Reduced recalcitrance and improved pulp properties in eucalypt woods pretreated with white-rot fungi and mild alkali

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# ABSTRACT

*Eucalyptus* *globulus* (EGB) is well-suited for kraft pulping due to its physicochemical properties, whereas more recalcitrant woods require harsher thermochemical conditions to attain identical delignification levels. The pulp and paper industry is increasingly seeking solutions to efficiently explore recalcitrant feedstocks, while reducing chemical usage during pulping and bleaching. The present work aimed at assessing if biological and mild alkali pretreatments are able to reduce eucalypt wood recalcitrance and increase kraft pulping and bleaching efficiency. EGB, *E*. *nitens* (ENT) and *E*. *urograndis* (EUG) woodchips were pretreated with white-rot fungi (WRF) and/or 0.1 M NaOH. Results suggested that these pretreatments mainly act by breaking ester bonds involved in cell wall polymer crosslinking, promoting de-acetylation, lignin detachment, increased cellulose accessibility and woodchip impregnation during pulping. Combined WRF and mild alkali pretreatments substantially reduced wood recalcitrance and improved pulp properties. Residual lignin content (K#) was reduced by up to 9% in EGB, 15% in ENT and 16% in EUG. Whereas ClO2 consumption during bleaching was reduced by up to 16% in EGB, 19% in ENT and 13% in EUG. These pretreatments can substantially reduce chemical requirements and lead to higher relative yields, ultimately reducing costs and environmental impacts of kraft pulping and bleaching.

**Keywords**: Cell wall; *Eucalyptus*; Lignin; Pretreatment; Pulping; White-rot fungi.

# INTRODUCTION

Breeding and clonal management of *Eucalyptus* spp. have improved biomass production of these short rotation hardwood forests1. In Southern European countries such as Portugal, eucalypt wood is the main raw material for the pulping industry, with *Eucalyptus* *globulus* Labill. largely dominating the market2. *E*. *globulus* is considered by specialists as the ideal fiber source for printing and writing (P&W) papers, thanks to its physicochemical properties, which confer exceptional key papermaking properties, such as short wood fibers, and the paper produced has excellent specific volume, good formation, porosity and dimensional stability, high opacity and a smooth surface1. Nevertheless, incorporation of wood from other *Eucalyptus* species and hybrids, such as *E*. *nitens* Maiden or *E*. *urograndis* (*E*. *urophylla* S.T.Blake × *E*. *grandis* W.Hill) is still required to achieve production capacity in Portuguese mills. *E*. *nitens* can be used in reforestation in colder geographic regions but the lower wood density implies a loss of industrial productivity. Nonetheless, paper produced with *E*. *nitens* has lower specific volume (bulk) and a significant lower quality for many end-uses3. *E*. *urograndis* has a high biomass production potential and is commercially planted in emerging markets, such as Brazil, South Africa, or China4. However, it requires harsher kraft cooking conditions (higher temperature and/or alkali charge) due to wood chemical composition, such as higher lignin content and lower syringyl/guaiacyl ratio5. The plant cell wall is primarily composed of cellulose, hemicelluloses, pectins and the aromatic polymer lignin6. Wood fibers are bound together with a lignin matrix. This polymer provides mechanical support to the plant, and provides hydrophobicity to the cell wall, enabling vascular transport of water and nutrients, also playing a protective role against biotic and abiotic stressors. Lignin is synthesized mainly from *p*-coumaryl, coniferyl, and sinapyl alcohols, each of them originating different types of lignin units: *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units, respectively7,8. In hardwoods such as eucalypts, lignins are mostly composed by S-units, followed by G-units, with H-units being minor components9. Lignin monomers are polymerized by free radical coupling mechanisms in reactions mediated by peroxidases and laccases, generating a very complex polymer7,8.

Increasing public concern about environmental issues is having a rather large impact on the pulp and paper industry, leading to an increase in research into more sustainable pulping practices. Pulping consists of treating wood to separate fibers, mainly by chemical and mechanical processes. However, to make high quality papers, the hydrophobic lignin must be removed. Therefore, chemicals are used in chemical pulping to solubilize lignin, leading to pulps containing liberated fibers. Kraft pulping (using an aqueous solution of NaOH and Na2S as active chemicals) is the most common process for P&W paper production. The harshness of pulping conditions is dependent on wood recalcitrance, as higher chemical loads and/or temperatures are required for efficient pulping of feedstocks with a higher lignin content. Certain biological systems may be used to assist in the pulping of the wood by using isolated ligninolytic enzymes10. However, several factors may limit direct enzyme utilization, such as low yields in enzyme production, or enzymatic inhibition during lignocellulose hydrolysis by interaction with lignin or lignin-carbohydrate complexes11. An alternative to the use of isolated enzymes is the direct application of white-rot fungi (WRF) for the pretreatment of woods used for pulping12,13. In this approach, wood is treated with lignin-degrading fungi prior to pulping, in a process often called biopulping – which is increasingly being viewed as a viable option to decrease the costs and impacts of conventional chemical pulping14. Several WRF have been used for biopulping applications, including *Ceriporiopsis* *subvermispora* (Pilát) Gilb. & Ryvarden, *Ganoderma* *lucidum* (Curtis) P. Karst., *Phanerochaete* *chrysosporium* Burds., *Pleurotus* *ostreatus* (Jacq.) P. Kumm. and *Trametes* *versicolor* (L.) Lloyd15-18. These fungi are able to produce a set of lignin-degrading enzymes (including a wide range of peroxidases and laccases), and deliver these deep into the woodchips, via their hyphae, while also maintaining the conditions necessary for the enzymatic reactions19-21. Consequently, WRF may selectively degrade lignin, making them ideal for applications where lignin must be altered or removed22. Namely, as pretreatments applied to woody feedstocks, prior to kraft pulping, with the aim of reducing chemical charges used during cooking and bleaching. Fungal and other biological pretreatments are attractive as they rely on mild operating conditions, involve relatively low operational costs and are environmentally friendly23,24. Nonetheless, they are less studied than thermochemical pretreatments, possibly because industry often finds slower processing rates unattractive. However, this issue can be addressed by continuous flow processing, rather than batch process systems25.

WRF-mediated biopulping has the potential of decreasing energy requirements and the amount of used chemicals, in comparison to conventional kraft pulping17. Moreover, it has been demonstrated – namely using beech wood (*Fagus* sp.) in Japan26–, that combined WRF and mild alkali pretreatments may further enhance pulp quality. It is known that even mild alkali concentrations can break crosslinking between lignin and carbohydrate portions of the cell wall and release small-molecule compounds, increase lignocellulosic biomass degradability27-30. However, there are still limited examples where these approaches have been adapted to different feedstocks and geographic contexts, and their impact and relevance, validated in industrial settings.

The main objective of our work was to address these gaps and develop sustainable biopulping methodologies to produce papermaking grade pulps from *Eucalyptus*, by the application of WRF and mild alkali-mediated pretreatments. We also aimed at assessing if these pretreatments can offset the higher recalcitrance of *E*. *urograndis* in relation to *E*. *globulus*. The digestibility and composition of the pretreated biomass and pretreatments effluents were analyzed, to characterize the effect of the pretreatments at a molecular level. After this initial characterization phase, biopulping in an industrial setting was implemented, involving woodchip pretreatment at a larger scale, followed by kraft pulping and bleaching (Fig. 1). This second phase of our study allowed for a more direct assessment of the impact of the tested pretreatments on the production of high-quality pulps.

