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Cinnamyl alcohol dehydrogenase downregulation in poplar wood increases saccharification after dilute acid pretreatment: a key role for lignin revealed by a multimodal investigation

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Abstract

This study is the first to apply dilute acid pretreatment (DAP) under different severity conditions to poplar wood genetically modified for the *cinnamyl alcohol dehydrogenase* (*CAD1*) gene, which is involved in the lignin biosynthesis pathway. The carefully selected pretreatment conditions resulted in glucose yields that were 15 points higher for the *hpCAD* poplar line than for the wild-type (WT) wood after 48 h of enzymatic hydrolysis. To explain this higher saccharification rate, the chemical, spectral and structural changes in WT and *hpCAD* wood were analyzed in relation to the severity of the pretreatment process. Although few differences were found at the chemical level, variations in autofluorescence and cell deformation were more significant: at high severity, the cells of *hpCAD* wood observed by nanotomography were more easily deformed, but their middle lamella was more resistant than those of WT wood. All these differences are possibly explained by changes in the molecular structure of lignin in *hpCAD* wood, leading to the formation of more hydrophobic shorter monomer chains with fewer lignin–carbohydrate interactions.

Keywords Lignocellulosic biomass, Biorefinery, *CAD* gene, Genetic modification, DAP, Monosaccharide, Enzymatic hydrolysis

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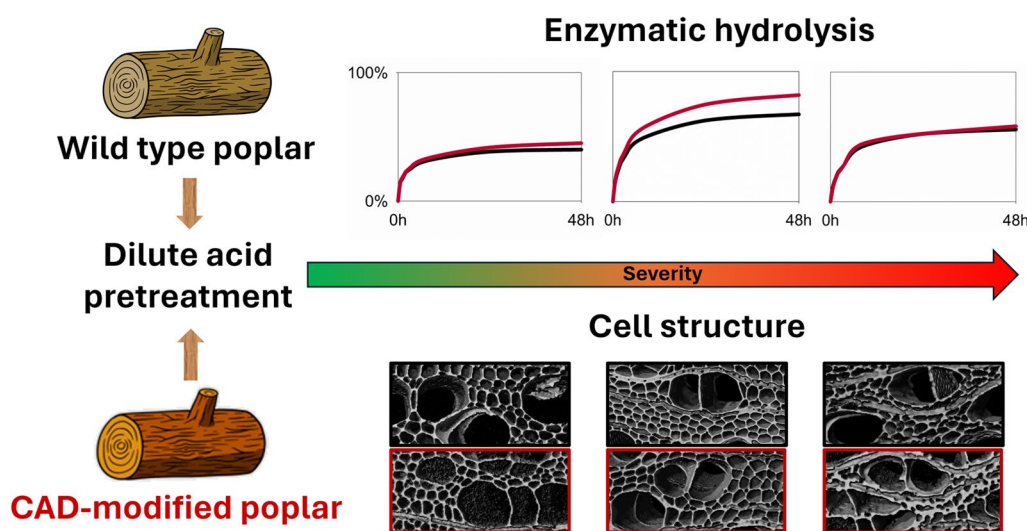
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Graphical Abstract



Introduction

In 2023, the European pulp and paper industry represents 175,000 direct jobs in Europe, with an annual turnover of approximately 100 billion euros and contributes to 20 billion euros to the European Union GDP (Gross Domestic Product) [1]. Because of its economic importance, the process of making pulp from wood has been the subject of numerous studies since the 1990s, with the aim of reducing the consumption of chemicals, water and energy needed to separate lignin from cellulose fibers [2, 3]. To further reduce the economic and ecological costs of paper manufacturing, research has focused on genetic engineering to help separate lignin from cellulose [3]. Lignin is a complex polymer composed of three hydroxycinnamyl alcohol subunits, S, G and H (syringyl, guaiacyl and *p*-hydroxyphenyl, respectively), also referred to as monolignols. Their proportions depend on the tree species and on genetic variability within tree species [4, 5]. Depending on the subunits involved, different types of bonds are formed between these monolignols to create lignin, which directly impacts wood resistance to the delignification process. For example, ether β -O-4 bonds are the most common and labile, whereas the carbon-carbon 5-5 bonds formed between G subunits are much stronger [4, 5]. One way to improve wood delignification is to genetically modify lignin to change the subunit composition and the type of bonds involved in the polymer, making the kraft pulping process simpler and less costly [3].

This strategy can involve downregulating the activity of any enzyme in the lignin biosynthetic pathway,

leading to the development of modified wood genotypes [6, 7]. Among these modifications, downregulation of the *cinnamyl alcohol dehydrogenase* (*CAD1*) gene has shown interesting results, with easier delignification, a 6% reduction in the amount of chemicals used and a 2–3% increase in pulp yield [3, 8]. The role of the enzyme encoded by this gene is to catalyze the final step in the biosynthesis of monolignols from their aldehyde form, called hydroxycinnamaldehyde, to their corresponding hydroxycinnamyl alcohol form [9–11]. This gene can be downregulated via a silencing approach with *CAD1* hairpin RNAi (*hpCAD*), which reduces CAD activity to 15% of that of normal poplar [9]. Reducing the activity of the CAD enzyme results in high incorporation of hydroxycinnamaldehyde into lignin. Compared with the *wild-type* (WT) poplar line, the *hpCAD* poplar line has a 35-fold increase in the level of sinapaldehyde [10, 11]. The S/G (Syringyl/Guaiacyl) ratio is also reduced, and the lignin content is slightly decreased by 8–10%, while red coloration of the xylem tissue is observed [10].

These modifications, initially intended for the paper industry at the research stage, are now being studied in biorefineries. Changing the properties of lignin could reduce the recalcitrance of lignocellulosic biomass (LB), thus increasing the yield of enzymatic hydrolysis [3]. The first results showed that the *hpCAD* poplar line did not naturally have a greater saccharification yield than WT poplar did: pretreatment remains necessary [10]. Alkaline pretreatment increased cellulose conversion by 12 to 27% in the *hpCAD* poplar line compared with the WT, depending on the amount of NaOH used (6.25 mM

or 62.5 mM, respectively [10]). However, despite these good results, alkaline pretreatment is not currently the most economically viable option for wood pretreatment. Numerous studies have reported very good economic yields of steam explosion and dilute acid pretreatment (DAP) [12–14]. Although DAP has already been tested without success on the *hpCAD* poplar line [10], this study was limited to a single pretreatment condition at 90 °C, 180 min and 4% H₂SO₄ [10, 15]. However, these time and temperature treatment conditions, which are suitable for alkaline pretreatment [16], are very different from those of standard DAP (120 to 200 °C for 60 min [17]). Therefore, extending the DAP conditions to explore its effect on the saccharification yield of the *hpCAD* poplar line is relevant. The aim of this study was, therefore, to compare the saccharification rates of WT and *hpCAD* poplar lines subjected to a large set of DAP conditions and to relate these results to their chemical, spectral and structural properties.

