any of the beneficiaries is replaced for a particular reason, please explain and indicate the name and contact details of the new contact person.

Not applicable

APPENDIX F

CTP

Grant agreement no: KBBE-2009-3-244362
Optimized pre-treatment of fast growing woody and nonwoody Brazilian crops by detailed characterization of chemical changes produced in the lignin-carbohydrate matrix

36th Month Periodic Report

Date: 15 January 2013
Partner 6 (CTP)

Grant agreement no: **KBBE-2009-3-244362**

Project acronym: LIGNODECO

Project title: Optimized pre-treatment of fast growing woody and nonwoody Brazilian crops by detailed characterization of chemical changes produced in the lignin-carbohydrate matrix

Funding Scheme: Collaborative project (small or medium-scale focused research project)

Thematic Priority: KBBE-2009-3

Period covered: From 1 January 2012 to 31 December 2012

Date of preparation: 15 January 2013

Start date of project: 1 January 2010 Duration: 36 months

Partner name: **UFV**
Author 1 *Auphélia Burnet (CTP)*
Author 2 *Michel Petit-Conil (CTP)*

1. SUMMARY OF THE WORK

After the selection of five hybrid eucalyptus clones among nineteen and an elephant grass among three, different deconstructions were performed by the partners of the project in order to manufacture a special paper pulp, bio-fuels and bio-products:

- Alkaline deconstruction (kraft cooking, Soda/ Anthraquinone, Soda/ Anthraquinone/Oxygen),
- Organosolv deconstruction
- Enzymatic deconstruction using oxido-reductases or hydrolases

Different analyses were carried out on these pulps by all the partners. CTP was in charge of measuring the morphological characteristics of different pulps obtained after alkaline deconstruction using a Soda-AQ or Kraft process. These measurements were performed before and after an ODPD bleaching sequence. Among the five selected hybrid eucalyptus clones, the *E. globulus* species seemed the most interesting: its fibres were the longest, the most flexible with a high bonding potential and the pulps contained few vessels only (positive effect on the paper mechanical properties and on the paper printability). The Soda-AQ cooking seemed more interesting than the Kraft cooking with fewer vessels.
The other analyses performed by the partners of this project have also demonstrated that the *E. globulus* species were the most interesting for the production of a special paper pulp. The cooking and bleaching results carried out in laboratory were validated at pilot scale.
The Soda-AQ pulp could replace the Kraft pulp. This pulp was less polluting (sulfur-free process); the unbleached pulp brightness was higher with lower vessels content. Conversely, the mechanical properties of this bleached pulp were slightly lower. Nevertheless, according to UFV laboratory trial, the introduction of xylans in this pulp improved these properties.

The enzymatic deconstructions using oxido-reductases or hydrolases performed in laboratory were also validated on the CTP's pilots. Nevertheless, the productions of bioethanol at big scale were not conclusive.

2. PROJECT OBJECTIVES FOR THE PERIOD

The objectives of this period were to perform enzymatic deconstruction using oxido-reducates and hydrolases and alkaline deconstruction, bleaching and refining at pilot scale in order to validate the laboratory results.

3. WORK PROGRESS AND ACHIEVEMENTS DURING THE PERIOD

3.1 Progress on WP1

Task 1.2: General characterisation of the lignocellulosic feedstocks
During WP1, different morphological and dimensional analyses (MorFi, CyberMetrics and **MorFi** wall thickness) were performed on Eucalyptus hybrids samples **and** on elephant grasses in order to help in the choice of the most promising raw materials to be used in WP2 for deconstruction pre-treatments.
Eighteen wood samples from seven year old eucalypt hybrids, including a number of double/triple/fourth crossings, were collected from an experimental station located near the city of Belo Oriente, Minas Gerais, Brazil.
The wood samples were "pulped" with the conventional method using acetic acid/hydrogen peroxide in order to individualise the fibres for analysis.

Table 1 : Eucalypt hybrids selected for the project.

1	U1xU2	*E. urophylla (Flores IP) x E. urophylla (Flores IP)*	10	G1xGL2	*E. grandis (Coffs Harbour)x E. globulus (R)*
2	U2xC1	*E. urophylla (Timor) x E. camaldulensis (VM1)*	11	DGxC1	*[E. dunnii* (R) x *E. grandis (R)] x E. camaldulensis (VM1)*
3	G1xUGL	*E. grandis (Coffs Harbour) x [E. urophylla (R) x E. globulus (R)]*	12	U2xGL1	*E. urophylla (Timor) x E. globulus (R)*
4	U1xUGL	*E. urophylla (Flores IP) X [E. urophylla (R) x E. globulus (R)]*	13	DGxGL2	*[E. dunnii* (R) x *E. grandis (R)] x E. globulus (R)*
5	U1xC2	*E. urophylla (Flores IP) x E. camaldulensis(VM2)*	14	U1xD2	*E. urophylla (Flores IP) x E. dunnii (R)*
6	C1xC2	*E. camaldulensis (VM1) x E. camaldulensis (VM1)*	15	IP	E. urophylla (IP) x E. grandis (IP)
7	DGxUGL1	*[E. dunnii* (R) x *E. grandis (R)] x [E. urophylla (R) x E. globulus (R)]*	16	VC	Veracel Celulose
8	DGxU2	*[E. dunnii* (R) x *E. grandis (R)] x E. urophylla (Timor)*	17	CC	Cenibra Celulose
9	C1xUGL	*E. camaldulensis (VM1) x [E. urophylla (R) x E. globulus (R)]*	18	U xG	*E. urophylla x E. grandis*

Based on dimensional and morphological characterization of the fibres and the vessels, eucalypt hybrid [*E. dunnii* (R) x *E. grandis* (R)] x *E. urophylla* (Timor): DGxU2 seemed to be the most interesting raw material for pulp manufacture. It presented the lowest vessels content and the longer and flexible fibres with a high bonding potential.
Longer fibres develop better the strength properties of the final paper. Vessels negatively influence pulp quality and could generate speckles and picking problems during printing of the final paper.

The other eucalyptus hybrids selected for the deconstruction work due to their high forest productivity and their chemical composition had also interesting characteristics:

- ***E. grandis* (Coffs Harbour) x [*E. urophylla* (R) x *E. globulus* (R)]: G1xUGL sample had long fibres and low content of broken fibres and fines: the bonding potential was higher than this of U1xU2 sample.**
- ***E. urophylla* (Flores IP) x *E. urophylla* (Timor): U1xU2 sample had long flexible fibres and low content of broken fibres and fines.**
- ***E. grandis* (IP) x *E. urophylla* (IP): IP fibres had a low cell wall thickness and high hydrogen bonding potential.**

According to morphological characteristics, elephant grass *Pennisetum purpurem* (150 days) (EG1) chosen for WP2, had the longest fibres with highest hydrogen bond potential and lowest vessels content than both other samples.

Table 2 : High-productivity elephant grasses selected for the project.