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**Figure 1.** Schematic diagram of the employed experimental study design.

# MATERIALS AND METHODS

*Eucalyptus* biomass

Woodchips from *Eucalyptus* *globulus* (EGB, from Aveiro, Portugal), *E*. *nitens* (ENT, from North of Spain) and *E*. *urograndis* (EUG, from Brazil) were provided by an industrial partner. The present study is divided into two phases (Fig. 1). In the first, pretreated samples were prepared at laboratory-scale with the aim of defining methodologies, characterizing processes and biomass composition (characterization phase). In the second (biopulping phase), larger amounts of pretreated samples were produced and pulped using standard industrial procedures. For the characterization phase (Fig. 1), samples were milled using a Retsch SR3 Rotor Beater Mill (Retsch, Germany) to a particle size in the range of 0.18 mm – 0.85 mm (mesh sizes 80 and 20). For the biopulping phase, samples were used in woodchip form, as explained in the following sections.

Preparation of alcohol insoluble residue

Alcohol insoluble residues (AIR) were prepared as previously reported31 and used for subsequent analyses. For each sample, approximately 1 g of ground biomass was extracted sequentially as follows: with 30 mL 70% (*v*/*v*) aqueous ethanol (99.9%, Manuel Vieira & Cª, Portugal), first for 12h and then twice more for 30 min in a shaking incubator set at 40 °C/150 rpm; three times with 20 mL of a 1:1 (*v*/*v*) solution of chloroform (99.9%, Carlo Erba Reagents, France) and methanol (99.8%, Honeywell, Germany), and shaken at 150 rpm, for 30 min at 25 °C; and finally, three times with 15 mL acetone (99.5%, PanReac, Spain), shaken at 150 rpm for 30 min, at 25 °C. Between each step of the extraction, the material was collected by centrifugation at 25000×*g* for 10 min and the supernatants were discarded. Following the third acetone step, the samples were left to dry overnight in a fume hood.

Inoculum preparation and fungal pretreatment

*Ceriporiopsis* *subvermispora* (CES), *Ganoderma* *lucidum* (GAN), *Phanerochaete* *chrysosporium* (PHN), *Pleurotus* *ostreatus* (PLE) and *Trametes* *versicolor* (TRA) were used as white-rot fungi (WRF) for biological pretreatments. As described elsewhere32, morphological examination and molecular analysis, targeting internally transcribed spacer (ITS) regions, allowed the identification of the fungal species used in this study. Fungal inocula were prepared by culturing the individual WRF strains at 23 °C on 2.9% potato dextrose agar (PDA; CM0139, Oxoid, England, UK), in Petri dishes (Ø 90 mm × 15.8 mm; Deltalab, Spain). After 10 days, liquid inocula were prepared. Potato dextrose broth (PDB) was produced by boiling 125 g of peeled potatoes per 1000 mL deionized water, and adding 5 g Bacto dextrose (Difco 0155-17, BD Biosciences, MI, USA) and 0.75 g Bacto yeast extract (212750, BD Biosciences) to the filtered supernatant at room temperature. From each WRF strain, the total mycelium was removed from 5 entirely colonized PDA Petri dishes (Ø 90 mm) and transferred to a blender, containing 500 mL PDB. The mixture of PDB and the mycelium was blended into small pieces and subsequently allowed to regrow for 5 days in a shaking incubator (23 °C; 110 rpm).

Under the characterization phase (Fig. 1), to serve as solid media for WRF growth, the *Eucalyptus* biomass was prepared as follows: approximately 1.5 g of previously dried and milled but not organic solvent-washed (non-AIR) biomass was added to 5 mL deionized water and autoclaved in glass culture tubes capped with hydrophobic cotton. Once at room temperature, for each combination of WRF (GAN, TRA and PLE) and *Eucalyptus* spp. (EGB and ENT), 1.25 mL of the corresponding liquid inoculum was added to the autoclaved biomass under aseptic conditions, in triplicate. Additionally, non-inoculated biomass samples (NF controls; no fungi), with an equal volume of deionized water added, were included to serve as negative controls. These slurries were vortex mixed and left incubating statically at 24 °C in the dark for 30 days. WRF-pretreatment methodologies were adapted from procedures reported elsewhere33,34. After incubation, aliquots of the pretreatment effluents were collected, immediately flash-frozen with N2 and then freeze-dried before subsequent chemical characterization. The solid pretreated biomass was washed twice with deionized water, dried at 40 °C for 48h, and stored for subsequent analyses and alkali pretreatment. For neutral sugars and lignin characterizations, AIR samples were produced from this WRF-treated biomass (as described above).

For the biopulping phase (Fig. 1), larger biomass quantities were used. Approximately 1000 g of woodchips fromEGB, ENTand EUG were added to 20 L reactors with 240 mL of liquid inoculum (for TRA, CES and PHN) and 960 mL of water. For negative controls, 1200 mL of water were added to the biomass without any inoculum. The woodchips were vigorously mixed with the added liquid inocula and/or water, and left incubating statically at 24 °C in the dark for 30 days. After incubation, the solid pretreated biomass was washed twice with deionized water, air dried at room temperature for 7 days, and stored for subsequent alkali pretreatment and pulping.

Mild alkali pretreatment

In both the characterization and the biopulping phases, a portion of the non-WRF treated (NF) and WRF-treated solid fractions were subjected to a mild alkali (ALK) treatment, with 10 mL 0.1 M NaOH (97%, Merck, Germany) per 1 g of biomass (dry weight) for 24h at 21 °C. Subsequently, aliquots of the pretreatment effluents were collected, immediately flash-frozen with N2 and then freeze-dried before subsequent chemical characterization. The pretreated solids were washed 3 times in 0.025 M acetic acid/potassium acetate buffer (KOAc; pH=5.6; glacial acetic acid, ≥99%, Chem-Lab, Belgium; CH3COOK, ≥99%, Merck) and twice with deionized water. Samples were then dried, at 40 °C in the characterization phase, and air dried at room temperature in the biopulping phase, before subsequent analyses and pulping.

During the characterization phase, neutral sugars and lignin analyses were performed on these alkali-treated samples only after AIR preparation, as described above. In the biopulping phase, some of the treatments involved the application of the mild alkali pretreatment before the WRF pretreatment. In these cases, woodchips were initially treated with 0.1 M NaOH (24h at 21 °C), washed 3 times in 0.025 M KOAc buffer (pH=5.6) and twice with deionized water, and air dried at room temperature, before undergoing WRF pretreatment. For a list of pretreatments employed during the present study, see Tables 1 (characterization phase) and 2 (biopulping phase). A list of all types of pretreated biomass, including those which did not undergo kraft pulping, is included in Supporting Information Table S1. Furthermore, for the purpose of characterizing the isolated effect of mild alkali on structural compounds, the alkaline pretreatment (0.1 M NaOH; 24h; 21 °C) was employed on AIR samples prepared from non-pretreated samples. These samples are subsequently referred to as AIK.