Materials and methods

Origin of biomass

Poplar wood was collected from the INRA 717-1B4 clone, a *Populus tremula* × *Populus alba* hybrid (WT samples), and from the *hpCAD*19 line by silencing the *CAD1* gene via *CAD1* hairpin RNAi [10]. Five WT cuttings and five *hpCAD* cuttings were grown for 2 years in the greenhouse at INRAE Orléans-Val de Loire (France) before harvest. At the time of harvesting, the samples had an average diameter of 23 mm, which could vary depending on the type of sample (WT or *hpCAD*) and the height of the measurement in relation to the base of the plant (see Results). Once harvested, each plant was cut into 3 parts of approximately 30 cm and labeled: WT 1 to 5 or *hpCAD* 1 to 5, bottom/middle/top. The samples were then stored in an oven at 35 °C to limit internal humidity until use. Minor compounds such as extractives, inorganics and protein were not extracted before the treatment. However, their quantity remains low [18] and have little influence as the pH is imposed in DAP is the main factor acting on the degradation of the main lignocellulosic components [17].

Sample preparation

Twelve 30 cm samples (six from the WT poplar line and six from the *hpCAD* poplar line) were selected for their homogeneous diameter, straight growth and absence of knots. The samples were first debarked and sanded by hand to remove any remaining bark. To fit the dimensions of the pretreatment reactor, each of the 12 samples was then precisely cut into 30 8-mm-thick cylinders via a microcutting machine (Secotom-50, Struers, Denmark) and numbered from 1 (bottom) to 30 (top). For

each pretreatment condition, six cylinders of the same number (1–30) from the six different samples (WT or *hpCAD*) were used in ascending order. It is assumed that wood properties vary little along the longitudinal dimension, and since this dimension was respected during cutting, the cylinders from the same sample were considered identical, thus homogenizing the results of the pretreatment.

Moisture content

Three randomly selected WT or *hpCAD* cylinders were weighed and dried in an oven at 105 °C for 24 h to determine the moisture content (NewClassicMF, Mettler Toledo, Switzerland).

Dilute acid pretreatment (DAP)

A unique in-house equipment was used to apply the different DAP conditions (temperature and time) [19]. The entire pretreatment procedure and the equipment used were described in detail in a previous publication [20], and only the number of samples in the reactor and their dimensions changed (see Supporting Information). The six selected cylinders (WT or *hpCAD*) were saturated with acid solution before being placed in the reactor. Upon completion of the pretreatment, the liquid fraction at the bottom of the reactor was frozen at –20 °C prior to chemical analysis, and any solid residue was immediately weighed and stored at 5 °C. One of the pretreated cylinders was used to recover the extractives remaining inside, whereas the other five underwent cycles of acid neutralization and washing before drying at 35 °C for 5 days and then storing at 25 °C until analysis, again following the previously published protocol [20].

In addition to the nonpretreated control, five DAP conditions were selected (Table 1). The choice of these conditions was based on two previous studies in which a complete dataset of changes in WT poplar wood exposed to DAP was generated and used [20, 21]. These conditions were selected to cover a large range of combined

Table 1 Time, temperature, acid conditions and corresponding CSFs of the six conditions

Time (min)	Temperature (°C)	Acid (%wt)	CSF
–	–	–	0
10	140	0.5	1.2
20	150	2.0	2.4
30	160	3.0	3.0
60	170	2.0	3.5
35	180	3.0	3.7

Control conditions: raw wood without pretreatment (CSF = 0); gray conditions were performed in triplicate

severity factors (CSFs, Eq. 1), which take into account the cumulative effects of the DAP reaction time, temperature and acid level [20]:

$$\text{CSF} = \log_{10} \left(t \times e^{\frac{T - T_r}{14.75}} \right) - \text{pH} \quad (1)$$

where t is the reaction time (min), T is the operating temperature (°C), T_r is the reference temperature (100 °C) and pH is the temperature of the liquid phase used to soak the biomass.

For example, a minimum severity of 1.2 is required for xylose to appear in solution, and the maximum yield is reached at CSF 2.4 [20, 21]. This severity level also marks the start of the degradation of xylose into furfural and just before the rapid degradation of cellulose, which is observed at CSF 3.0, with the appearance of the furfural peak. The last two selected conditions, CSF 3.5 and 3.7, allowed the formation of pseudolignin, and the degradation products of C6 into levulinic and formic acids were observed. Given the particular interest of CSFs 2.4 and 3.0, which are approximately moderate in severity, represented by the rapid degradation of cellulose and the formation of numerous inhibitors, these conditions were carried out in triplicate and were subjected to enzymatic hydrolysis (Table 1). The other conditions had either too much hemicellulose (CSF 1.2) or too low a sugar yield (CSF 3.5 and 3.7) for enzymatic hydrolysis to be of interest [21].

Chemical and spectral analysis

One of the treated cylinders was kept intact for structural analysis, while the others were ground via a liquid nitrogen ball mill (2 cycles of 2 min at 30 s⁻¹, intermediate cooling for 30 s at 5 s⁻¹; CryoMill, Retsch, Germany) to a final average granulometry of 130 µm. All chemical and spectral analyses of the solid and liquid residues resulting from DAP were carried out via methods described in previous publications [20, 21]. The content of the various chemical compounds is expressed per 100 g of raw wood: %DMi, dry initial matter, in which the dry matter is obtained from the sample mass and its moisture content.

Nanotomography

The sample structure was analyzed via X-ray nanotomography (EasyTOM XL 150/160 model, RX-solution, France). The crucial advantage of tomography is its ability to allow the cross-section of the sample to be observed and analyzed without any artifact due to sample preparation that is inherent to any microscopic technique. The tomography setup featured a nanosource (LB6 filament), a CCD detector with a resolution of 2016×1336 pixels, and a voxel size of 0.5 µm. Each frame had an exposure time of 2 s, averaged over 5 frames per scan. The

X-ray voltage was set at 65 kV with a working current of 200 µA. With these acquisition parameters, each scan lasted approximately 4 h, generating 1568 projections. 3D reconstruction from the 2D projections was executed via X-act software (v22.06, RX Solutions, France), followed by further analysis via Avizo (v2019-2, Thermo Fisher Scientific, Waltham, MA, USA). Initially, 3D volume visualization of the stacks was performed via the volume rendering tool. Subsequently, subvolumes were extracted from the original datasets [size (voxel) 1200×1000×500] and processed with a 3D median filter to reduce noise. Auto thresholding (high) was then applied to distinguish the cell walls from the background, followed by calculation of the cell wall thickness via a thickness map and global analysis tools.

To investigate the impact of DAP on vessel shape, auto-thresholding (low) was applied to 2D stacks. Various steps were implemented to enable a more precise and detailed analysis of the features: the fill holes function (XY planes, 4-neighborhood connectivity) was utilized to fill holes within cells and vessels. The opening function (type 3, XY planes, 3 pixels), a combination of erosion followed by dilation, was subsequently applied. The separate object function (Chamfer-conservative, XY planes, Marker extent 2, output type split) computes the best-fit contours and separates the overlapped particles. Following this, labeling was applied to assign cells and vessels as individual objects, allowing quantification of their 2D area, perimeter, and Feret diameters, which are the distances between two parallel tangents on opposite sides of the object along different directions [22] (we used here 12 diameters with a step of 15°), via the label analysis function. Cells with an area of less than 20 µm² were considered noise and discarded from the calculations, whereas cells with an area greater than 500 µm² were considered vessels. Considering only the vessels that are not cut by the image borders, the ratio between the maximum diameter and minimum diameter was then calculated for each vessel, providing insight into its deformation.