EG 1	*Pennisetum purpureum (150 days)*
EG 2	*Pennisetum americanum (60 days)*
EG 3	*Pennisetum americanum (150 days)*

3.2 Progress on WP2

Task 2.3: Enzymatic deconstruction using hydrolases

Before performing enzymatic deconstruction at pilot scale in the MSD Pressafiner, the different partners decided to carry out mechanically pre-treatment in the MSD Pressafiner on the elephant grass *Pennisetum purpurem* (150 days) (EG1) without addition of enzymes at the end of the screw. This treated raw material was sent to IRNAS and CIB in order to perform different enzymatic tests and

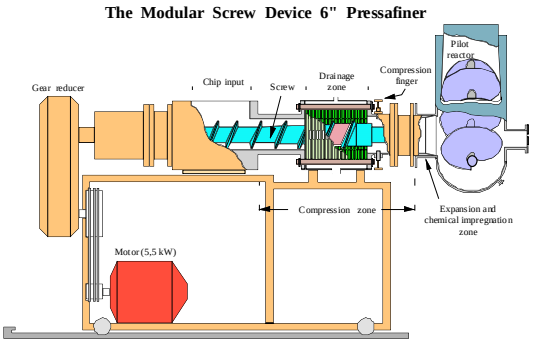


Figure 28 : MSD 6'' Pressafiner (Modular Screw Device Pressafiner)

to determine the best conditions to be applied for further treatments. These optimal conditions were also tested in the MSD Pressafiner at big scale (cf paragraph 3.5).

3.3 Progress on WP3

Task 3.1: Fibre morphology and strength

The different eucalyptus raw materials, selected in WP1, were treated with Kraft (as a reference) or Soda/ Anthraquinone cooking process in order to obtain pulps at kappa 20 and 15. Morphological characteristics of the corresponding pulps were measured in order to determine which eucalyptus pulp had the best fibres morphological characteristics and which process was the most interesting.

The comparison of morphological analysis results obtained before and after cooking was not possible because the chemical treatment applied on the wood to determine the initial fibres characteristics affected the fibres (there was already a chemical impact). However, results obtained on the Soda/ Anthraquinone pulps confirmed tendencies observed on the raw materials, except for the vessels.
DGxU2 eucalypt hybrid seemed to be one of the most interesting raw material for pulp manufactured with Soda-AQ process.
E. globulus raw material was also interesting for the pulp manufacture. After Soda-AQ cooking, the fibres were the longest, the most flexible with a high bonding potential and this pulp contained fewer vessels.

On the other hand, after Kraft cooking, tendencies observed on pulps were not similar as tendencies observed on the raw materials.
IP and *E. globulus* eucalyptus woods were the most interesting raw materials for Kraft pulp manufacture. IP pulp presented the longest and flexible fibres with a high bonding potential, and *E. globulus* pulp has the lowest vessels content and the longest fibres.

The best process depended on the raw materials. Soda-AQ cooking seemed to be the most interesting process for the manufacture of *E. globulus* and DGxU2 pulps. This cooking induced a reduction of vessels content and an improvement of the fibre flexibility and seemed to reduce the broken fibres content compared to the Kraft process.
On the other hand, Kraft cooking was the most interesting process for the manufacture of IP eucalyptus pulp. The IP Kraft pulp contained longest fibre with bonding potential and lower vessels content than the IP pulp manufactured with Soda-AQ process.

The morphological analysis on elephant grass pulp (EG1) showed that Soda-AQ process was slightly more interesting than Kraft cooking. This process allowed reducing vessels content in the pulp and fibres were more flexible. However, hydrogen bonding potential was higher after Kraft process.

3.4 Progress on WP4

Task4.1: Pulp characteristics and papermaking evaluation

Morphological characteristics of the bleached eucalyptus pulps were studied in order to determine which eucalyptus pulp had the best morphological characteristics and to determine the influence of bleaching after the different cooking processes.

Cooking process (Soda-AQ or Kraft) and kappa number of unbleached pulp (20 or 15) did not seem to have impact on mean area-weighted fibre length, mean fibre curl index, broken fibres content, fines content of the bleached pulps.
On the other hand, vessels content was lower after Soda-AQ cooking than after Kraft cooking and a higher delignification of unbleached pulp (reduction of kappa number 20 to 15) induced (i) a

reduction of mean fibre width, the hydrogen bonding potential,(ii) the increase in fibrillation of the bleached fibres and (iii) reduction of vessels content in the bleached pulp.
Consequently, Soda-AQ process seemed to be the best process for the production of special pulp, due to reduction of vessels content in the pulp which could reduce speckles and picking problems during the printing of the paper.
However, it was more difficult to determine the best kappa number of the unbleached pulp because the reduction of fibre width, the hydrogen bonding potential should have a negative impact on mechanical properties of the final paper. However, the increase in fibrillation should improve mechanical properties and the reduction of vessels content should decrease speckles and picking problems.
***E. globulus* seems to be the best eucalyptus species for pulp manufacture because this pulp contained the longest fibres (this pulp should have the best mechanical properties) and fewer vessels (probably less speckles and picking problems).**

After bleaching, morphological characteristics of elephant grass EG1 pulps (such as fibre length, relative bonded area index, fines content and vessels content) were equivalent for each pulp. On the other hand, the reduction of kappa number during cooking reduced the flexibility of the bleached fibres. Consequently, Soda-AQ process seemed as interesting as Kraft process for the production of elephant grass pulp. Bleached pulp manufactured at kappa number 20 should have better mechanical properties than pulp at kappa number 15.
The bleaching of the pulp reduced the fibre length, broken fibres content, the hydrogen bonding potential, flexibility of the fibres, vessels content and increased fines content.

3.5 Progress on WP5

Task 5.2: CTP enzymatic pre-treatment and bleaching pilot plant trials

Enzymatic deconstruction using hydrolases and oxido-reductases
Enzymatic deconstructions were performed at pilot scale with optimal conditions determined by the other partners.

Enzymatic deconstruction using hydrolases
The enzymatic deconstruction using hydrolases was performed on the selected elephant grass (*Pennisetum purpureum* (150 days) (EG1)). This non woody lignocellulosic feedstock was mechanically treated on the MSD Pressafiner (Figure 28) with energy consumption of 72.2 kWh/t in order to open the raw material structure and facilitate the penetration of the enzymes (cellulase, called AS) into the structure.
This trial was compared with similar trial without addition of enzyme. These trials were performed in the following conditions.

	Control	AS
Elephant grass, kg	2	
Cellulase NS22086, ml/kg of raw material	100	
Temperature,°C	50	
Retention time, h	24	
L/W	13.3	13.3
Final pH	6.3	4.5
Yield,%	88.4	86.4

Table 3 : Conditions using during enzymatic deconstruction with hydrolases

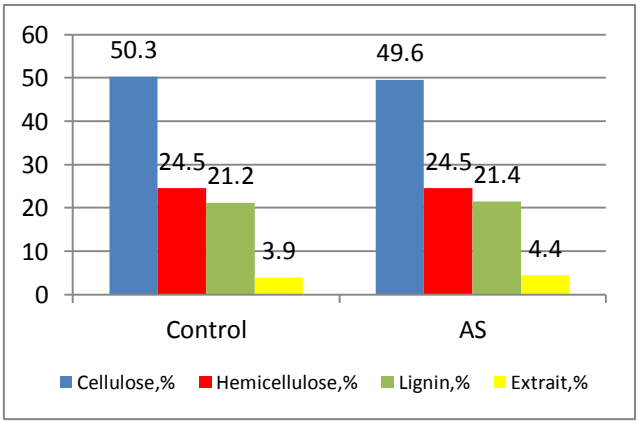


Figure 29 : Chemical composition of the elephant grass pulp after enzymatic deconstruction using hydrolases

According to Figure 29, percentage of cellulose was slightly lower in the AS pulp than in the control pulp.
Both pulps were sent to VTT in order to perform saccharification and fermentation for the production of bio-ethanol. However, according to results obtained by VTT, the enzymatic treatment was less efficient at pilot scale than in laboratory.