**Table 1.** Description of all treatments employed on the *Eucalyptus* biomass used for the characterization phase.

|  |  |  |  |
| --- | --- | --- | --- |
| **Sample number** | ***E*. *globulus* (%)** | ***E*. *nitens* (%)** | **Treatment a** |
| 1 | 100 | 0 | NF |
| 2 | 100 | 0 | GAN |
| 3 | 100 | 0 | PLE |
| 4 | 100 | 0 | TRA |
| 5 | 100 | 0 | NF + ALK |
| 6 | 100 | 0 | GAN + ALK |
| 7 | 100 | 0 | PLE + ALK |
| 8 | 100 | 0 | TRA + ALK |
| 9 | 0 | 100 | NF |
| 10 | 0 | 100 | GAN |
| 11 | 0 | 100 | PLE |
| 12 | 0 | 100 | TRA |
| 13 | 0 | 100 | NF + ALK |
| 14 | 0 | 100 | GAN + ALK |
| 15 | 0 | 100 | PLE + ALK |
| 16 | 0 | 100 | TRA + ALK |
| a Treatments: NF, no fungi (negative control, incubated only with deionized water); GAN, *Ganoderma* *lucidum*; PLE, *Pleurotus* *ostreatus*; TRA, *Trametes* *versicolor*; ALK, samples treated with 0.1 M NaOH for 24h at 21 °C after fungal treatment. | | | |

**Table 2.** Description of sample types and treatments employed on the eucalypt woodchips in the biopulping phase, before applying kraft cooking.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Sample a** | ***E*. *globulus* (%)** | ***E*. *nitens* (%)** | ***E*. *urograndis* (%)** | **Treatment b** |
| G0 | 100 | 0 | 0 | NT |
| G1 | 100 | 0 | 0 | NF |
| G1k | 100 | 0 | 0 | NF + ALK |
| G2 | 100 | 0 | 0 | CES |
| Gk4 | 100 | 0 | 0 | pre-ALK + TRA |
| N1 | 0 | 100 | 0 | NF |
| N1k | 0 | 100 | 0 | NF + ALK |
| N3k | 0 | 100 | 0 | PHN + ALK |
| N4 | 0 | 100 | 0 | TRA |
| N4k | 0 | 100 | 0 | TRA + ALK |
| Nk4 | 0 | 100 | 0 | pre-ALK + TRA |
| U1 | 0 | 0 | 100 | NF |
| U1k | 0 | 0 | 100 | NF + ALK |
| U2k | 0 | 0 | 100 | CES + ALK |
| U3 | 0 | 0 | 100 | PHN |
| U3k | 0 | 0 | 100 | PHN + ALK |
| U4 | 0 | 0 | 100 | TRA |
| U4k | 0 | 0 | 100 | TRA + ALK |
| Uk4 | 0 | 0 | 100 | pre-ALK + TRA |
| Mix A | 70 | 0 | 30 | G0 + U4 (TRA) |
| Mix B | 70 | 0 | 30 | G0 + U4k (TRA + ALK) |
| Mix C | 70 | 0 | 30 | G0 + Uk4 (pre-ALK + TRA) |
| a Abbreviations: G, *Eucalyptus* *globulus*; N, *E*. *nitens*; U, *E*. *urograndis*; Mix, 70% of non-treated *E*. *globulus* with 30% of treated *E*. *urograndis*.  b Treatments: NT (0), raw woodchips without any treatment; NF (1), no fungi (negative control, incubated only with deionized water); ALK (k), samples treated with 0.1 M NaOH for 24h at 21 °C (after fungal treatment, where applicable); pre-ALK, samples treated with 0.1 M NaOH for 24h at 21 °C before fungal treatment; CES (2), *Ceriporiopsis* *subvermispora*; PHN (3), *Phanerochaete* *chrysosporium*; TRA (4), *Trametes* *versicolor*. For a list of all employed pretreatments, including samples that did not undergo kraft pulping consult Supporting Information Table S1. | | | | |

Fourier-transform infrared spectroscopy

Attenuated total reflectance Fourier-transform mid-infrared (FTIR-ATR) spectroscopy was performed on all samples from the characterization phase (Table 1), following a previously reported procedure31. Duplicate spectra were collected in the 4000 – 400 cm-1 range, in a Bruker Optics Vertex 70 FTIR (Bruker Optik, Germany) spectrometer purged with CO2-free dry air and equipped with a Bruker Platinum ATR single reflection diamond accessory. A Ge on KBr substrate beam splitter and a liquid nitrogen-cooled wide band mercury cadmium telluride (MCT) detector were used. Spectra were averaged over 32 scans at a resolution of 2 cm-1, and the 3-term Blackman-Harris apodization function was applied. The Bruker Opus 8.1 software was applied to: i) remove eventual H2O and CO2 contributions, and ii) correct for the frequency dependence of the electric field penetration depth in ATR (considering a mean refractive index of 1.25). The spectral data were imported into MatLab (v. R2021b, MathWorks, MA, USA) and all computations – vector normalization to unit length (2-Norm), and baseline correction according to the automatic weighted least squares algorithm (polynomial order = 2) – were carried out using the Eigenvector PLS Toolbox (v. 9.0, Eigenvector Research, WA, USA) and in-house developed routines, as previously described35,36. Finally, the spectra were restricted to the fingerprint region (1800 – 800 cm-1), averaged (per replicate) and plotted.

Digestibility assay

WRF-treated and mild alkali-treated samples from the characterization phase (Table 1) were included in a digestibility assay, with four technical replicates for each sample, using a previously reported automatic platform37. Briefly, enzymatic hydrolysis was performed using the commercial Cellic CTec2 enzymatic cocktail (Novozymes, Denmark) in 0.025 M acetic acid/sodium acetate buffer (NaOAc; pH=4.5; glacial acetic acid; CH3COONa ∙ 3H2O, ≥99%, Merck) at 50 °C. Cocktails were prepared so that cellulase loadings were 8 filter paper units (FPU) per g of biomass in the NaOAc buffer. Digestibility was measured after 8h by colorimetric detection of reducing sugar equivalents, according to an adapted 3-methyl-2-benzothiazolinone hydrazone (MBTH) method, as described elsewhere37,38. Values were expressed as nmol of reducing sugars per mg of biomass dry weight (nmol mg-1).

Neutral monosaccharides

Acid hydrolysis and neutral monosaccharide determinations were performed as previously described39,40 on all samples from the characterization phase (Table 1). Briefly, 100 μL of 72% (*w*/*w*) aqueous H2SO4 (95%, Thermo Fisher, Germany) was added to 10 mg of each sample. Sealed tubes were left at 30 °C for 1h. Samples were diluted to 4% H2SO4 (*w*/*w*) with deionized water and autoclaved at 121 °C for 1h. Once at room temperature, hydrolysates were neutralized using CaCO3 (≥99%, Merck), and the tubes were centrifuged (2000×*g* for 10 min) to produce particulate-free supernatants. Carbohydrate separation and detection was achieved using high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). An ICS-5000 ion chromatography system (Dionex, CA, USA) was operated at 45 °C using a Dionex CarboPac SA10 column with a CarboPac SA10G guard column. An eluent generator, comprising a Dionex EGC 500 eluent generator cartridge, prepared 0.001 M KOH for 14 min isocratic elution at 1.5 mL min-1. d-(–)-arabinose (99%), d-(+)-glucose (anhydrous, 99%) and d-(+)-xylose (≥98%) calibration standards supplied by Thermo Fisher, were used for monosaccharide identification and quantitation.