Enzymatic hydrolysis

Enzyme hydrolysis of the samples was carried out via the commercial enzyme cocktail Cellic® CTec2 (Novozymes, Denmark). The three samples tested (CSF 0, CSF 2.4 and CSF 3.0) were pretreated and hydrolyzed in triplicate, except for CSF 3.0. Owing to the important mass loss caused by the pretreatment, only a sufficient quantity of material was available for two replicates instead of three. For each triplicate, 500 ± 10 mg of powder was weighed and suspended in 10 mL of Milli-Q water at 50 °C overnight. The enzyme was then added at 10 FPU (filter paper unit) to initiate hydrolysis, which was conducted for 48 h at 50 °C with stirring. Samples of 700 µL were taken at

different times: 0, 0.5, 1, 2, 4, 8, 24 and 48 h. The enzymes were immediately inactivated in a water bath at 105 °C for 5 min. Then, the samples were centrifuged at 3220×g for 5 min, and the supernatant was frozen at −20 °C until analysis. All the samples were then filtered (polytetrafluoroethylene, 0.45 μm) and injected into an HPLC system (SIL-20A HT, Shimadzu, Japan). Hydrolyzed glucose and xylose were measured by a refractive index infrared detector (RID-10A 230 V, Shimadzu, Japan) on an Aminex hpX-87H column (300×7.8 mm, Bio-Rad, US) with a microguard cation H precolumn (30×4.6 mm, Bio-Rad, US).

Calculations

The enzymatic and total yields of glucose and xylose were calculated via Eqs. 2 and 3, respectively. The enzymatic yield (G_{ENZ} for glucose and X_{ENZ} for xylose in g of hydrolyzed monosaccharide/100 g of residual polysaccharide after DAP) corresponds to the conversion of residual polysaccharides into simple sugars after pretreatment, and the total yield (G_{TOT} for glucose and X_{TOT} for xylose in g of hydrolyzed monosaccharide/100 g of initial polysaccharide) to the monosaccharide ratio obtained in relation to the amount of initial polysaccharide in the raw sample:

$$G_{ENZ} \text{ or } X_{ENZ} = \frac{\text{MonoS} \times V}{m} \times \frac{1}{\text{PolyS}} \times 100 \quad (2)$$

where MonoS is the monosaccharide concentration after enzymatic hydrolysis in g/L, V the reactor volume (0.01 L), m the mass of the pretreated sample (0.5 ± 0.01 g) and PolyS the average polysaccharide content in the pretreated sample in g of residual polysaccharide/g of pretreated sample:

$$G_{TOT} \text{ or } X_{TOT} = \frac{\text{MonoS} \times V}{m} \times \frac{(1 - \text{ML})}{\text{PolyS}_{\text{raw}}} \times 100 \quad (3)$$

where ML represents the average mass loss of the pretreated sample and $\text{PolyS}_{\text{raw}}$ the average polysaccharide level in the raw sample (WT or *hpCAD*) in g of initial polysaccharide/g raw sample.

Results

Chemical composition

The composition of raw WT poplar wood is 42.7% cellulose, 23.1% hemicelluloses (18.1% xylose and 5.0% other sugars) and 19.6% lignin, with the remainder being mainly extractives and minerals. The *hpCAD* poplar line contains 41.5% cellulose, 23.2% hemicelluloses (18.5% xylose and 4.7% other sugars) and 18.5% lignin. Only lignin was significantly greater by 1.1 points in WT wood than in *hpCAD* wood (Student's test, p value < 0.05), whereas the contents of the other

compounds were not significantly different. After DAP, the solid and liquid fractions were analyzed. For the latter, no significant differences were observed between WT and *hpCAD*, either in the degradation of polysaccharides to simple sugars at medium CSF or in the production of degradation products at higher CSF (see Supporting Information). Some variations were observed at very high severities (CSF 3.5 and 3.7), but no repetition was made to confirm or invalidate these results. With the high standard deviations obtained for the other conditions due to methodological approximations, it is not possible to conclude that significant differences exist. In the solid fractions, very few differences were found, albeit with much lower standard deviations. Indeed, the hemicellulosic fractions rapidly degraded from the first CSF stage and had similar profiles, whereas the cellulosic fraction remained at a constant level until CSF 2.4–3.0 (Fig. 1A–C). The cellulose in *hpCAD* seemed to resist CSF 3.5 better than that in the WT. However, the cellulose was completely degraded at CSF 3.7 for both samples (Fig. 1C). The levels of WT and *hpCAD* lignin also followed the same pattern as the CSF increased with, once again, a difference at CSF 3.5, where *hpCAD* lignin remained below 20%, whereas WT lignin increased to 25%. For both types of wood, the lignin content reached 30% at CSF 3.7 (Fig. 1D). However, as the CSF 3.5 condition was not replicated, it is not possible to draw conclusions from these data.

Spectral evolution

A noticeable color difference was observed between native WT and *hpCAD* wood, with the latter being darker (see Supporting Information). This difference remained constant up to CSF 3, despite the darkening of the wood color due to pretreatment. After CSF 3, both types of wood were almost completely black, making it impossible to distinguish between WT and *hpCAD*. Spectrofluorescence analyses were performed to investigate lignin autofluorescence and organization in the samples: large differences were observed between the WT and *hpCAD* woods (Fig. 2). Although the data remained constant and identical for both types of wood above CSF 2.4 (Ex 350 nm, Em 420 nm, intensity close to zero), the milder conditions showed contrasting trends. *hpCAD* wood reached its fluorescence maximum at significantly higher emission and excitation wavelengths than did WT wood under CSF 0 and CSF 1.2 conditions (Fig. 2A, B). Specifically, the maximum intensity wavelength for *hpCAD* wood at CSF 1.2 (Ex 375 nm, Em 470 nm) was greater than that for the WT (Ex 350 nm, Em 420 nm) (Fig. 2A, B). Figure 2C depicts the maximum fluorescence intensity (peak intensity, expressed as AU=arbitrary units)

