Biofuel Research Journal 50 (2026) 2698-2710

Biofuel Research Journal

Journal homepage: www.biofueljournal.com

Original Research Paper

Improved sugar yield upon steam explosion and enzymatic saccharification of field-grown poplar downregulated for cinnamyl alcohol dehydrogenase 1

Jordi Geerts[1], Tim Croes[1], Paulien Geertje Corlijn Leemans[1], Aurore Richel[2], Arnaud Besserer[3], Nicolas Brosse[3], Barbara De Meester[4,5], Wout Boerjan[4,5,*], Iris Cornet[1,*]

[1]*Faculty of Applied Engineering, University of Antwerp Groenenborgerlaan 171, 2020 Antwerpen, Belgium.*

[2]*Department of Industrial Biological Chemistry, University of Liège – Gembloux Agro-Bio Tech Passage des Déportés No. 2, B-5030 Gembloux, Belgium.*

[3]*LERMAB, Université de Lorraine Campus, Bd des Aiguillettes, 54506 Vandœuvre-lès-Nancy, France.*

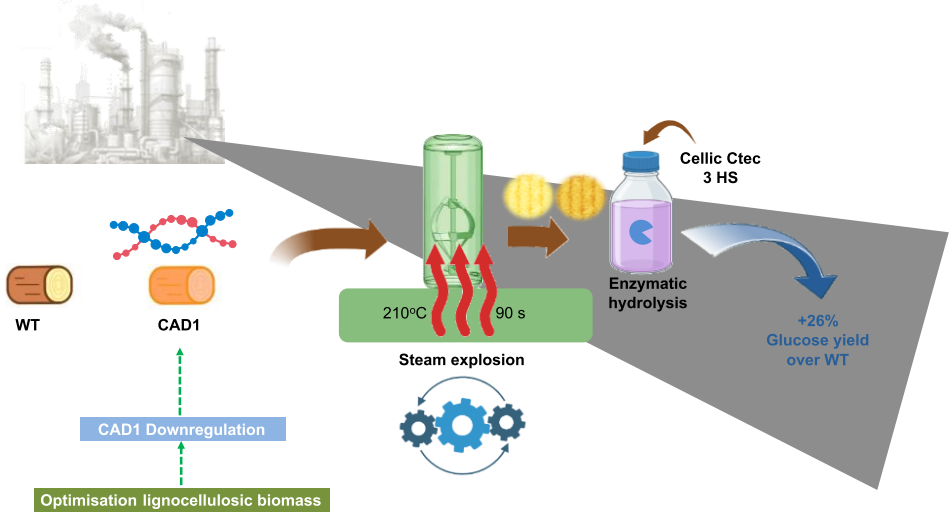
[4]*Department of Plant Biotechnology and Bioinformatics, Ghent University Technologiepark 71, 9052 Ghent, Belgium.*

[5]*Centre for Plant Systems Biology, VIB; Ghent, Belgium Technologiepark 71, 9052 Ghent, Belgium.*

HIGHLIGHTS

- Wood from three-year-old, field-grown, *CAD1*-downregulated poplar was subjected to STEX.
- CAD wood delignified 25% compared to 15% in the control.
- Saccharification yielded 26% more glucose than the control wood.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 27 February 2026
Received in revised form 26 April 2026
Accepted 3 May 2026
Published 1 June 2026

Keywords:
Cinnamyl alcohol dehydrogenase
Lignin engineering
Steam explosion
Enzymatic hydrolysis
Lignocellulose

ABSTRACT

Genetic modification of lignocellulosic biomass can enhance its processability by reducing its recalcitrance to pretreatment and enzymatic conversion to chemicals such as biofuels. Downregulation of cinnamyl alcohol dehydrogenase 1 (*CAD1*) in poplar results in the increased incorporation of sinapaldehyde in the lignin polymer. Here, we investigated steam explosion as a pretreatment strategy for the enzymatic hydrolysis of field-grown *CAD1*-downregulated and wild-type (WT) poplar. Steam explosion at 210°C for 90 s resulted in a significantly higher lignin reduction of 25% for CAD wood, compared to 15% for WT wood. Under limited conditions at 8.4% solid loading and 8 filter paper units of Cellic Ctec3 HS per gram dry weight, enzymatic hydrolysis resulted in a significantly higher average glucose yield of 76% for CAD wood compared to 60% for WT wood. Our data show that steam explosion pretreatment of *CAD1*-modified poplar is a promising strategy to improve enzymatic hydrolysis without the need for additional chemicals.