Lignin content determination

Acetyl bromide soluble lignin percentages (ABSL%) were determined in duplicate for all samples from the characterization phase (Table 1), following previously reported procedures41,42. Briefly, 500 μL of a freshly prepared 25% (*v*/*v*) solution of acetyl bromide (≥98%, Thermo Fisher) in glacial acetic acid was added to 10 mg of each sample. Sealed tubes were left at 50 °C for 3h. Subsequently, 2 mL of 2 M NaOH and 350 μL of 0.5 M hydroxylamine hydrochloride (99%, Thermo Fisher) were added. Final volumes were adjusted to 10 mL with glacial acetic acid and centrifuged (2000×*g* for 10 min) to produce particulate-free supernatants. From there, 200 μL were transferred to UV-transparent 96-well plates (UV-Star, Greiner Bio-One, Austria). Absorbance at 280 nm was measured with a plate reader (Multimode Plate Reader 2300 EnSpire, Perkin Elmer, MA, USA). Blank controls were included and their absorbance at 280 nm was set as absorbance baseline. Lignin dry weight percentages were calculated as follows: *ABSL*%=(*A280*/(*SAC*×*PL*))×(*VR*/*WS*)×100%; where *A280* is the absorption reading at 280 nm; *PL* is the pathlength determined for the 96-well microplates with a volume of 200 μL per well used during the analysis (0.556 cm); *VR* is the reaction volume (liters); *WS* is the sample weight (g); and *SAC* is the specific absorption coefficient of 23.6 g-1 L cm-1, as previously reported for hardwoods43.

Characterization of pretreatment effluents

The freeze-dried pretreatment effluents from WRF-treated, mild alkali-treated, and non-inoculated negative controls (characterization phase; Table 1), were reconstituted in HPLC grade methanol (99.8%, Thermo Fisher). These were then kept at -20 °C for 24h and then centrifuged (14000×*g* for 5 min). The resulting supernatants consisted of clarified methanolic extracts, containing phenols of interest, but free of most sugars and other water-soluble compounds. Samples were analyzed by reverse-phase high performance liquid chromatography equipped with a photodiode array detector and coupled with an electrospray ionization tandem mass spectrometer (HPLC-PDA-ESI-MSn) on a Thermo Finnigan system (Thermo Electron Corp, MA, USA), as described elsewhere44. Separation of compounds was carried out on a Waters C18 Nova-Pak column (3.9×150 mm, particle size 4 µm) at 30 °C with a flow rate of 1 mL min-1 and injection volume of 10 µL. The mobile phase consisted of water with (A) 0.1% formic acid (≥99%, Chem-Lab), and (B) HPLC grade methanol with 0.1% formic acid, with B increasing from 5 to 65% in 30 min. Eluting compounds were detected with a Finnigan PDA Plus detector between 240 and 400 nm and a Finnigan LTQ linear ion trap with an ESI source. MS parameters were as follows: sheath gas flow 30, auxiliary gas flow 15 and sweep gas zero (arbitrary units), spray voltage -4.0 kV in negative and 4.8 kV in positive ionization mode, capillary temperature 320 °C, capillary voltage -1.0 and 45 V, respectively, tube lens voltage -68 and 110 V, respectively, and normalized collision energy (CE) typically 35%. Data were acquired and processed using Xcalibur software (v. 3.0.63, Thermo Fisher). Compounds of interest were characterized and/or tentatively identified by their UV and MS spectra or identified by direct comparison with standards or fragmentation patterns reported elsewhere45.

Kraft pulping process, pulp characterization and bleaching

A rotary digester (made by Tavares & Tavares S.A., Portugal; automated by APINEQ Lda., Portugal) was used at the facilities of an industrial partner, containing 4 sealed minireactors of 2 L capacity each, submerged in heated pressurized water. The rotation provided agitation of the liquor-woodchips mixture inside the minireactors. Temperature was digitally controlled. In each reactor, 200 g (dry basis) of woodchips were added with a liquor to wood ratio of 4:1. The woodchips consisted of the previously pretreated samples, as well as three different combinations of 70% raw EGB woodchips without any treatment (G0), mixed with 30% pretreated EUG (Table 2). All pulping trials were performed by the kraft process, using the same conditions: 160 °C, 1h, 1 °C min-1 heating rate, 30% sulfidity and 19% active alkali charge (Na2O basis). Only a set of selected pulps were produced and bleached by the sequence D0EpD1D2. D stands for ClO2 and Ep means alkaline extraction reinforced with hydrogen peroxide. The bleaching assays were performed in polyethylene bags submerged in a water bath shaker, with a consistency of 10%, in the conditions presented in Table 3. The total ClO2 charge was split into 65, 25 and 10% for D0, D1 and D2 stages, respectively. After pulping, yield, and Kappa number (TAPPI T 236) were determined. Unbleached and bleached pulps were characterized in terms of intrinsic viscosity (International Organization for Standardization, ISO 5351), diffuse reflectance factor (ISO 2469)/ISO brightness and brightness stability (post color number, PC#)46. Bleachability was calculated as the difference between diffuse reflectance factor after and before bleaching divided by the total chlorine dioxide charge applied in the bleaching sequence (active chlorine basis).

**Table 3.** Bleaching conditions of kraft pulps obtained from woodchips presented in Table 2.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Bleaching stage** | **Temperature (°C)** | **Time (min)** | **NaOH charge (%)** | **H2O2 charge (%)** |
| D0 | 70 | 25 | 1.77 | 0.44 |
| EP | 80 | 90 |
| D1 | 70 | 150 |
| D2 | 80 | 90 |

Statistical analysis

All univariate descriptive statistics, analyses of variance and Tukey’s range tests were performed using Statistica (v. 8.0; StatSoft, OK, USA).

# RESULTS AND DISCUSSION

Digestibility of *Eucalyptus* biomass pretreated with dilute alkali and WRF

Wood recalcitrance factors, such as high lignin content, low syringyl/guaiacyl ratio and high extractive content, which reduce pulping and bleaching efficiency, can also inhibit cellulolytic enzyme activity and reduce cellulose accessibility47. Thus, the digestibility assay performed during the characterization phase of our study (Fig. 1), is a fast and simple approach to evaluate the effect of the pretreatments on biomass recalcitrance, using relatively small amounts of sample.

AIR samples, without and with the mild alkali pretreatment (AIK) were included in the assays, to determine the effect of the pretreatment on extractive-free biomass and to assess its digestibility. When treated with the alkali, the digestibility of AIR, expressed as nmol of reducing sugars per mg of biomass dry weight (nmol mg-1), is significantly increased (*P*≤0.05; Fig. 2), from 105.0 to 452.7 for EGB, and from 89.7 to 250.2 for ENT (Supporting Information: Table S2). These results represent 4.3-fold and 2.8-fold sugar yield increases, respectively for EGB and ENT. In WRF-treated EGB, GAN causes a significant (*P≤*0.05) 2.1-fold increase in relation to the NF control (Fig. 2). With ENT biomass, the maximum increase in digestibility is observed with PLE (1.2-fold). When WRF and mild alkali pretreatments are combined, the best digestibility result for EGB is seen with GAN, as a 3.5-fold increase is observed in relation to the NF controls (Fig. 2). For ENT, both GAN and TRA cause a 1.6-fold increase in relation to the NF controls. These results clearly indicate that not only the alkali pretreatment but also the WRF can effectively improve the digestibility of *Eucalyptus* biomass. Indeed, the best digestibility results are seen when both the WRF and the mild alkali are used. Conversely, although the differences are not significant, in some cases the digestibility is lower in samples treated only with WRF than in the controls (Fig. 2). This may be explained by the release of compounds during fungal action which inhibit the cellulolytic enzymes used in the digestibility assay. Pretreatment-derived soluble compounds can inhibit the action of hydrolytic enzymes through steric hindrance of binding sites29,48,49. The use of the alkaline pretreatment after the WRF may have the added benefit of washing-off these potentially inhibitory molecules.