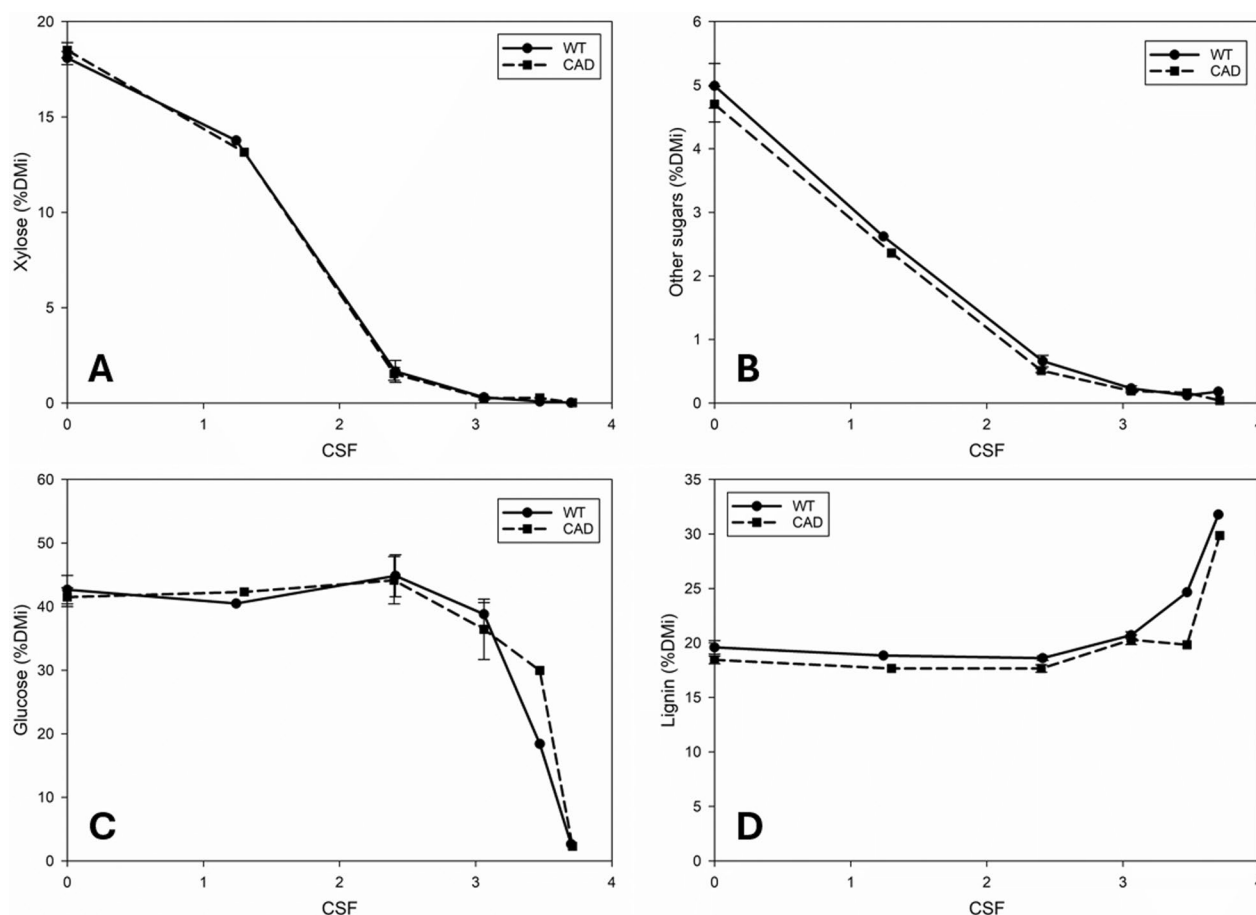


Fig. 1 Changes in the contents of compounds in the WT and *hpCAD* poplar lines subjected to dilute acid pretreatment for CSFs from 0 to 4. **A** Evolution of the xylose fraction in hemicelluloses; **B** evolution of the nonxylose fraction in hemicelluloses; **C** evolution of the glucose fraction in cellulose; **D** evolution of lignin. All rates are expressed as g per 100 g of dry raw wood. Single samples for CSF 1.2, 3.5 and 3.7, triplicate samples for control (CSF 0), and CSF 2.4 and 3.0

determined by scanning all the excitation and emission wavelengths on the 3D spectra of the samples. The fluorescence intensity maxima were lower for *hpCAD* wood than for WT wood, reaching a maximum difference at CSF 1.2 (1352 vs 3980).

Structural changes

Modification or degradation of the macromolecular organization of the cell wall not only affects its thickness but also alters the shape and dimension of the cellular structure [19, 23]. Therefore, examining the evolution of wood morphology was considered a relevant marker for investigating potential differences between WT and *hpCAD* wood samples under increasing DAP severity. While the cellular structure of the wood does not undergo any significant deformation, the reduction of cell wall thickness is a direct indicator of the cell wall alteration (shrinkage due to the degradation of macromolecules). When the cellular structure itself is affected,

the cell lumens can disappear, which give rise to an artifact in the image processing: the software affects a double wall thickness to a completely collapse cell. A regular and comparable decrease of the cell wall thickness was observed for the two wood types from CSF 0 to 2.4 (Fig. 3). After CSF 2.4, the wall thickness of the WT wood stabilized, whereas the wall thickness of the *hpCAD* wood significantly increased up to CSF 3.5. For WT wood, this increase occurred only at CSF values greater than 3.0, and the cell wall remained thinner than that in *hpCAD* wood, whereas at CSF 3.7, the cell wall thickness was similar in both types of wood. Significant structural changes were evident at severities above 3.0, with, in *hpCAD* wood, flattened vessels and a decreased number of fibers that were also smaller in size. At CSF 3.7, it was almost impossible to identify the wood structure due to generalized cell collapse. The observed increase in cell wall thickness that follows the initial decrease is indeed an artifact caused by cell collapse: when the cell lumen