* Corresponding authors at:
E-mail addresses: iris.cornet@uantwerpen.be (Iris Cornet); wout.boerjan@psb.vib-ugent.be (Wout Boerjan)

Please cite this article as: Geerts J., Croes T., Leemans P.G.C., Richel A., Besserer A., Brosse N., De Meester B., Boerjan W., Cornet I. Improved sugar yield upon steam explosion and enzymatic saccharification of field-grown poplar downregulated for cinnamyl alcohol dehydrogenase 1. Biofuel Research Journal 50 (2026) 2698-2710. DOI: 10.18331/BRJ2026.13.2.4.

Contents

Abbreviations, Nomenclatures, and Symbols

AIL	Acid insoluble lignin
ASL	Acid soluble lignin
Bn	Block, referring to the position of the trees in the field trial, with 'n' the number assigned to the block.
CAD 1	Cinnamyl alcohol dehydrogenase 1
CAD 4	Poplar line hpCAD 4
CAD 24	Poplar line hpCAD 24
CAD wood	Cinnamyl alcohol dehydrogenase 1 deficient poplar wood
CoD	Coefficient of dispersion
C3'H	Coumarate 3' hydroxylase
C4H	Cinnamate 4-hydroxylase
F5H	Ferulate 5-hydroxylase
G	Glucose concentration [g/L]
G_m	Maximum glucose concentration, calculated [g/L]
G_{max}	Maximum glucose concentration, model estimation [g/L]
m_{dw}	Mass dry wood [g]
r_{0G}	Initial reaction rate [g/L/h]
r_{0Y}	Relative initial reaction rate [(m/m%)/h]
S_0	Severity factor [-]
SRC	Short rotation coppice
STEX	Steam explosion
S/G ratio	Syringyl to Guaiacyl ratio
t	time [min]
T	Temperature [°C]
V_{total}	Total Volume [L]
WT	Wild type
Y_G	Glucose yield [m/m%]

1. Introduction

The significant CO_2 emissions and broader environmental impacts associated with fossil-derived fuels and chemicals necessitate reducing their use. Consequently, the production of biofuels from renewable resources has increased substantially over recent decades. For instance, the global energy production from bioethanol and biodiesel rose from 201 TWh in 2004 to 1425 TWh in 2024 (Ritchie et al., 2025). However, life-cycle assessments have demonstrated that not all biofuels deliver net climate benefits. In particular, first-generation biofuels derived from food crops have been associated with higher net greenhouse gas emissions than the fossil fuels they replace, depending on system boundaries and land-use change assumptions. These crops usually require intensive cultivation and high levels of fertiliser. They also sequester less carbon in the soil than perennial woody systems and compete with food production for land and resources (Ma et al., 2023; Sandford et al., 2024).

In contrast, second-generation lignocellulosic feedstocks, such as short-rotation coppice (SRC) poplar, offer a more sustainable alternative. SRC poplar requires comparatively low management intensity and fertiliser inputs, can be harvested on multi-year rotations without replanting, and can be cultivated on marginal or reclaimed land, including former gravel extraction sites (NNFCC, 2010). These characteristics make SRC poplar a promising feedstock for sustainable biofuel production. Despite their advantages, second-generation biofuels and biochemicals often occupy a smaller market share than their first-generation counterparts. The global market share of, e.g., second-generation cellulosic bioethanol was valued at 885.82 million USD in 2022, while the total bioethanol market is estimated at around 83.4 billion USD (Polaris Market Research, 2023; Statista, 2024). The main reasons for this relatively small market share compared with first-generation products are the technological barriers related to the natural recalcitrance of lignocellulosic materials when they are converted into fermentable sugars. This results in lower product yields and higher production costs (Hemansi et al., 2019).