Our results confirm other reports where a similar trend for increased digestibility, under identical WRF and mild alkali pretreatments, was observed in hardwoods such as *Acacia* spp.30. In EGB, it has been reported that a combined pretreatment, consisting of a *Trametes* sp. and autohydrolysis, led to a greater increase in biomass digestibility than for autohydrolysis alone50. Furthermore, cornstalk pretreated with a combination of WRF and alkali often is less recalcitrant than when single pretreatments are employed51, indicating that these pretreatments can act synergistically when combined. As for the exclusive effect of biological pretreatment, our results are also supported by reports that the digestibility of *E*. *grandis* is increased when its biomass is pretreated with WRF52. However, for the alkaline pretreatment alone, most reports on eucalypts employ harsher alkaline solutions in combination with high temperatures53, which have a more profound impact on wood composition and structure, leading to some loss or structural alteration of cellulose and hemicelluloses54-56. In addition, these approaches are also more costly and generate noxious and difficult to treat residual effluents, due to higher alkaline loadings. Conversely, in other types of lignocellulosic biomass, such as switchgrass, it has been reported that milder alkali pretreatments are highly efficient, as they utilize lower alkali concentrations, have higher solid recovery percentages, while substantially reducing biomass recalcitrance57.

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**Figure 2.** *Eucalyptus* *globulus* and *E*. *nitens* biomass digestibility. Values represent mean nmol of reducing sugar released per mg of biomass dry weight (nmol mg-1) after 8h incubation in a hydrolytic enzyme mixture. Abbreviations: AIR, non-pretreated alcohol insoluble residues; AIK, alcohol insoluble residues pretreated with mild alkali (0.1 M NaOH; 24h; 21 °C); WRF, white-rot fungi; GAN, *Ganoderma* *lucidum*; PLE, *Pleurotus* *ostreatus*; TRA, *Trametes* *versicolor*; NF, samples incubated without any fungi. Significant differences (*P*≤0.05) in relation to the controls (AIR or NF; striped bars) are marked with a "\*". Error bars represent the standard error of the sample replicates.

Pretreatment effects on the composition of *Eucalyptus* biomass

The variation observed in digestibility is likely to be a consequence of compositional differences between the eucalypt woods being tested, and the different modes of action of the pretreatments. To better understand these differences, various analytical approaches were used to characterize the composition of the pretreated biomass and the liquid pretreatment effluents (characterization phase; Fig. 1).

FTIR-ATR spectroscopy provided an overall chemical profile of the samples before and after the pretreatments, thus revealing the main compositional modifications effected by the pretreatments on *Eucalyptus* biomass. For both EGB and ENT, when comparing alkali-pretreated and non-pretreated biomass (Fig. 3), the most evident differences between the spectra of AIK and AIR samples are observed in the 1736 – 1730 cm-1 (*a*) and 1240 – 1235 cm-1 (*b*) regions. By including these organic solvent-washed samples, it was possible to determine the effect of the mild alkali pretreatment on the cell wall composition, without the interference from non-structural compounds. Band *a*, centered at 1735 cm-1 and ascribed to C=O stretching in hemicelluloses58-60, showed reduced intensities in alkali pretreated biomass. Concomitantly, the intensity of band *b*, associated to acetyl and carbonyl vibration in xylan60-62, is also reduced. Similar trends are observed in the non-AIR (not organic solvent-washed) *Eucalyptus* biomass treated with WRF and mild alkali (Fig. 4). Also in these samples, spectral differences are mainly noticeable in the *a* and *b* regions, which became less intense after the mild alkali pretreatments. It is known that substantial amounts of acetate are released when lignocellulosic biomass is treated with dilute alkaline solutions, which hydrolyze ester bonds63,64. Furthermore, most of the released acetyl groups are likely to originate from xylans, as *O*-acetylated xylan is the main source of acetylesters in the cell wall65,66. Particularly for eucalypt wood, it has been reported that in EGB and EUG xylans the degree of acetylation can reach 51%67. This agrees with our FTIR-ATR results, indicating that the pretreatments produce a decrease in signals associated to xylan and acetyl groups, seemingly through de-esterification reactions.

Main neutral sugars and lignin contents were determined to assess the effect of the pretreatments on individual cell wall fractions. To exclude contributions from non-structural compounds, the pretreated samples were organic solvent-washed (AIR) before carrying out these analytical procedures. Glucose, xylose and arabinose are the main neutral sugars in the biomass of the *Eucalyptus* spp. studied (Fig. 5, Supporting Information: Table S3). EGB typically contains higher amounts of all neutral sugars when compared to ENT. As shown in the NF controls, EGB samples had an average of 43.0% glucose, 14.9% xylose and 1.5% arabinose, as opposed to 39.7% glucose, 14.3% xylose and 1.4% arabinose, for ENT. Conversely, lignin contents are higher in the control samples of ENT (20.9%) than in EGB (19.2%). Post hoc Tukey’s tests were performed to assess how glucose, xylose, arabinose and lignin contents of the pretreated samples differ from those of control samples. For all pretreatments (WRF, ALK and WRF+ALK), no statistically significant differences (*P*>0.05) were detected when compared with NF controls. This demonstrated that despite the observed increase in sample digestibility (Fig. 2), the pretreatments do not cause significant degradation or loss of cell wall structural carbohydrates. Indeed, it was observed that the content of individual monosaccharides can be higher (although not statistically significant) in alkali-pretreated samples than in non-pretreated, although this is likely to be due to an increase of efficiency of the acid hydrolysis after the pretreatment. This preservation of constituent molecules ensures that the pretreatments do not extensively damage the cellulose fibers and maximize potential biorefinery applications of the biomass. By contrast, there is a tendency for decreased lignin content in pretreated biomass, especially when the alkaline pretreatment is used (Fig. 5; Supporting Information: Table S3). This decrease in lignin is not significant, but may consist of ester-linked hydroxycinnamic acids, which can act as synthetic precursors and represent small proportions of the lignin macromolecule68-70. Lower lignin contents are desirable for *Eucalyptus* biomass used in the pulp and paper industry because it permits the use of less drastic process conditions.

The liquid fractions derived following the fungal and mild alkali pretreatments, including the NF control samples (non-inoculated), were analyzed by liquid chromatography with detection by photodiode array and tandem mass spectrometry (HPLC-PDA-ESI-MSn). These analyses showed the appearance of a few new products following treatment with WRF, and these varied with the species of *Eucalyptus* – *e*.*g*., a compound with a molecular weight of 166 amu, absorbance maximum of 272 nm, and eluting at approximately 14.2 min, was detected in EGB but not in ENT samples. In general, all effluent samples appeared to contain low levels of compounds, which led to low signal-to-noise ratios, thus preventing accurate compound quantification in this study. However, an empirical examination of the chromatograms revealed that the main detected peaks had responses several orders of magnitude below that observed for a 0.005 M standard of the monophenol *p*-coumaric acid, analyzed under the same experimental conditions (Supporting Information: Figs. S1 – S3). Thus, it may be inferred that the effluents from pretreated samples do not contain high concentrations of secondary metabolites with potentially harmful effects on the environment. Nonetheless, the data did show that ellagic acid is the predominant compound in many samples (Table 4), particularly in controls and following alkali treatment, despite relative abundances varying depending on *Eucalyptus* species. Methylellagic acid and its deoxyhexose derivative were also detected in smaller quantities in the samples. When the WRF pretreatments were applied, ellagic acid and its derivatives were detected at reduced levels in ENT samples, but not in EGB samples. Ellagic acid and its derivatives have been extensively reported to occur in the wood71-74, bark75,76, fruits and leaves45,77 of EGB and other eucalypt species. Structurally, ellagic acid is a phenolic lactone dimer of gallic acid, that may be derived from ellagitannins, which in turn are esters of hexahydroxydiphenic acid and monosaccharides78. When ellagitannins are hydrolyzed by alkali, their ester bonds are broken, thus releasing hexahydroxydiphenic acid, which is spontaneously lactonized to ellagic acid79,80. Furthermore, as with other tannins, ellagitannins may occur in plant biomass in soluble (extractives) or insoluble forms (bound to the cell wall through weak interactions)81. These phenomena may explain the presence of ellagic acid and its derivatives in the pretreatment effluents. It is plausible that soluble ellagitannin fractions are readily removed from the biomass by the aqueous incubation media. Conversely, insoluble ellagitannin may be released and hydrolyzed during the mild alkali and WRF pretreatments, which are known to promote the hydrolysis of ester linkages in plant biomass30,82,83.