Enzymatic deconstruction using oxido-reductases

The enzymatic deconstruction using oxido-reductases was performed on the selected eucalyptus hybrid clone (*E. globulus*). This raw material was defiberised in a pressurised refiner with energy consumption of 393 kWh/t in order to open the structure and facilitate the penetration of the enzymes (figure 3).
After this defibering step, the feedstock was treated with laccases and a mediator before an extraction stage in a spefic reactor (figure 4) This treatment was repeated four times and was compared with similar treatment without mediator and without laccases and mediator:

- LM-Ep-LM-Ep- LM-Ep-LM-Ep
- L-Ep-L-Ep- L-Ep-L-Ep
- Control-Ep-Control-Ep- Control-Ep- Control -Ep

Figure 30: Pressurised refiner to destructure the eucalyptus wood

Figure 31: Reactor to carry out enzyme treatment

The conditions used in the reactor were presented in the Table 4.

	LM	L	Control	Ep
Temperature,° C	50			80
Retention time, h	20			1.5
Consistency,%	10			10
Buffer, mM	50			-
O_2, bar	2			-
Laccase 51003, ml/kg of raw material	50		-	-
Methyl syringate,%	3	-	-	-
NaOH,%	-	-	-	1
H_2O_2,%	-		-	3

Table 4 : Conditions used during enzymatic deconstruction with oxido-reductases

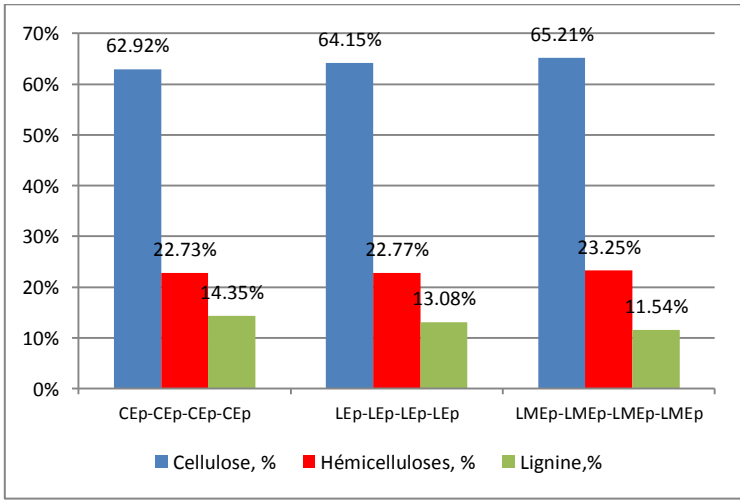


Figure 32 : Chemical composition of the eucalyptus pulp after enzymatic deconstruction using oxido-reductases

Laccase –mediator treatment induced a reduction of lignin (~-20%).

Microscopy examination and fibre morphological characteristics of the pulps obtained before and after the different treatments were analysed.

Figure 33: Microphotograph of defiberised pulp

Figure 34: Microphotograph of control pulp

Figure 35: Microphotograph of laccase-treated pulp

Figure 36: Microphotograph of laccase/mediator-treated pulp

According to FiguresFigure 33 to 9, treated pulps contained higher fine elements and fibrillated fibres than refined pulp (pulp before treatment). However, the comparison between the different treated pulps was difficult and MorFi analysis did not show difference between these pulps.

C*hemical deconstruction by alkaline processing*

Manufacture of unbleached pulp with elephant grass

Unbleached elephant grass pulp was manufactured at pilot scale in the CTP cooking pilot plant (Figure 37).
Normally, this production should be performed with conditions determined by Suzano in laboratory. However, the delivered raw material was not in sufficient quantity to carry out the cooking trial with a Liquor/Wood ratio of 4. A cooking ratio of 7 was only possible with this feedstock. In these conditions, liquor was able to recirculate on the elephant grass in the digester.

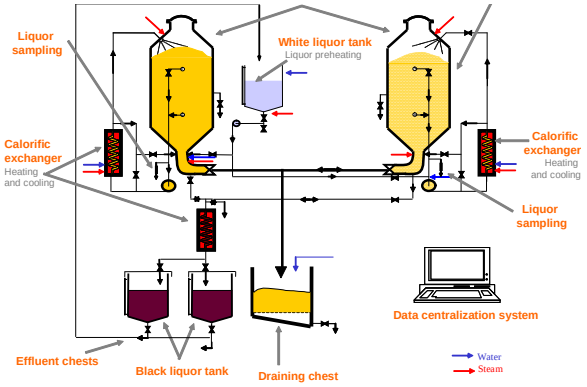


Figure 37 : Cooking pilot plan

Consequently, trials were performed in laboratory in order to determine the conditions necessary to obtain a Soda/ Anthraquinone elephant grass pulp at kappa number of 20.

Table 5 presents the conditions determined.

The reduction of active alkali (17.5 versus 15. 5) and the increase in L/W ratio allowed to obtain similar results as Suzano ones (Table 5).
Then these new conditions were used at pilot scale (Table 5). However, after pilot trial, kappa number of the elephant grass pulp was lower than pulp produced in laboratory (kappa number: 17).

	Suzano conditions	CTP conditions
Temperature, °C	170	
Time to reach temperature, min	90	
Time at temperature, min	50	
AA,%	17,5	15.5
L/W	4	7
AQ, %	0.05	0.05
Kapp number after cooking	20	

Table 5 : Cooking conditions determined at the laboratory for cooking trial

This problem came from the raw material. A part of the raw material was contaminated by fungi. Laboratory trial was performed with uncontaminated feedstock (Figure 38) whereas pilot trial was carried out with uncontaminated (Figure 38) and contaminated raw material (Figure 39).This difference of raw material was identified only after pilot trial when raw material used during pilot trial was compared with feedstock used at laboratory.

Figure 38 : Uncontaminated elephant grass chips

Figure 39 : Contaminated elephant grass chips

Cooking with the contaminated elephant grass (Figure 39) was performed in laboratory with same conditions as first laboratory and pilot trials (Table 5). After cooking, kappa number of this pulp was 13.5. These results confirmed that contaminated raw material modified the behavior during cooking, facilitating the lignin extraction

This unbleached elephant grass pulp produced at pilot scale was sent to UFV for xylan extraction. The extracted xylans should be used during the refining of Soda/ Anthraquinone *E. globulus* pulp. However, the transport of raw material from France to Brazil took a very long time and did not allow to produce the xylans from this pulp. Consequently, UFV manufactured the needed quantity of xylans at laboratory before sending to CTP.

Manufacture of unbleached pulp with eucalyptus

The manufacture of unbleached pulp with eucalyptus selected in WP1 (*E. Globulus*) was performed at pilot (Figure 37) with conditions determined by Suzano (Table 6). Kappa numbers obtained at pilot was close to those obtained at laboratory.
Analyses were performed on pulp (Figures 13 and 14) and on black liquor (Table 7) .

	Soda/AQ	Kraft
Temperature, °C	170	
Time to reach temperature, min	90	
Time at temperature, min	50	
Alkali charge,%	21.5	16
L/W	4	4
AQ, %	0.05	0
Kappa number after cooking	21	21

Table 6 : Cooking conditions used at pilot scale

Brightness of Soda-AQ pulp was higher than this of Kraft pulp, maybe due to the lower formation of cathecol groups. However, polysaccharides seemed more degraded during Soda/ Anthraquinone process (Figure 40).