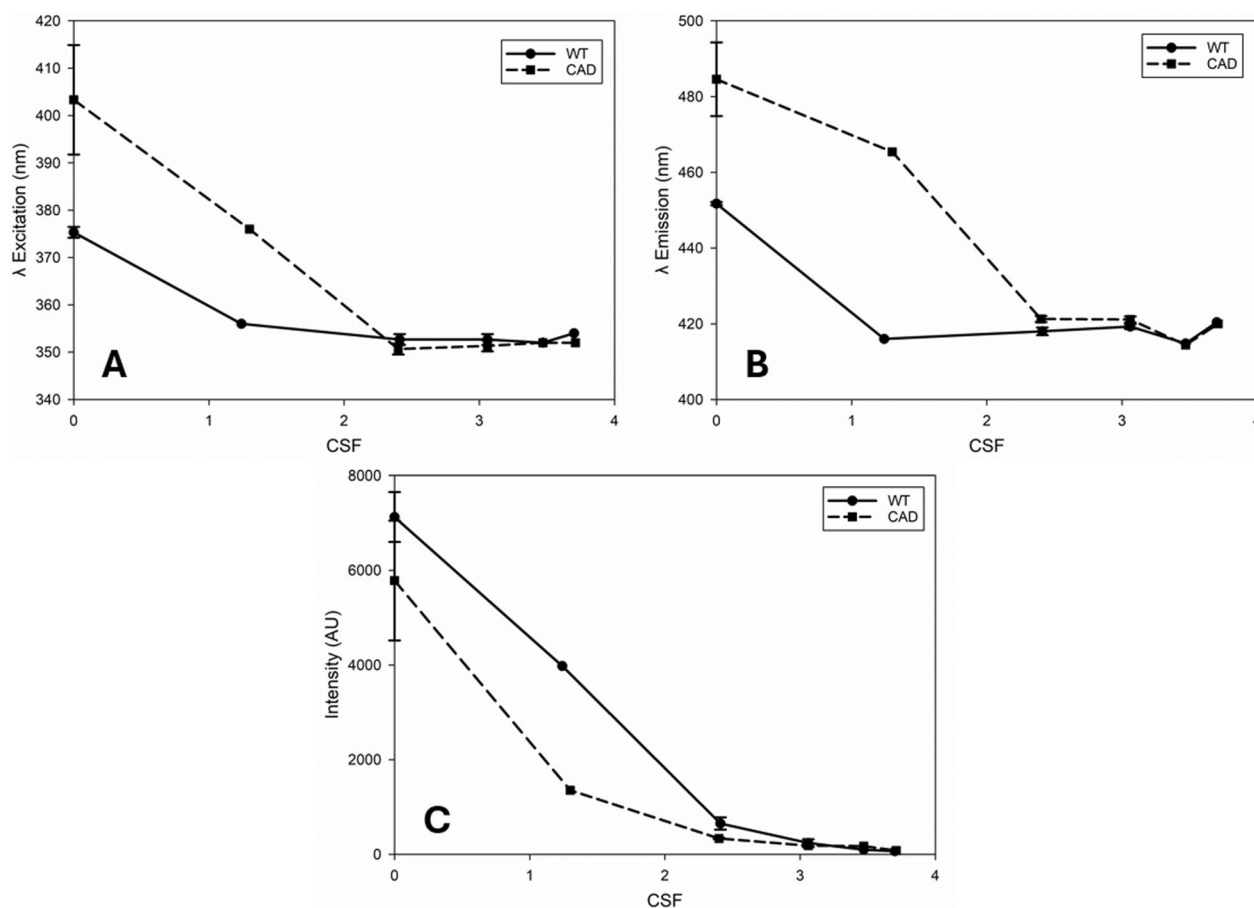


Fig. 2 Evolution of fluorescence parameters in WT and *hpCAD* poplar lines subjected to dilute acid pretreatment for CSFs from 0–4. **A** Change in excitation wavelength for maximum fluorescence intensity; **B** change in emission wavelength for maximum fluorescence intensity; **C** change in fluorescence intensity (arbitrary unit, AU). Single samples for CSF 1.2, 3.5 and 3.7, triplicate samples for control (CSF 0), and CSF 2.4 and 3.0

is no longer detected by automatic thresholding, the collapsed cell is considered a unique thick cell wall. The severity level at which the cell wall thickness increases is, therefore, a clear indication of the onset of severe cell collapse.

This observation was confirmed by other morphological measurements (Fig. 4). Both the relative surface area occupied by the vessels and the Feret ratio (the ratio between the maximum and minimum Feret diameters obtained), which is a good proxy of cell deformation, increased sharply from CSF 2.4 but were quite stable before. However, some differences were observed between WT and *hpCAD* woods, with the latter having a relative vessel surface area proportion 10 points greater at CSF 2.4 (49 vs 39%). At CSF 3.0, the relative vessel surface area of both woods increased to 85% (Fig. 4A). In fact, this increase is an artifact due to the increasing number of collapsed fibers. At higher severities, the surface area occupied by *hpCAD* vessels stabilized, whereas that of WT vessels decreased sharply. Indeed, at the

highest severity (CSF 3.7), the cellular structure is completely destroyed in WT wood with no measurement possible, while *hpCAD* wood still has some vessels identified by image processing (Fig. 4C).

Vessel deformation, which occurred mainly from CSF 2.4, showed little difference between WT and *hpCAD* wood. Feret ratios revealed that the mean, median and range values increased sharply with increasing severity above 2.4, but there were no statistically significant differences between WT and *hpCAD* (Fig. 4B). The majority of Feret's ratios for CSFs below 2.4 have a rather narrow distribution between 1.2 and 1.7, whereas the distribution range for cells with CSFs above 2.4 is between 1.0 and 3.5. This indicates the presence of vessels that have been severely deformed by pretreatment, alongside other vessels that retain their spherical structure despite increasing severity.

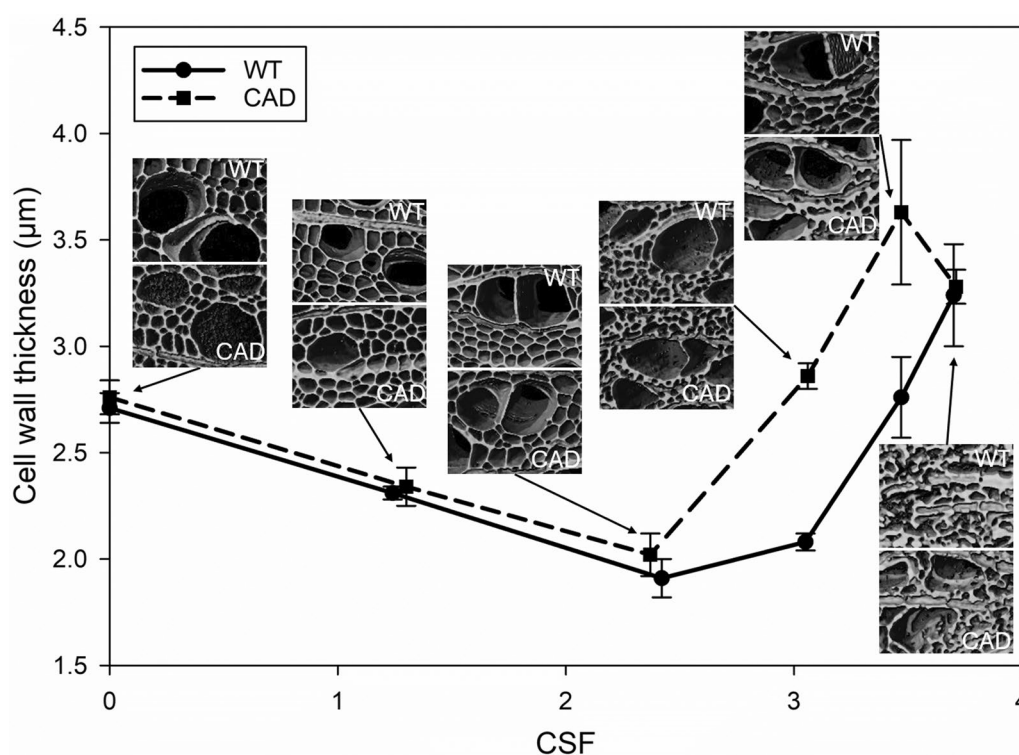


Fig. 3 Changes in cell shape and wall thickness for WT and *hpCAD* samples pretreated with dilute acid at CSF 0–4

Enzymatic hydrolysis

The three conditions studied (raw=CSF 0, 150 °C/20 min/2% H_2SO_4 =CSF 2.4 and 160 °C/30 min/3% H_2SO_4 =CSF 3.0) were compared using Student's *t*-tests to check the significance of the results. The xylose yields XYL_{ENZ} and XYL_{TOT} were similar between the WT and *hpCAD* samples (see Supporting Information). XYL_{ENZ} was significantly lower for CSF 0 than for CSF 2.4 and 3.0 (39%, 57% and 74% at 48 h on average, respectively). CSF values of 2.4 and 3.0 did not significantly differ because of the very high standard deviation observed with a CSF of 3.0. For XYL_{TOT} , the trend is reversed as the CSF 2.4 and 3.0 conditions decreased drastically (5% and 1% at 48 h on average, respectively), with very low standard deviations, while remaining similar between WT and *hpCAD*. These very low yields are linked to the low level of residual hemicelluloses after DAP under these conditions.