To significantly improve the market competitiveness of second-generation bioethanol and biochemicals in general, technological innovation is required. A well-supported strategic route is to optimise the starting material, i.e., the lignocellulosic matrix itself, by breeding or genetic engineering (Bryant et al., 2020; Vanhevel et al., 2024). Since lignin in the plant cell wall is the main contributor to the recalcitrance of lignocellulosic biomass towards biomass deconstruction (Fang et al., 2025), research to optimise bioethanol production has largely focused on genetic modification of the lignin biosynthesis pathway. All genes encoding enzymes in the monolignol biosynthetic pathway have been downregulated or overexpressed to alter lignin content or alter its composition in poplar. In addition, poplar has been transformed with heterologous genes to alter lignin structure and enhance saccharification (Sulis et al., 2023; Wang et al., 2025). These studies have shown that reducing lignin content often results

Please cite this article as: Geerts J., Croes T., Leemans P.G.C., Richel A., Besserer A., Brosse N., De Meester B., Boerjan W., Cornet I. Improved sugar yield upon steam explosion and enzymatic saccharification of field-grown poplar downregulated for cinnamyl alcohol dehydrogenase 1. Biofuel Research Journal 50 (2026) 2698-2710. DOI: 10.18331/BRJ2026.13.2.4.

in improvements in cellulose-to-glucose conversion rates upon saccharification.

Altering the lignin syringyl to guaiacyl (S/G) ratio also affects saccharification efficiency, with higher S/G ratios generally leading to better saccharification. However, the magnitude of this effect depends on the lignin amount, as was demonstrated by the analyses of poplar wood samples from a set of undomesticated *Populus trichocarpa* trees with contrasting lignin content and S/G ratios (Studer et al., 2011). Most of these studies were conducted using wood harvested from greenhouse-grown poplars, whereas only a limited number have investigated wood obtained from field-grown trees (De Meester et al., 2022b; Li et al., 2024). Here, the deconstruction and enzymatic hydrolysis of three-year-old field-grown cinnamyl alcohol dehydrogenase1 (*CAD1*)-downregulated poplar wood (CAD wood), genetically modified using an RNAi approach, were examined. This genetically modified wood, which was characterised by an increased incorporation of sinapaldehyde into the lignin polymer, resulted in an increased enzymatic hydrolysis after alkaline pretreatment (Van Acker et al., 2017; De Meester et al., 2022a). This effect has mainly been attributed to the fact that sinapaldehyde incorporation into the lignin leads to a higher proportion of β-ether moieties with conjugated γ-carbonyl functionalities, which are more susceptible to alkaline hydrolysis than the non-conjugated β-ether moieties (Lapierre et al., 1999; Van Acker et al., 2017). However, the increased incorporation of syringaldehyde and sinapaldehyde into the lignin polymer is also expected to (i) reduce the size of the lignin polymer chains, (ii) make lignin more hydrophobic, resulting in fewer non-covalent interactions with hemicellulose, and (iii) reduce the ether or ester bonding between hemicellulose and lignin, potentially reducing recalcitrant lignin-carbohydrate complexes (Mottiar et al., 2016; Van Acker et al., 2017).

Besides optimised substrates, efficient pretreatment of the woody biomass is essential to achieve economically viable monomeric sugar yields in the subsequent saccharification step, where cellulose and hemicellulose are converted into monomeric sugars (Hoang et al., 2023; Gautam et al., 2024). Significant improvements in saccharification yield have been reported for genetically engineered poplar wood following alkali, acid, green liquor, ozone, or hot water pretreatments (Bryant et al., 2020). However, research into the pretreatment and saccharification of CAD genetically engineered poplar wood is limited. The focus has primarily been on its alkaline pretreatment (Van Acker et al., 2017; De Meester et al., 2022) and soda pulping (Segmehl et al., 2018), as well as acidic conditions, such as acidic bleaching (Segmehl et al., 2018) and dilute acid pretreatment (Van Acker et al., 2017; du Pasquier et al., 2025). To the best of our knowledge, the present study is the first to examine steam explosion pretreatment (STEX) of CAD wood. STEX is one of the most energy-efficient and environmentally friendly pretreatment techniques. It requires less energy for particle size reduction compared to grinding or milling, and it usually requires only water (steam) without added chemicals (Shrotri et al., 2017; Hoang et al., 2023). Although the production of compounds that inhibit the subsequent fermentation process is often cited as a disadvantage of STEX, it is one of the most promising lignocellulose pretreatment methods and is already being applied industrially (Oliveira et al., 2013). Research on STEX pretreatment of wood derived from genetically modified trees has thus far been limited to greenhouse-grown poplar trees in which either *FERULATE 5 HYDROXYLASE* (*F5H*) was overexpressed (C4H::F5H trees) to increase the S/G ratio, or p-*COUMARATE 3' HYDROXYLASE* (*C3'H*) was downregulated (RNAi-C3'H trees) to reduce lignin content (Mansfield et al., 2012).