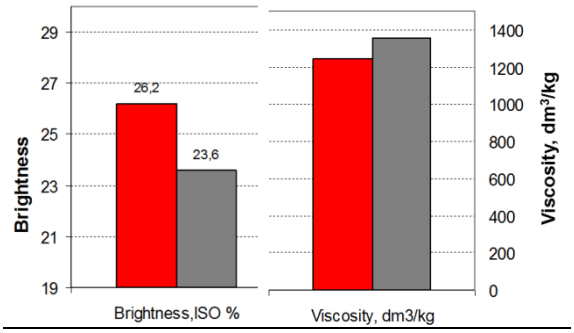


Figure 40 : Brightness and viscosity of unbleached *E. Globulus* pulp manufactured with Soda/AQ or Kraft process at pilot scale

Soda-AQ and kraft pulps had similar morphological fibres characteristics, except for curl index and vessels content.
Soda/Anthraquine had lower vessels content.

Soda/Antraquinone pulp seemed slightly more flexible than kraft fibre. Flexible fibre will develop better the strength properties of the final paper.

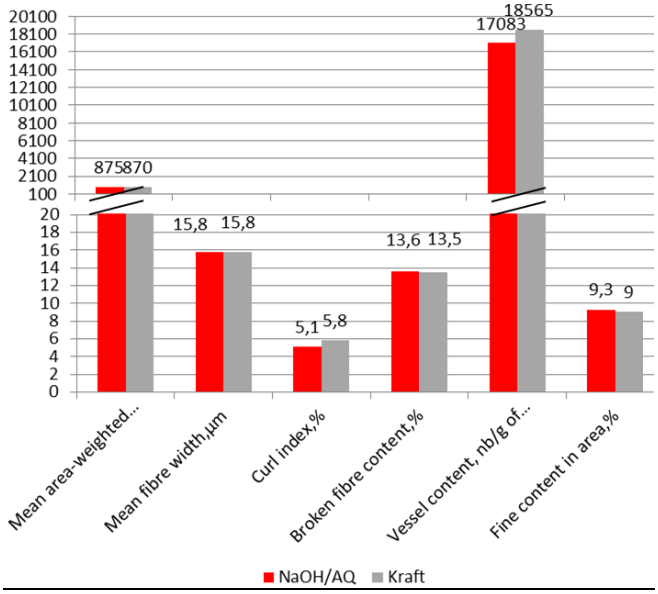


Figure 41 : Morphological fibres characteristics of unbleached *E. Globulus* pulp manufactured with Soda/AQ or Kraft process

Table 7 shows results obtained on black liquor for the both processes. Black liquor of Soda-AQ cooking contained lower sulfur ion than Kraft process.

Table 7 : Black liquor analysis of Soda-AQ and Kraft cooking performed at pilot scale

	Heating value, MJ/kg.dry	C,%	H,%	N,%	S,%	Na,%	K, mg/kg	Si, mg/kg	Cl, mg/kg
Soda-AQ	15.1	43.70	4.18	0.05	0.16	14.40	1018.5	519.57	102
Kraft	15.9	43.98	4.15	0.04	2.84	14.60	922.6	705.57	91

Bleaching

The unbleached *E.globulus* pulps were bleached in the CTP bleaching pilot plant (Figure 42).

Normally, during the trial, conditions determined by UFV should be used. However, during the first O stage, problem occurred with sodium hydroxide pump and consequently lower caustic soda content was added.

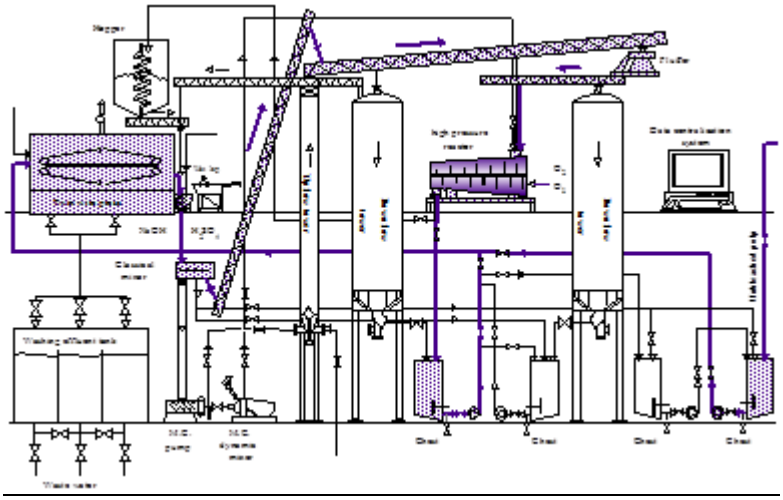


Figure 42 : CTP Bleaching pilot plant

This reduction of sodium hydroxide content during O stage induced a lower delignification. Consequently, chloride dioxide content was increased during both D stages in order to compensate the lower delignification. Different analyses were performed after the bleaching sequence on both pulps (Figures 16 to 18).

Brightness of the pulps and morphological fibres characteristic were similar after bleaching sequence, except curl index (Figure 44). After bleaching, Kraft pulp fibres seemed slightly more flexible than Soda/ Anthraquinone pulp fibres.

Opacity of pulp produced with Kraft process was lower than Soda-AQ pulp one.

	O	D	P	D
Consistancy,%	10	10	10	10
Temperature,°C	100	85	85	70
Time,min	60	120	120	120
O_2,kg/odt	20	-	-	-
Kappa factor	-	0.2	-	
ClO_2,kg/odt	-	**9.9**		**12.0**
H_2O_2, kg/odt	-	-	5.0	
NaOH,kg/odt	20	-	8.0	
pH		2.5		3.5

Table 8 : Bleaching conditions used at pilot scale on the *E.globulus* Soda/AQ and kraft pulps with .

For viscosity measurement, trend observed in unbleached pulp was confirmed in bleached pulp. Soda-AQ process degraded more the polysaccharides than Kraft cooking, inducing lower mechanical properties of the final paper (Figure 18). Nevertheless, laboratory trial performed by UVF showed that the introduction of xylans in pulp allowed to improve mechanical properties.