The study of glucose yields (GLU) revealed contrasting results in favor of *hpCAD* wood (Fig. 5). For the GLU_{ENZ} saccharification yield (Fig. 5A), the WT and *hpCAD* samples behaved similarly under extreme conditions, CSF 0 and CSF 3.0, throughout hydrolysis. In contrast, for CSF 2.4, the *hpCAD* sample presented a greater yield than the WT sample did (*p* value < 0.05) starting from 4 h of hydrolysis, reaching a final yield of 77% at 48 h, which

was 14 points greater than that of the WT sample. For the WT samples, the glucose saccharification yield was almost identical for CSF 2.4 and CSF 3.0 throughout hydrolysis, except at 24 h. In contrast, the yield of the *hpCAD* samples was almost consistently significantly greater for CSF 2.4 than for CSF 3.0 from 30 min to 48 h (*p* value < 0.05).

The GLU_{TOT} yield (total yield, Fig. 5B) was consistently lower than the GLU_{ENZ} yield (saccharification yield, Fig. 5A) at CSF 3.0 and identical at CSF 0 with no pretreatment. The slightly higher GLU_{TOT} yield compared with the saccharification yield at CSF 2.4 was not significantly different (77 to 82% for *hpCAD* at 48 h) and was due to the uncertainty of the chemical measurements. Finally, the average GLU_{TOT} yield for CSF 2.4 was significantly greater in the *hpCAD* samples than in the WT samples by 5 points at 4 h, 8 points at 8 h, 12 points at 24 h and 15 points at 48 h (82% versus 67% at 48 h).

Discussion

The *hpCAD* mutant improved saccharification after mild DAP

To the best of our knowledge, this study is the first to show a significant 15% improvement in the total glucose yield at 48 h for the *hpCAD* poplar line compared with the WT under DAP, which was carried out at

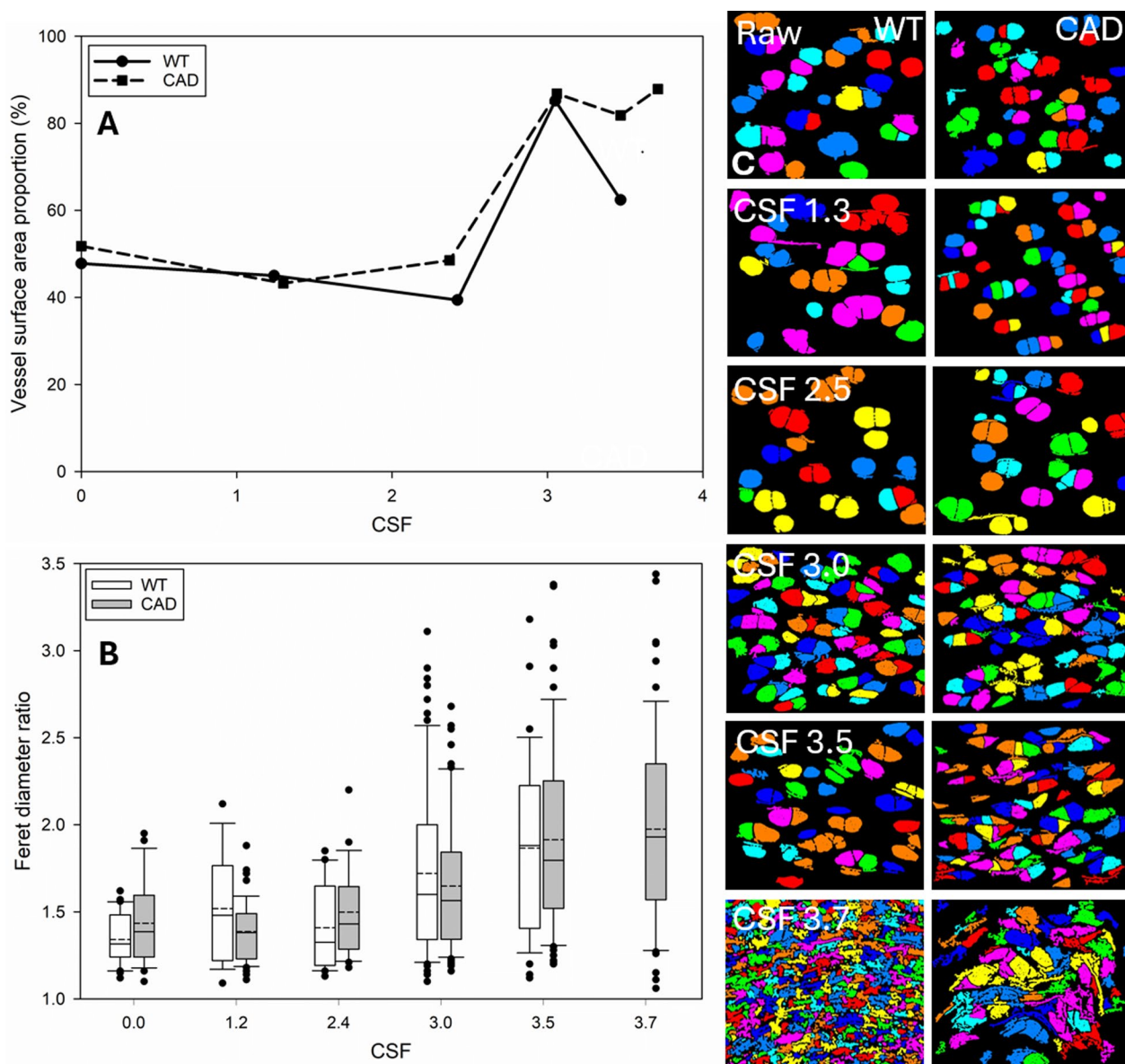


Fig. 4 Morphological modifications of WT and *hpCAD* vessels subjected to DAP on a 2D section of the 3D scan. **A** Evolution of the relative surface area occupied by the vessels; **B** evolution of the distribution of the Feret diameter ratios of the vessels, a proxy of cell deformation. The first quartile Q1 is the lower limit of the box, the dotted and solid lines inside the box indicate the mean and the median, respectively, and the third quartile Q3 is the upper limit of the box. The low mustache with the dots below represents the first decile and the lowest 10% of values, and the high mustache with the dots above represents the ninth decile and the highest 10% of values. **C** Representation of the deformation of whole vessels on 2D sections. Depending on the samples, the number of vessels identified on the standard surface area varies from 17 to 86