The biomass and effluent compositional data strongly suggest that the employed pretreatments promote the hydrolysis of ester linkages in the biomass. This can be accomplished either by saponification of ester bonds by the alkali, or by the action of a diversity of fungal esterases known to be secreted by WRF82,84-88. Much of the self-crosslinking between cell wall polysaccharides and lignin is mediated by ester bonded substituents, such as feruloyl esters6. By breaking these ester bonds, the pretreatments may increase biomass porosity and cause some lignin detachment89,90. Furthermore, acetylester ornamentation is critical for the interaction between xylan and cellulose91, thus the partial removal of these substituents during the pretreatments may also make cellulose more accessible and less recalcitrant. It has been reported that even 0.15 M NaOH solutions are highly efficient at promoting hardwood de-acetylation (80% reduction of the acetyl content of the biomass)92. From an industrial point of view, the removal of acetyl groups is very relevant, as higher degrees of wood acetylation demand the use of chemically harsher processes during kraft pulping for paper production66. Mild alkali can thus lead to increased surface areas and cellulose accessibility, reducing biomass recalcitrance, without the need for more aggressive pretreatments focused on lignin removal63. Indeed, it has been reported that lignin structural features can be more relevant to lower the energy and chemical loads required for industrial processing of lignocellulosic biomass, than merely total lignin content93. Furthermore, harsher pretreatments not only rely on higher chemical and energy consumption, but also lead to unwanted consequences, such as exacerbating peeling reactions, whereby cellulose degrees of polymerization are reduced by the action of OH- under high concentrations and temperatures94,95. Ultimately, it has also been proposed that fungal pretreatments can erode and perforate biomass particles, as the hyphae spread over the substrate, leading to increased porosity and surface area of the materials96,97. This physical aspect of biological pretreatments may represent an advantage over purely chemical processes, thus contributing to a synergistic effect between the two types of pretreatments.

**Table 4.** Compounds detected in pretreatment effluents by HPLC-PDA-ESI-MSn.

|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  |  | **tR (min)** |  | **λmax (nm)** |  | **Mr** |  | **MS2 fragments\*** |  | **Tentative identification** |  |
| **NF** |  | 11.9 |  | 277 |  | 292 |  | **247** |  |  | *Eucalyptus* *globulus* |
|  | 19.7 |  | 254, 363 |  | 302 |  | 284, **257**, 229, 185 |  | Ellagic acid |
|  | 22.7 |  |  |  | 316 |  | **300** |  | Methylellagic acid |
| **WRF** |  | 13.2 |  | 264 |  | 282 |  | **237**, 219,201,171, 123 |  |  |
|  | 14.2 |  | 272 |  | 166 |  | **121** |  |  |
|  | 19.3 |  | 258, 306 |  | 244 |  | 215, **196**, 175 |  |  |
| **ALK** |  | 15.5 |  | 280 |  | 434 |  | 415, 400, 389, **374**, 356, 343 |  |  |
|  | 19.9 |  | 254 363 |  | 302 |  | 284, **257**, 229, 185 |  | Ellagic acid |
|  | 22.4 |  | 263, 365 |  | 462 |  | **315** |  | Methylellagic acid rhamnoside |
|  | 22.8 |  |  |  | 316 |  | **300** |  | Methylellagic acid |
| **WRF+ALK** |  | 14.2 |  | 272 |  | 166 |  | 121 |  |  |
|  | 19.4 |  | 258, 306 |  | 244 |  | 215, **196**, 175 |  |  |
| **NF** |  | 11.9 |  | 277 |  | 292 |  | **247** |  |  | *Eucalyptus* *nitens* |
|  | 19.8 |  | 254, 363 |  | 302 |  | 284, **257**, 229, 185 |  | Ellagic acid |
| **WRF** |  | 9.3 |  | 280 |  | 182 |  | **137**, 119, 109 |  |  |
|  | 10.7 |  | 321 |  | 180 |  | 161, **135** |  | Caffeic acid |
|  | 11.9 |  | 273, 353 |  | 292 |  | **247** |  |  |
|  | 19.8 |  | 254, 363 |  | 302 |  | **257**, 229, 185 |  | Ellagic acid |
|  | 20.7 |  | 274 |  | 220 |  | 204, **191**, 173 |  | Methylellagic acid |
| **ALK** |  | 11.9 |  | 277 |  | 292 |  | **247** |  |  |
|  | 19.8 |  | 254, 363 |  | 302 |  | 284, **257**, 229, 185 |  | Ellagic acid |
|  | 22.4 |  |  |  | 462 |  | **315** |  | Methylellagic acid rhamnoside |
|  | 22.7 |  |  |  | 316 |  | **300** |  | Methylellagic acid |
| **WRF+ALK** |  | 19.8 |  |  |  | 302 |  | 284**, 257**, 229, 185 |  | Ellagic acid |
|  | 22.4 |  |  |  | 416 |  | **315** |  | Methylellagic acid rhamnoside |
|  | 22.7 |  |  |  | 316 |  | **300** |  |  |
| \* Negative mode, base peak in bold.  Abbreviations: NF, no fungi (negative control, incubated only with deionized water); WRF, samples pretreated only with *Trametes* *versicolor*; ALK, samples pretreated only with 0.1 M NaOH for 24h at 21 °C; WRF+ALK, samples pretreated with *T*. *versicolor* followed by 0.1 M NaOH for 24h at 21 °C. | | | | | | | | | | | |

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**Figure 3.** Mean FTIR-ATR spectra (1800 – 800 cm-1) of *Eucalyptus* *globulus* and *E*. *nitens* non-pretreated (AIR) and mild alkali-pretreated (AIK; 0.1 M NaOH; 24h; 21 °C) alcohol insoluble residues. Spectral bands of interest are marked as: *a* (1736 – 1730 cm-1) and *b* (1240 – 1235 cm-1).

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**Figure 4.** Mean FTIR-ATR spectra (1800 – 800 cm-1) of *Eucalyptus* *globulus* and *E*. *nitens* pretreated biomass (not organic solvent-washed, non-AIR). Mild alkali refers to samples pretreated with 0.1 M NaOH (24h; 21 °C). Abbreviations: NF, control samples incubated without any fungus; GAN, *Ganoderma* *lucidum*; PLE, *Pleurotus* *ostreatus*; TRA, *Trametes* *versicolor*. Spectral bands of interest are marked as: *a* (1736 – 1730 cm-1), and *b* (1240 – 1235 cm-1).