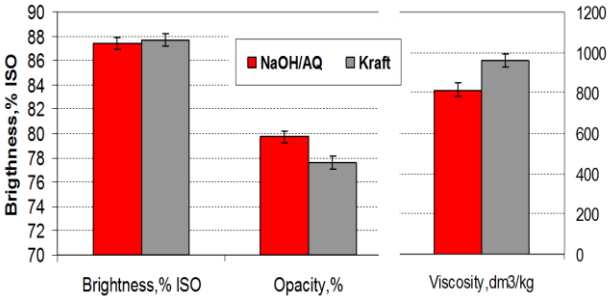


Figure 43 : Brightness, opacity and viscosity of bleached *E. Globulus* Soda/AQ or Kraft pulps

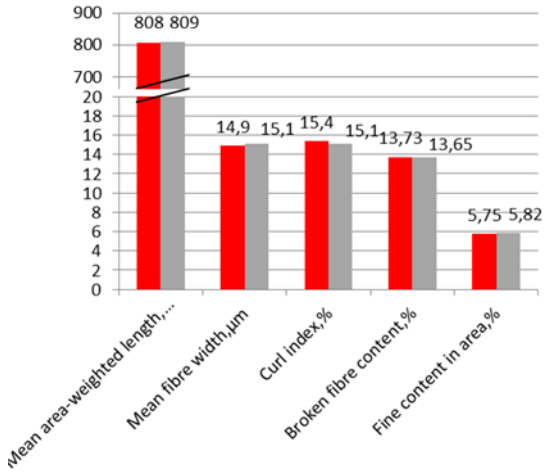


Figure 44 : Morphological fibres characteristics of bleached *E. Globulus* Soda/AQ or Kraft pulps

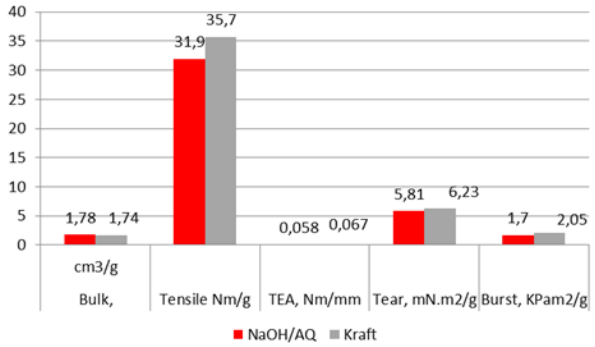


Figure 45 : Mechanical properties of bleached *E. Globulus* Soda/AQ or Kraft pulps

Task 5.3: Pulp refining pilot trials

The bleached Soda/Antraquinone and Kraft pulps were refined at pilot scale.

In the project research programme, Suzano was responsible of this task. However, due to the lower quantity of *E. globulus* wood, this task was transferred to CTP. Suzano needed 80 kg (o.d) of pulp to perform refining demonstration whereas CTP needed only 5 kg (o.d) of pulp.

Refining trials were performed with a simple disc refiner (Figure 19).

Before comparing refining trial on the bleached Soda-AQ and Kraft pulps, different tests were performed in order to identify the best refining conditions.

Three refining intensities by adapting the specific edge load (0.1, 0.2 or 0.4 W.s/m) were tested in conditions described in Table 9.

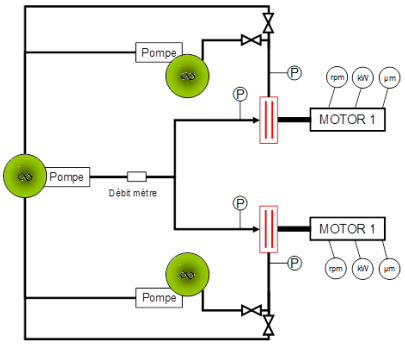


Figure 46 : Simple disc refiner pilot

Temperature,°C	40
Pulp consistency,%	4
Flow,m^3/h	7.5
Rotation speed, tr/min	1500

Table 9 : Refining conditions

	angle	a (1/16")	b (1/16")	c (1/16")	Length of cut blade (km/tr)	Length of cut blade at 1500 tr/mn (km/s)	Refining intensity, Ws/m
Rotor	5	2	2	3	0.82	20.50	0,4
Stator	5	2	2	3	0.82	20.51	0,4
Rotor	5	1.5	1.5	2	1.77	44.25	0.2
Stator	5	1.5	1.5	2	1.77	44.25	0.2
Rotor	15	1	2	5	3.80	95.50	0.1
Stator	15	1	2	5	3.80	95.50	0.1

Table 10 : Refining disc characteristic

The following figures present mechanical properties of bleached Soda-AQ pulp refined with different refining intensities versus energy consumption. Properties were also examined in annex 1 versus drainage index.

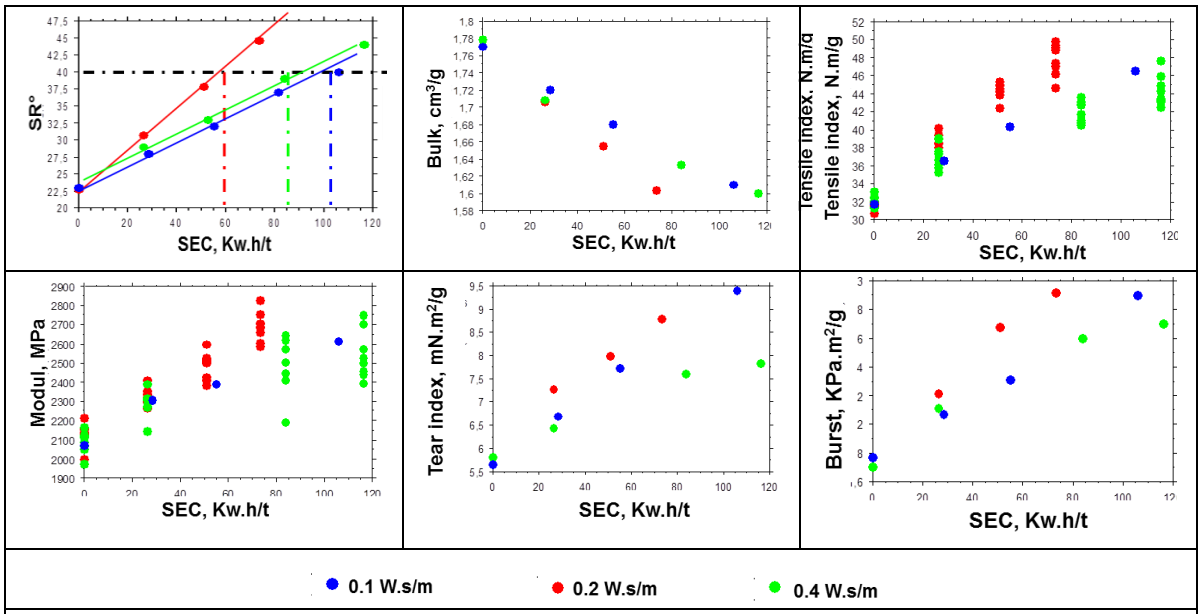
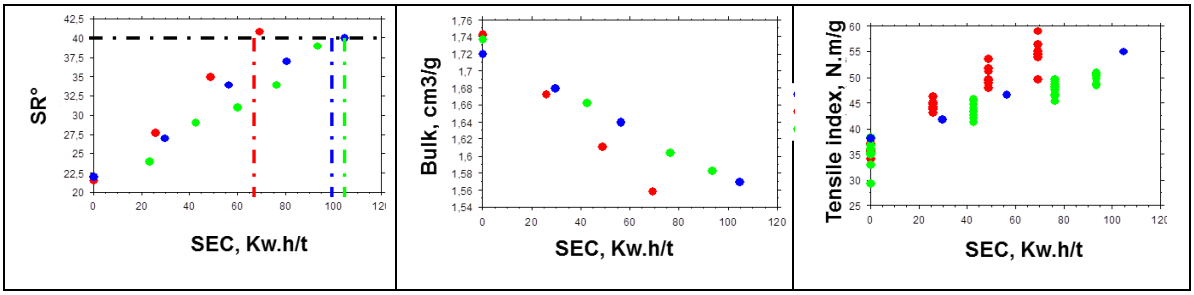


Figure 47 : Mechanical properties of bleached Soda/AQ pulp refined with different refining intensities.

For similar energy consumption, the best mechanical properties were obtained with 0.2 Ws/m refining intensity, because the eucalyptus chemical pulp fibres were quite amenable to fibrillation and therefore to refining.