150 °C for 20 min with 2%wt H_2SO_4 (CSF 2.4). Few studies have focused on the pretreatment and saccharification of *hpCAD* mutants on poplar wood [9, 10, 24, 25] or other types of biomass [26–30]. Among these studies, most focused on alkaline pretreatment, which results in good saccharification yields [10]. Only three studies used DAP [10, 27, 29], with two indicating higher cellulose conversion rates of 5–10% [27] and 15% [29] after

48–72 h of hydrolysis on switchgrass and rice, respectively. These results, although obtained for different biomasses, showed a yield increase in the same order for poplar, given that the DAP conditions used are also close enough: 121 °C, 20–40 min, and 1.8–2.7%wt H_2SO_4 (CSF 1.5–2.0) [27, 29]. The only study of DAP focused on the *hpCAD* poplar line [10] used a single low-severity condition of CSF 1.9, with very low-temperature conditions

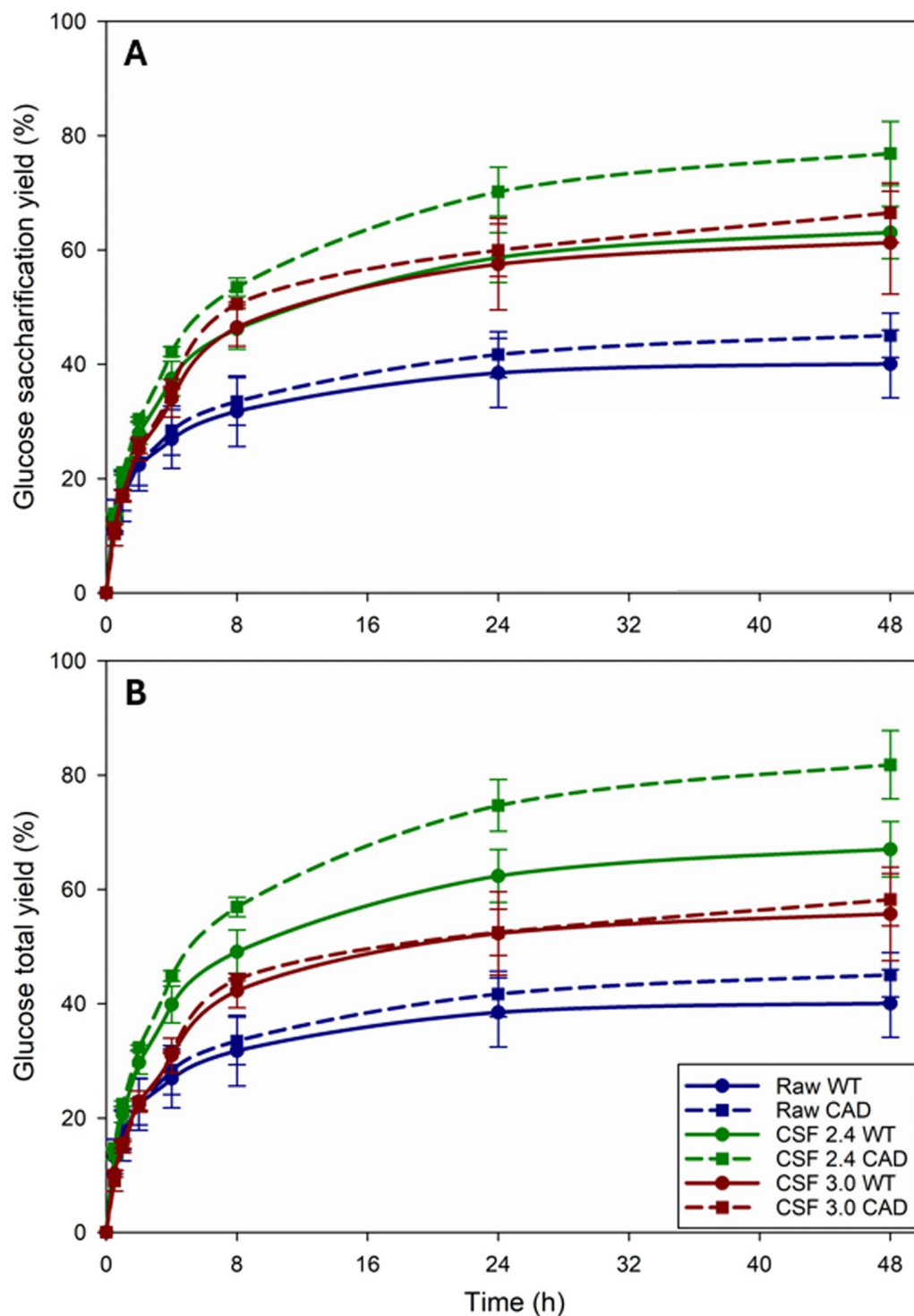


Fig. 5 Glucose yield after enzymatic hydrolysis for 48 h for triplicate WT and *hpCAD* poplar samples exposed to DAP. **A** Glucose yield for the enzymatic hydrolysis step only; **B** total glucose yield after pretreatment and enzymatic hydrolysis

(90 °C, 180 min and 4% H₂SO₄). This easily explains the low enzymatic yields obtained: the conditions used were not sufficient to modify the polymer biomass network,

since they were far from the usual DAP conditions, which are more likely to be approximately 120 to 200 °C and under 60 min [17].

Specific DAP conditions are needed

This improvement in yield for *hpCAD* wood is very significant for one of the hydrolyzed conditions at CSF 2.4 (20 min, 150 °C, 2%wt H₂SO₄), but the CSF 0 and CSF 3.0 conditions showed no differences between WT and *hpCAD*. This means that there is a narrow range of conditions and severities of DAP that allows the hydrolysis potential of *hpCAD* wood to be exploited. At lower severities, cellulose is not sufficiently accessible, and at higher severities, the degradation of biomass is too advanced. Previous studies on other biomasses with good saccharification results have used CSFs ranging from 1.5 [29] to 2.0 [27]. It would, therefore, be possible that similar or even better yields could be obtained for even less severe conditions. An optimization study from CSF 1.5–2.5, similar to that used to determine the conditions employed here [20], would be necessary to explore this possibility.

Cell wall composition does not explain saccharification differences

Among the different biomass properties that might influence saccharification, the chemical composition was the first to be investigated. However, up to CSF 3, the global chemical composition is similar between WT and *hpCAD*, except for a 2-point difference in lignin content for native *hpCAD* wood. This difference, which is consistent with the literature [10], does not change between the pretreatment conditions and therefore cannot be attributed to a variation in the degree of cellulose conversion. However, the interactions between these compounds differ: the incorporation of sinapaldehyde into lignin involves a rearomatization mechanism that leads to a reduction in the number of hydroxyl groups in lignin, increasing its hydrophobicity and limiting non-covalent interactions with carbohydrates [10]. The same mechanism may also reduce the formation of recalcitrant lignin–carbohydrate complexes [10], which may partly explain the improved hydrolysis results obtained.