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**Figure 5.** Mean percentage (%) composition of alcohol insoluble residues (AIR) prepared from pretreated biomass from *Eucalyptus* *globulus* and *E*. *nitens*. Abbreviations: NF, control samples incubated without any fungus (striped bars); WRF, samples pretreated only with *Trametes* *versicolor*; ALK, samples pretreated only with mild alkali (0.1 M NaOH; 24h; 21 °C); W+K, samples pretreated with *T*. *versicolor* followed by mild alkali. No significant differences were detected (*P*>0.05) in relation to the NF controls. Error bars represent the standard error of the sample replicates.

Impact of the pretreatments on the properties of pulps obtained from kraft cooking and bleaching

In addition to EGB, EUG was included in pulping and bleaching assays (biopulping phase; Fig. 1). EUG wood has high recalcitrance to pulping, which we aimed at mitigating by applying WRF and mild alkali pretreatments. For the WRF pretreatments, TRA was tested, in addition to CES and PHN, given that these three fungal species have been dominantly used in biopulping applications17. Furthermore, TRA and PHN are the most extensively studied WRF, especially in what concerns their ability to degrade lignin98. For each of the applied pretreatments, the percentage of recovered solids was calculated (Supporting Information: Table S4). When the WRF or the mild alkali pretreatments are applied individually, typically more than 99% of the biomass is recovered. As for the combined WRF and mild alkali pretreatments, the mean biomass dry weight losses are different, depending on the order by which the treatments are performed. When the alkali is applied after the WRF, the losses are close to 1.4% of total dry weight. When the order is inverted and the alkali precedes the WRF, losses increase to about 4%. Higher losses, when the mild alkali is applied before WRF, may result from more extensive removal of soluble compounds from the biomass during the KOAc buffer washes employed after the alkali pretreatment. These washes are required to lower the pH and create suitable conditions for fungal growth.

Pretreated woodchips underwent kraft cooking and bleaching to determine how the resulting pulp yields and quality compared to a control pulp prepared from untreated (NT) EGB woodchips (G0; Table 2), identical to those used at the industrial scale. The same kraft pulping conditions were employed in all trials (160 °C, 1h, 1 °C min-1 heating rate, 30% sulfidity and 19% active alkali charge, Na2O basis). Among the EGB biomass, the pulps derived from the NF controls (G1) and from woodchips treated with CES (G2), had the highest Kappa number (K#; proportional to the residual lignin content), indicating no improvement from the pretreatment with this fungus alone (Table 5). Conversely, both G1k and Gk4 pulps exhibited a K# of about 1.4 units lower than the G0 control (a 9% reduction) and higher or equal bleachability (brightness gain per chlorine dioxide charge). The ClO2 consumption required to reach an ISO brightness of 90% was also lower in G1k and Gk4 pulps – ~3.5 kg active Cl2/100 kg pulp, which is a 15% reduction in relation to the value of G0, 4.13 –, thus reducing bleaching costs. When comparing pre and post bleaching intrinsic viscosities (Table 5), the pulp produced from G1 controls had higher values (initial: 1280 mL g-1; final: 1038 mL g-1) than those obtained for G1k (initial 1210 mL g-1; final: 956 mL g-1) and Gk4 (initial: 1235 mL g-1; final: 1027 mL g-1). This suggests that the alkaline treatment, alone or in combination with TRA, may cause a small amount of cellulose degradation. Nonetheless, this possible degrading effect does not seem to be severe, given that the intrinsic viscosities of G1k and Gk4 are still comparable to the values obtained for the G0 pulp which did not undergo any kind of pretreatment (initial: 1228 mL g-1; final: 1098 mL g-1).

Regarding the set of pulps produced from ENT, all pretreatments promoted a decrease of pulp K# and an increase of its bleachability, when compared to N1 controls (Table 5). However, the predominant effect of the pretreatments seems to be due to the alkali component. This is supported by the fact that the mean K# of pulps derived from alkali-pretreated woodchips was 13.4 as opposed to 14.8 obtained from N4, which was only biologically pretreated (TRA). Nonetheless, the higher reflectance (initial brightness) of the unbleached pulp N3k, and the fact that this was the pulp which required the lowest ClO2 charge (3.18; 19% reduction in relation to N1), denotes a synergistic action between the mild alkali and the WRF (PHN). Furthermore, pulp parameters vary depending on the order in which the pretreatment components are applied, as woodchips treated with alkali before WRF gave better results than the inverse. This can be verified by comparing pretreatments where mild alkali was applied either after (N4k) or before (Nk4) treatment with the same WRF (TRA). By pre-applying the alkali, the resulting pulps had lower K# and ClO2 charge, as well as higher bleachability and brightness stability (lower PC#), than when the alkali was applied after the WRF. However, this improvement in pulp quality obtained from treating the woodchips with mild alkali before fungal treatment can lead to lower overall yields. It is plausible that by using mild alkali before the WRF treatment, certain inhibitory compounds are removed – such as acetyl substituents, which can cause steric hindrance and hamper the interaction of hydrolytic enzymes29,48,65 –, thus facilitating fungal action. Additionally, as the woodchips used in the biopulping phase were not previously decontaminated, it is possible that WRF colonization of the substrates occurred in competition with opportunist fungi. Most molds do not survive at a high pH value99 such as that of 0.1 M NaOH (pH = 13) used during our pretreatments. Therefore, when the mild alkali step is applied before the biological treatment, the high pH of the solutions may have a decontaminating effect, which after washing and neutralization, leads to a substrate partly devoid of biological competitors, facilitating the subsequent WRF colonization.

The pretreatments performed on EUG wood also resulted in higher delignification rates (lower K#) when compared to the U1 control (mean 13% reduction; Table 5). The results confirm that the effect of alkaline treatment is predominant, but a synergistic action between fungal and alkaline treatments was evidenced, comparing U3 with U3k, or U1k with U2k, U3k and Uk4. The combined treatment of PHN and mild alkali (U3k) allowed the production of an unbleached pulp with considerable advantages over the U1 control pulp. Specifically, it exhibited lower K# (16% reduction) and ClO2 charge (13% reduction), and higher reflectance, bleachability and brightness stability, without substantially affecting the overall yield and final intrinsic viscosity. These biopulping results agree with previous studies, although none contains the same level of aggregated information as herein presented. Lower K#, with no substantial difference in pulp yield, has also been reported in pulps derived from the hardwood *Drimys* *winteri* JR Forst & G Forster treated with *G*. *australe* (Fr.) Pat.100, and from ENT treated with CES101. Furthermore, higher unbleached pulp brightness and improved bleachability have been reported in *Eucalyptus* *tereticornis* Sm. pulps treated with CES102.

Given that EGB is one of the most valued species for paper production and there is a high demand for its wood, it can be advantageous to diversify the feedstocks by producing mixed pulps integrating woods from other sources, such as EUG. However, untreated EUG (U1) yields subpar pulps when compared with other untreated woods (Table 5), with higher lignin content and lower syringyl/guaiacyl ratio being the presumed main reasons for its lower performance5. Therefore, when EGB is mixed with EUG, the cooking conditions must be more aggressive to reach the same delignification degree, thus decreasing the yield, and increasing the consumption of the bleaching chemicals, in comparison with pure EGB pulps. Three mixed pulps were produced (Table 2), consisting of 70% untreated EGB (G0) woodchips, and 30% EUG woodchips pretreated with: TRA alone (U4, Mix A); TRA followed by the mild alkali pretreatment (U4k, Mix B); and mild alkali before TRA (Uk4, Mix C). As expected, these pulps present a higher consumption of ClO2 to reach an ISO brightness of 90%, caused by the higher K# of the unbleached pulp, compared to pure G0 (Table 5). However, when performing the calculation of K# weighing in the mass percentages of untreated EGB (G0) and EUG (U1), the theoretical K# is 16.3 (70%×14.8+30%×19.7). Mix A had a K# similar to the theoretical value, but lower K#s were measured for both Mix B (15.4; -6%) and Mix C (15.6; -4%). Additionally, Mix B required a slightly lower ClO2 charge, and had an overall yield and initial intrinsic viscosity comparable to those of the G0 control pulp. Therefore, a combined WRF and mild alkali pretreatment of EUG, followed by kraft pulping mixed with untreated EGB, can further mitigate cooking drawbacks associated with the recalcitrance of EUG, while maintaining milder cooking conditions as those used for EGB.