The following figures present mechanical properties of bleached Kraft pulp refined with different refining intensities versus energy consumption. Properties were also examined in annex 2 versus drainage index.



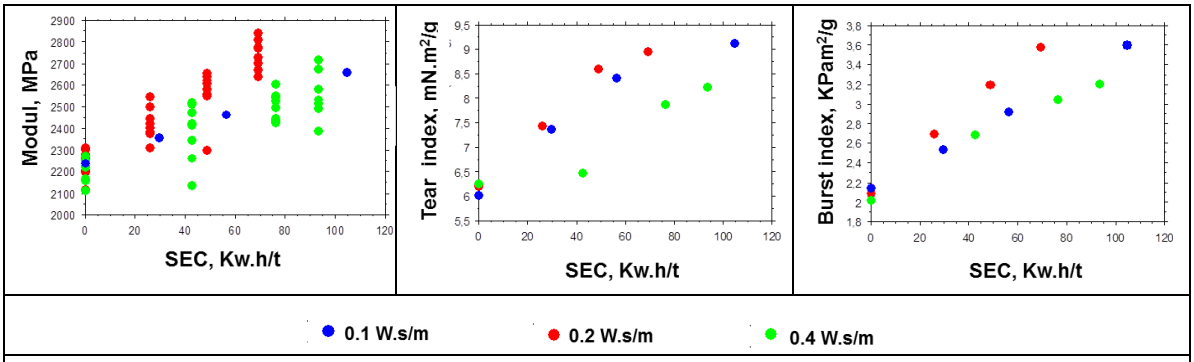


Figure 48 : Mechanical properties of b bleached Kraft pulp refined with different refining intensities.

Similar results were obtained for the Kraft pulp. The best mechanical properties were obtained with 0.2 Ws/m refining intensity.

The refining of Soda/Anthraquine was compared to the refining of Kraft pulp (Figure 49) in the best refining conditions (0.2 Ws/m refining intensity). Properties were also examined in annex 3 versus drainage index.

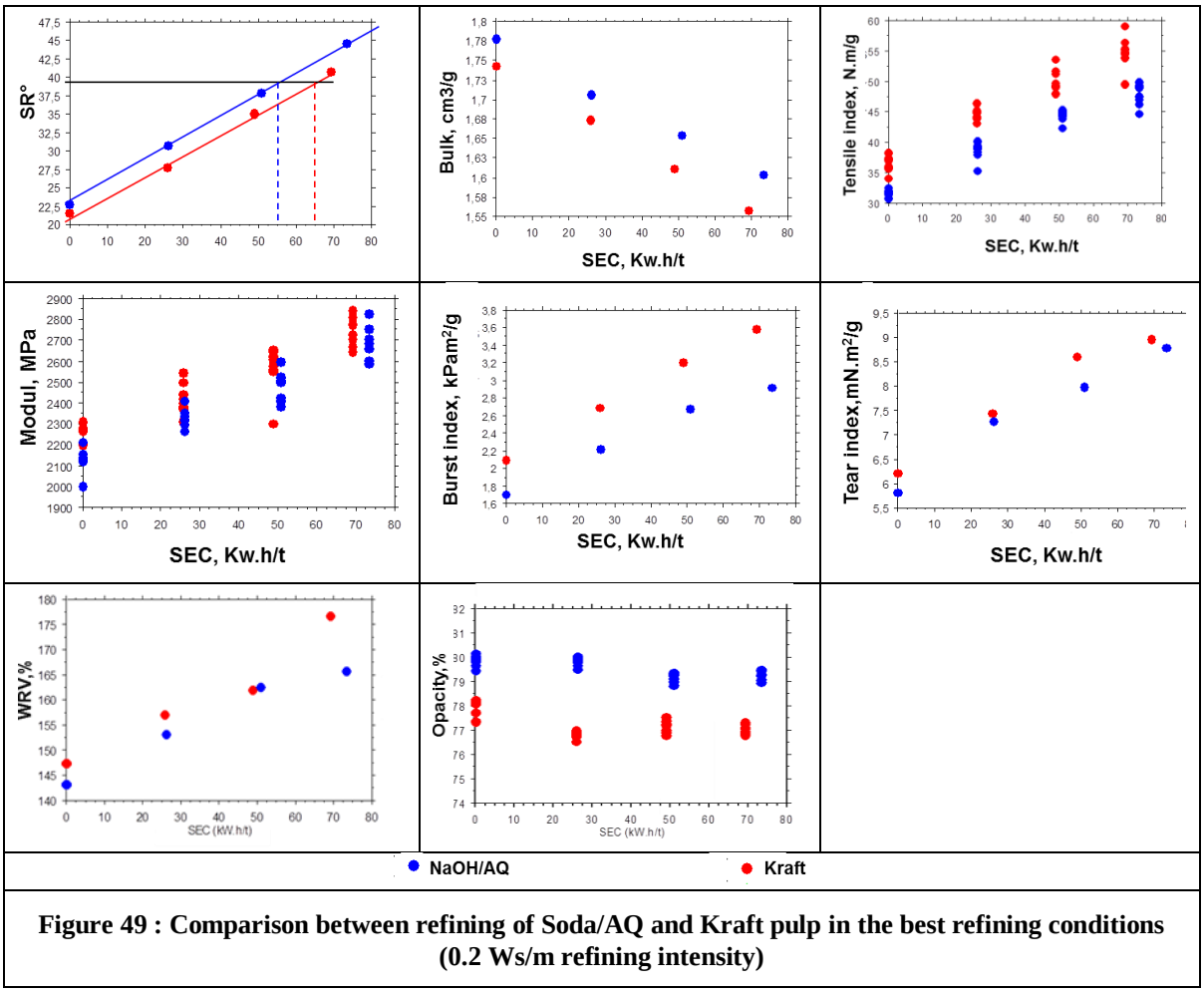


Figure 49 : Comparison between refining of Soda/AQ and Kraft pulp in the best refining conditions (0.2 Ws/m refining intensity)

Soda/AQ pulp consumed less energy than Kraft pulp to reach a given drainage index. Water retention value of Soda/Antraquinone pulp is lower and opacity is higher than Kraft pulp. However, mechanical properties of Soda-AQ pulp were lower than these of the Kraft pulp (Figure 22). Nevertheless, according to UFV results, the addition of xylans in Soda-AQ pulp allowed to improve mechanical properties and to compensate this difference.

Consequently, before refining, xylans from elephant grass pulp (EG1) were added to Soda-AQ pulp (3%). Refining was carried out on this pulp in the best refining conditions already determined (0.2 Ws/m refining intensity). Figure 50 presents mechanical properties of refined bleached Soda-AQ pulp with or without addition of xylans before refining.