Autofluorescence highlights differences in lignin organization

Significant changes in autofluorescence intensity were observed between WT and *hpCAD*, mainly at CSF 0 and CSF 1.2, where *hpCAD* fluorescence was notably lower. The massive incorporation of sinapaldehyde into *hpCAD* lignin to replace the syringyl (S) subunits, together with the slightly reduced amount of guaiacyl (G) [10, 11], led to a decrease in the S/G ratio (from 1.84 to 1.5 [10]). It has been previously shown that a change in the S/G ratio can modify the excitation and emission wavelengths of lignin [31]. In particular, a positive correlation was found between the number of ether bonds (β -O-4) and

lignin autofluorescence intensity [31]. Sinapaldehydes are more likely to form other ether bonds and carbon–carbon (β – β) bonds [10], so the decrease in β -O-4 bonds in *hpCAD* wood [11] may explain this loss in autofluorescence. However, these differences mainly explain the variations in fluorescence at low CSFs (0–1.2). The best hydrolysis rates for *hpCAD* wood were observed at CSF 2.4, where the fluorescence characteristics decreased due to advanced lignin degradation processes such as repolymerization [32]. At this level of severity, conclusions cannot be drawn about lignin interlinkages from its fluorescence and, therefore, about their influence on the saccharification yield of the biomass.

Lignin influences cell wall mechanical properties

Previous results revealed that the structural or mechanical properties of the raw *hpCAD* poplar line did not differ from those of WT wood [33]. However, depending on the severity of the pretreatment applied in this study, important structural differences were observed between the samples. First, structural analysis revealed that the cell wall thickness decreased in a similar manner between the WT and *hpCAD* samples up to CSF 2.4. This decrease was certainly due to the degradation of hemicelluloses, which occurred mainly at low severity levels in the same proportions for both WT and *hpCAD* wood. In terms of cell deformation or surface area occupied by vessels, major structural changes occurred mainly at CSF 3 and higher. This corresponded to the onset of cellulose degradation, and although this degradation appeared identical in WT and *hpCAD* wood considering glucose release, it left only lignin to provide the cell framework, which could explain the observed differences in collapse. Indeed, at CSF3, the cells begin to collapse together, artificially increasing the wall thickness, making accurate measurement of wall thickness above the CSF2.4 challenging. However, the collapse is significantly greater for *hpCAD* wood cells, suggesting that *hpCAD* wood may be less resistant to deformation than WT wood is. Finally, the reduction in the area occupied by vessels at CSF 3.5 and the disappearance of the cell structure at CSF 3.7 for WT wood indicate that the cells have been torn apart, whereas *hpCAD* wood has a constant vessel area and still has some identifiable structural features at these high CSFs.

All these results seem to indicate that the cellular structures of *hpCAD* and WT wood display different characteristics after high-severity DAP: *hpCAD* wood appears to be less rigid but more resistant, with accentuated deformation properties but reduced tearing. Lignin contributes to the strength of the middle lamella and is the only major component that differs between WT and *hpCAD*, which may explain why cells remain more tightly

bound together in the case of *hpCAD*. Indeed, one of the characteristics of the incorporation of sinapaldehyde into the lignin network is the low oxidizability of the terminal groups, which prevents polymer growth from these terminal units and leads to the formation of a disorganized lignin network with small lignin domains [11], which explains the better flexibility. In addition, *hpCAD* lignin appears to delay its conversion into humin at a very high CSF (Fig. 1D), implying that it is less reactive and retains its structure more effectively than WT lignin. These likely characteristics of *hpCAD* lignin (shorter and less reactive polymers with potentially fewer interactions with polysaccharides [10]) are possibly responsible for the different structural behaviors of *hpCAD* wood but may also be responsible for its better saccharification yield at CSF 2.4 than that of WT wood. Indeed, they can lead to a naturally more limited physical coverage of the lignin around the cellulose in *hpCAD* wood, which intensifies with increasing severity and repolymerization of lignin. This could result in greater accessibility to enzymes once hemicelluloses have been degraded at CSF 2.4, whereas beyond this point, the wood compounds undergo too advanced degradation.

Conclusion

The saccharification yield of *hpCAD* wood treated with DAP was 15 points greater than that of WT wood at the lowest of the two severity factor values tested in this study (CSF 2.4 and 3.0). Despite these differences in saccharification, there was minimal variation in the holocellulose levels between *hpCAD* wood and WT wood, with most differences resulting from lignin. Indeed, lignin is known to be a key compound contributing to lignocellulose recalcitrance: the main hypothesis is that modifications occurring in *hpCAD* lead to shorter and less reactive monomeric chains with fewer carbohydrate interactions. This likely results in limited physical coverage of the lignin around the cellulose, especially when lignin is altered and hemicelluloses degrade at CSF 2.4, allowing better cellulose accessibility and enhancing saccharification. Considering the results reported in the literature for other CAD-downregulated biomasses, DAP appears to be a very promising pretreatment in comparison with alkaline pretreatment. The optimum method of *hpCAD* poplar saccharification might be studied by exploring CSF values between 1.5 and 2.5 and performing a detailed structural characterization of the polymer interactions to evaluate the economic viability.

Abbreviations

LB	Lignocellulosic biomass
DAP	Dilute acid pretreatment
CAD	Cinnamyl alcohol dehydrogenase
WT	Wild type
CSF	Combined severity factor

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s13068-025-02623-8>.

Supplementary material.

Acknowledgements

The authors are grateful to Mr. François Gaudard for his help with the Klason lignin analyses and to Mrs. Juliette Floret for her help with enzymatic hydrolysis. J. Du Pasquier's PhD was funded by Communauté Urbaine du Grand Reims and Chaire de Biotechnologie de CentraleSupélec. Communauté Urbaine du Grand Reims, Département de la Marne, Région Grand Est and European Union (FEDER Champagne-Ardenne 2014–2020) are acknowledged for their financial support to the Chair of Biotechnology of CentraleSupélec and the Centre Européen de Biotechnologie et de Bioéconomie (CEBB).

Supporting Information

Sample pretreatment and analysis workflow, evolution and degradation of WT and *hpCAD* wood compounds in the liquid phase, colorimetry and enzymatic hydrolysis of hemicelluloses as a function of pretreatment severity (CSF).

Author contributions

The manuscript was written through the contributions of all the authors. All the authors approved the final version of the manuscript.

Funding

J. Du Pasquier's PhD was funded by Communauté Urbaine du Grand Reims and Chaire de Biotechnologie de CentraleSupélec. Communauté Urbaine du Grand Reims, Département de la Marne, Région Grand Est and European Union (FEDER Champagne-Ardenne 2014–2020) are acknowledged for their financial support to the Chair of Biotechnology of CentraleSupélec and the Centre Européen de Biotechnologie et de Bioéconomie (CEBB).

Data availability

The data will be available on request.

Declarations

Ethics approval and consent to participate

Not applicable.

Competing interests

The authors declare no competing interests.

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Received: 18 October 2024 Accepted: 11 February 2025

Published online: 06 March 2025

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