**Table 5.** Yield and chemical characterization of unbleached and bleached pulps from wood previously treated with WRF.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Sample a** | **Overall yield (%)** | **K# b** | **Initial intrinsic viscosity (mL g-1)** | **Final intrinsic viscosity (mL g-1)** | **Initial ISO brightness (Br) (%)** | **PC# c** | **ClO2 charge**  **(kg active Cl2/100 kg pulp)** | **Bleachability (∆Br/charge)** |
| G0 | 51.8 | 14.8 ± 0.1 | 1228 ± 1 | 1098 ± 9 | 37.6 | 0.35 | 4.13 | 12.7 |
| G1 | 52.3 | 14.9 ± 0.2 | 1280 ± 13 | 1038 ± 24 | 43.3 | 0.35 | 3.98 | 11.7 |
| G1k | 51.5 | 13.4 ± 0.1 | 1210 ± 26 | 956 ± 1 | 42.9 | 0.36 | 3.52 | 13.4 |
| G2 | 52.3 | 14.9 ± 0.1 | 1338 ± 21 | 1041 ± 2 | 43.3 | 0.46 | 4.01 | 11.7 |
| Gk4 | 52.7 | 13.5 ± 0.1 | 1235 ± 3 | 1027 ± 4 | 46.3 | 0.35 | 3.46 | 12.6 |
| N1 | 52.0 | 15.5 ± 0.2 | 1293 ± 8 | 976 ± 20 | 44.7 | 0.32 | 3.92 | 11.6 |
| N1k | 50.1 | 13.4 ± 0.2 | 1203 ± 6 | 944 ± 19 | 46.8 | 0.31 | 3.21 | 13.5 |
| N3k | 50.1 | 13.4 ± 0.1 | 1121 ± 2 | 959 ± 4 | 48.2 | 0.30 | 3.18 | 13.1 |
| N4 | 50.7 | 14.8 ± 0.1 | 1206 ± 3 | 999 ± 5 | 40.1 | 0.38 | 3.66 | 13.6 |
| N4k | 48.6 | 13.8 ± 0.1 | 1181 ± 21 | 878 ± 2 | 47.4 | 0.38 | 3.40 | 12.5 |
| Nk4 | 47.5 | 13.1 ± 0.1 | 1182 ± 2 | 971 ± 8 | 47.5 | 0.29 | 3.22 | 13.2 |
| U1 | 49.7 | 19.7 ± 0.2 | 1199 ± 11 | 962 ± 12 | 38.6 | 0.40 | 5.23 | 9.8 |
| U1k | 48.8 | 17.1 ± 0.2 | 1134 ± 2 | 841 ± 1 | 39.8 | 0.40 | 4.62 | 10.9 |
| U2k | 49.1 | 16.5 ± 0.2 | 1131 ± 2 | 859 ± 23 | 40.7 | 0.42 | 4.66 | 10.6 |
| U3 | 49.6 | 18.8 ± 0.2 | 1160 ± 7 | 943 ± 7 | 36.4 | 0.44 | 5.84 | 9.2 |
| U3k | 49.3 | 16.6 ± 0.2 | 1147 ± 1 | 963 ± 6 | 40.4 | 0.32 | 4.54 | 10.9 |
| Uk4 | 48.4 | 16.5 ± 0.1 | 1207 ± 17 | 899 ± 33 | 39.6 | 0.43 | 5.01 | 10.1 |
| Mix A | 50.8 | 16.2 ± 0.2 | 1224 ± 1 | 1066 ± 15 | 37.2 | 0.39 | 4.47 | 11.8 |
| Mix B | 51.8 | 15.4 ± 0.1 | 1236 ± 5 | 1067 ± 11 | 38.5 | 0.38 | 4.43 | 11.6 |
| Mix C | 51.6 | 15.6 ± 0.2 | 1243 ± 6 | 1055 ± 16 | 38.4 | 0.37 | 4.49 | 11.5 |
| a See Table 2 for description of treatments.  b Kappa number.  c Post color number. | | | | | | | | |

# CONCLUSIONS

The present study has contributed to the characterization of the mechanism of action of the pretreatments. Overall, the efficiency of the pretreatments mostly relies on a controlled de-esterification of the biomass, promoting the hydrolysis of crosslinks between cell wall polymers, causing some lignin detachment, and increased cellulose accessibility. Additionally, the removal of ester-linked substituents such as acetylester groups allows milder chemical processes during kraft pulping. Moreover, as WRF hyphae colonize the substrates and preferentially degrade lignin from cell walls103, the resulting mechanical and chemical structure gaps may increase surface areas of the biomass, and facilitate chemical impregnation during subsequent mild alkali pretreatments and pulping. Indeed, this may explain the synergy observed when the WRF and mild alkali pretreatments are combined. Conversely, when the alkali is applied first, inhibitory, and toxic compounds are removed104,105, which may facilitate and enhance WRF action. Moreover, the pretreatments partly remove wood extractives, thus reducing pitch deposits in pulp mills.

No substantial losses were observed in the concentrations of glucose, xylose and arabinose in the pretreated biomass, or in the intrinsic viscosities of the resulting pulps. These observations demonstrate that the pretreatments do not cause substantial losses or degradation of cell wall structural carbohydrates. The treatment of EUG with PHN, followed by mild alkali treatment (U3k), improved kraft pulping efficiency, unbleached pulp properties, and pulp bleachability – as ClO2 consumption was reduced –, when compared to non-pretreated wood (U1), the most recalcitrant of the tested woods. Furthermore, the mixture of pretreated EUG chips with untreated EGB can also be pulped with milder conditions. These savings in chemical reagents contribute to reducing costs and environmental impact. The effect of the tested biopulping approaches on these *Eucalyptus* species and wood mixtures is herein reported for the first time. Future research will focus on process optimization, as it is plausible that lower incubation times may also allow high pulping efficiency. Furthermore, the fact that WRF pretreatments may be conducted statically dismisses the need for moving fermenting chambers, or other complex solutions, potentially facilitating process adaptation to larger scale applications.

# ASSOCIATED CONTENT

This manuscript contains an attached file which includes: a list of all pretreatments employed on eucalypt woodchips during the biopulping phase (Table S1); results from the digestibility assay (Table S2) and compositional determinations (arabinose, glucose, xylose and lignin; Table S3) of *Eucalyptus* biomass; HPLC-PDA-ESI-MSn chromatograms of the pretreatment effluent samples from *E*. *globulus* (Figure S1) and *E*. *nitens* (Figure S2), and a *p*-coumaric acid standard solution (Figure S3); percentage of recovered *Eucalyptus* biomass after pretreatments (Table S4) (PDF).

# NOTES

The authors declare no competing financial interest.

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