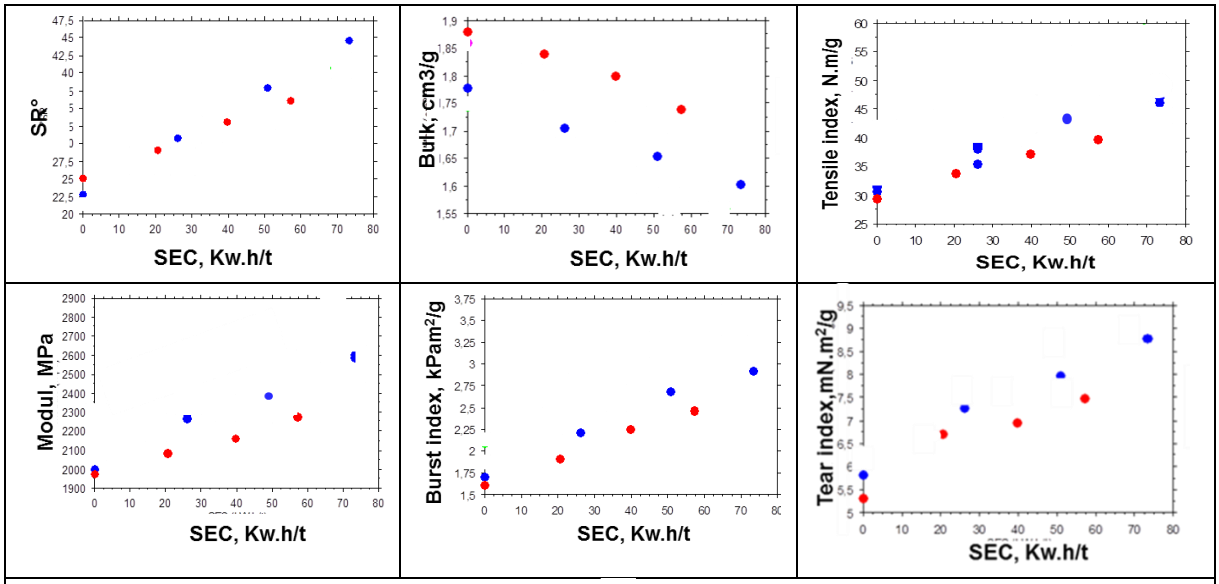


Figure 50 : Comparison between refining of Soda/AQ pulp with and without addition of xylans (3%/ pulp)

According to Figure 50, the addition of xylans reduced the mechanical properties. This strange result was due to the bad solubilization of xylans in the pulp (Figure 51).

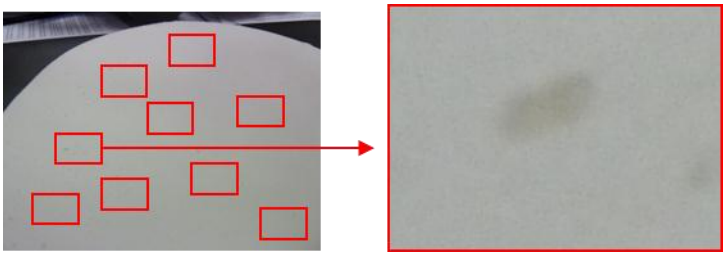


Figure 51 : Presence of xylan unsolibilized in the handsheet obtained after refining

Different techniques were tested to solubilize xylans before its addition in pulp:

- Mixing xylans with water at high speed
- Mixing xylans with water at high speed and at high temperature > 90°C
- Mixing xylans with water and ethanol
- Mixing xylans and sodium hydroxide with different concentrations. However the increase in sodium hydroxide was limited because at high pH, refining was very difficult to manage.

Consequently, other solution was tested to solubilize xylans in the pulp before refining. Bleaching stage (extraction stage) was performed on bleached Soda-AQ pulp and with addition of xylans.
The extraction stage was performed in the same conditions as O stage carried out during bleaching pilot trial (Table 8) without oxygen addition. However, after extraction stage, xylans were again present at the surface of the handsheet produced with this pulp.

Consequently another test with higher pH and retention time was performed (pH 12.5 and retention time of 90 min).
Mechanical properties of bleached Soda-AQ pulp after extraction stage carried out with or without addition of xylans were measured (Figure 52).
The addition of xylans in Soda/Antraquinone pulp during extraction stage did not improve mechanical properties. Insolubilized xylans were again present at the surface of the handsheet.

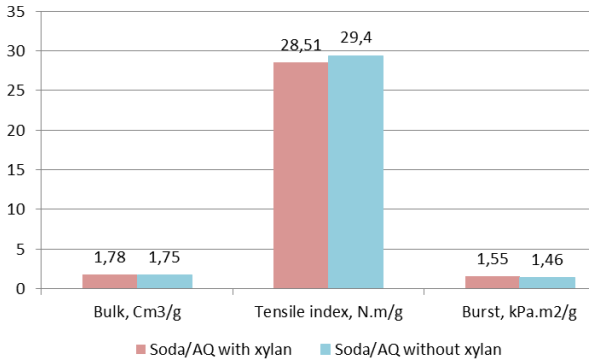


Figure 52 : Mechanical properties of bleached Soda/AQ pulp after extraction stage performed with or without addition of xylans (9% /pulp o.d)

Consequently another trial was performed in order to solubilize xylans before the extraction stage. Xylans were added with sodium hydroxide at pH 12.5 in similar conditions as previously used without pulp addition. Then, solution was heated at 98°C during 90 min. This solution contained also insolubilized xylans which were filtered.
Then, This solution was used to perform extraction stage with Soda-AQ pulp. Extraction stage was performed with hydrogen peroxide in order to avoid the formation of chromophoric groups during this stage. After extraction stage, mechanical properties of this pulp were compared to those of Kraft pulp treated in similar conditions without addition of xylans. However, mechanical properties of Soda-AQ pulp were lower than Kraft pulp.

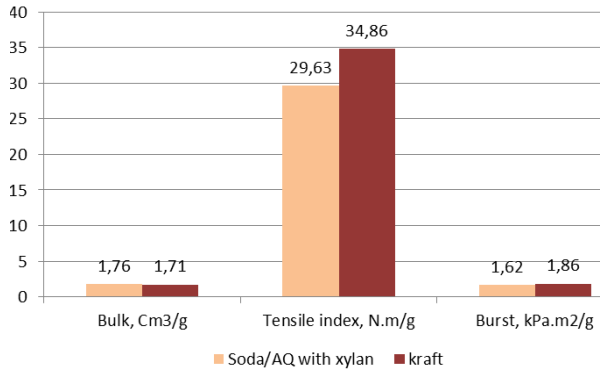


Figure 53 : Mechanical properties of bleached Soda/AQ pulp with xylans (9%/ pulp o.d) after extraction stage

During laboratory trial performed by UFV, xylans were well solubilized in the pulp. Xylans used in UFV laboratory were not dried. On the other hand, xylans used at CTP were dried in UFV laboratory. These bad results with xylans could be attributed to their drying. Consequently, it was not interesting to perform pilot refining trial with these dried xylans.

4. CONCLUSIONS

Alkaline demonstration trial at pilot scale validated results obtained at laboratory. Soda/ Anthraquinone pulp could replace Kraft pulp. It is a sulfur-free process which is interesting for limiting air pollution. However, Soda/ Anhraquinone pulp had lower mechanical properties than Kraft pulp. However, according to UFV laboratory trial, addition of xylans from elephant grass on Soda/Anthraquine pulp can improve mechanical properties.

The enzymatic deconstructions using oxido-reductases or hydrolases performed in laboratory were also validated at pilot scale. Unfortunately, the corresponding production of bioethanol was not conclusive.

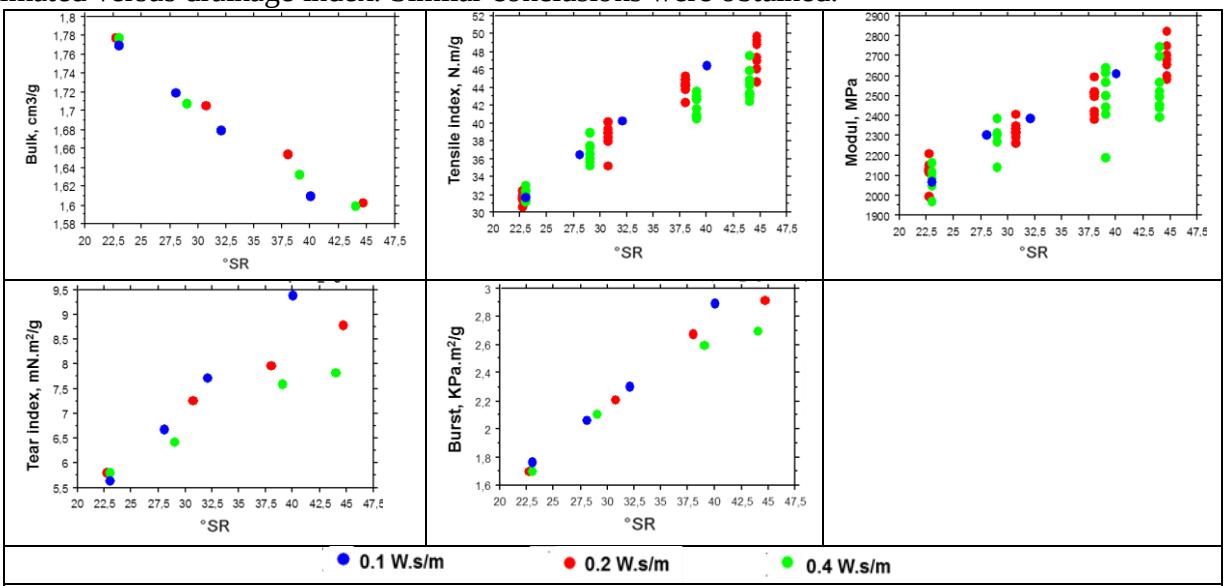
5. DELIVERABLES AND MILESTONES

During the last 18 months, CTP in collaboration with partners participated to the following deliverables and milestones:

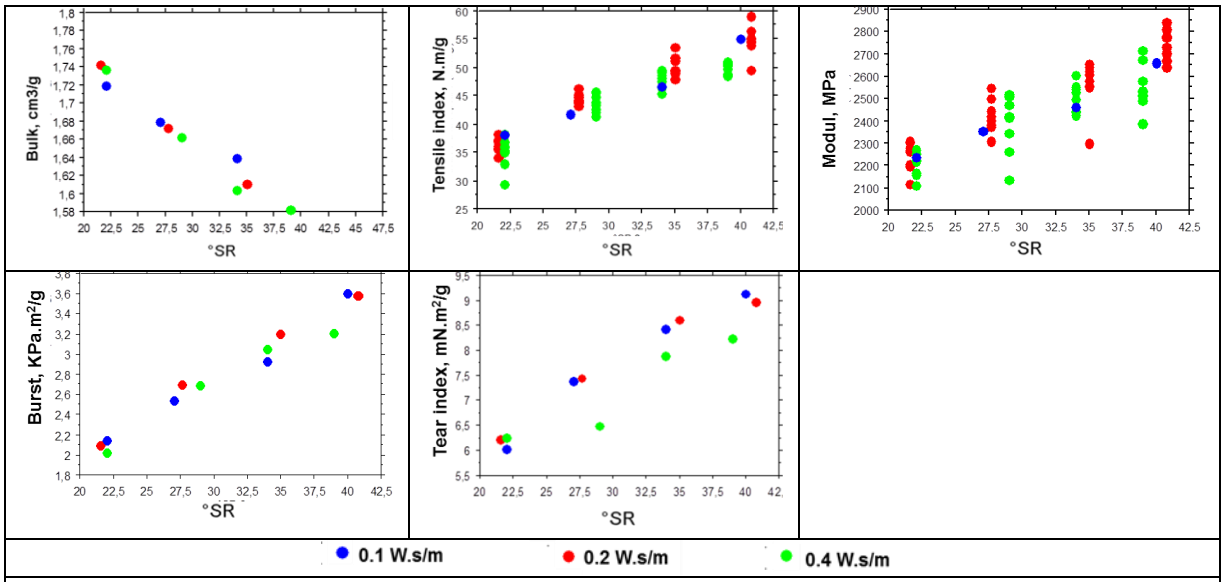
DL4.4: Procedure for improving eucalyptus pulp with grass xylan additive
DL5.2: Pilot-Scale enzymatic deconstruction trials and ECF pulp bleaching trials
DL5.3: Pilot-scale pulp beatability trials

ANNEX

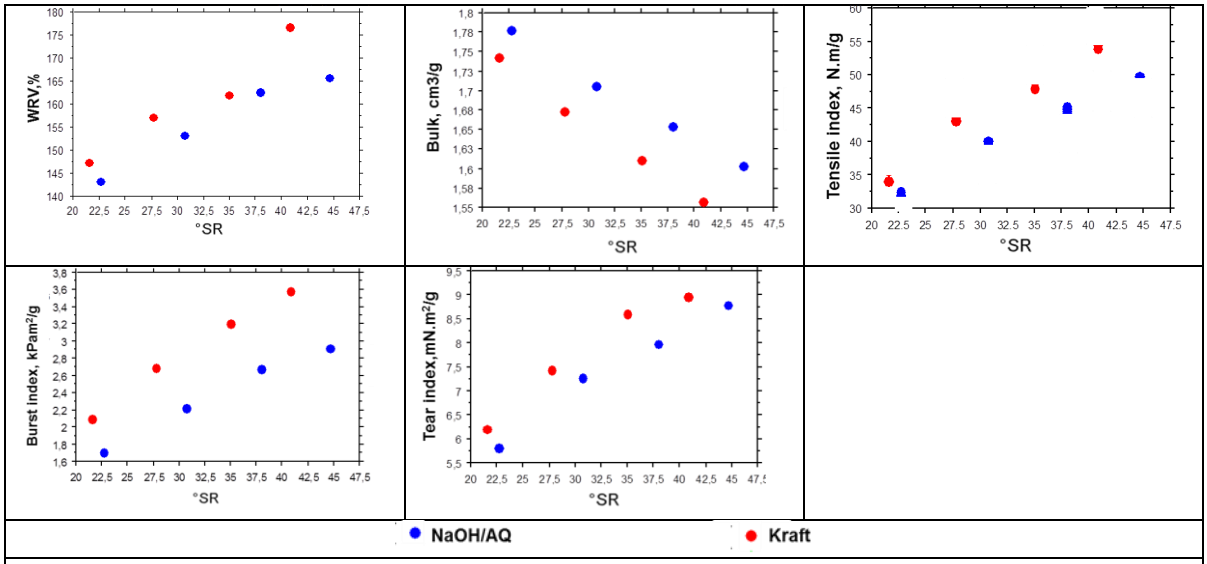
All results presented in Figures 20 to 22 (mechanical properties versus energy consumption) were also exanimated versus drainage index. Similar conclusions were obtained.



Annex 1 : Mechanical properties of bleached Soda/AQ pulp refined with different refining intensities



Annex 2 : Mechanical properties of bleached kraft pulp refined with different refining intensities



Annex 3 : Comparison between refining of Soda/AQ and Kraft pulp in the best refining conditions (0.2 Ws/m refining intensity)