In the present study, the stem biomass of field-grown *CAD1*-deficient poplars (CAD wood) and wild-type (WT) trees was pretreated using STEX. This was followed by enzymatic hydrolysis using a commercially applied enzyme complex, Cellic Ctec 3 HS. The data on the release of monomeric sugars were then compared with the data from the compositional and structural analyses of the wood. Mass balances were set up for the major constituents of lignocellulose. We showed that STEX pretreatment of CAD wood leads to significantly higher delignification than STEX of WT wood. Subsequent enzymatic hydrolysis yielded a significantly higher glucose yield compared to WT wood.

2. Materials and Methods

2.1 Plant material

The plant material comprised chopped whole stems and or branches (with bark) derived from the *hpCAD 4* and *hpCAD* 24 poplar lines and their respective WT trees, all harvested in winter. All of these were *Populus tremula* × *Populus alba* cv. 717-1B4. These genetically modified lines have been described before (Van Acker et al., 2017). Throughout the article, this stem biomass will be referred to as CAD 4, CAD 24 (or simply CAD wood), and WT wood. The trees were grown in a field trial as SRC culture for two consecutive rotations of one and three years, of which the three-year-old wood is used in this study (De Meester et al., 2022a). Three biological replicates of each of the two CAD lines and the WT line, corresponding to three different blocks of trees in the field trial, were analysed. Each biological replicate itself was composed of wood from 40 trees. Each tree contained, on average, 6 stems due to the coppice. The blocks from which the wood was harvested were block 1 (B1), B4, and B5 of the field trial for the WT, B4, B5, and B6 for the CAD 4 line, and B1, B2, and B3 for the CAD 24 line (De Meester et al., 2022a). The field plot is presented in **Figure S1**.

2.2 Preparation of the poplar stem biomass for steam explosion pretreatment

CAD 4, CAD 24, and WT woody stems were chipped in a wood chipper without prior debarking. The range in length of the resulting stem chips is shown in **Figure S2**. Fifty grams of the dry chips were placed in Schott bottles, and 1 L of demineralised water was added. The bottles were inverted three times to ensure contact between the wood and the water. The mixtures were kept at room temperature for 16 h. After pre-soaking, the stem chips were separated from the soaking liquid by sieving through a Steel mesh sieve with a pore size of 38 µm (Endecotts LTD, London, UK).

2.3 Steam explosion pretreatment

STEX was performed on pre-soaked stem chips from WT, CAD 4, and CAD 24 poplars using the STEX equipment of the BioWAVE research group at the University of Antwerp (ATI Sistemas, A Coruña, Spain). This equipment consists of a 7-L reactor and a steam generator capable of producing steam at pressures of up to 40 bar (**Fig. S3**). The maximum operating pressure of the reactor is 20 bar. Prior to sample treatment, the reactor is preheated by performing a steam explosion in the empty reactor to ensure that the first sample is treated under conditions identical to those of subsequent samples. For steam explosion pretreatment, the reactor is first opened and loaded with the pre-soaked chips. During the subsequent heating phase, from the preheating temperature to the target temperature, the reactor is heated using both an electric heating mantle and hot steam introduced from the steam generator. The sample is then kept at the desired temperature under high-pressure saturated steam for the selected incubation time, after which the explosion valve (ball valve) opens in less than a second, propelling the biomass into the expansion vessel, which is located directly below the reactor. This expansion vessel is connected to the outside and is under atmospheric pressure. This sudden explosive decompression disrupts the stem biomass chips at the cellular level. All steam explosions were performed at 210°C (19 bar) for 90 s, which is considered a high temperature and a short time (Ziegler-Devin et al., 2021). The short time frame was chosen to evaluate whether CAD and WT wood would react differently, while minimising energy input. To evaluate whether all stem chips were treated uniformly, reactor temperature and pressure were continuously recorded over time, and the resulting data were used to calculate the severity factor, S^0 [–], as defined by **Equation 1** (Jacquet et al., 2012):

$$S^0 = \log\left(\int \exp((T-100)/14.75)\, dt\right) \quad \text{Eq. 1}$$

where t represents time [min], and T is temperature [°C].

Please cite this article as: Geerts J., Croes T., Leemans P.G.C., Richel A., Besserer A., Brosse N., De Meester B., Boerjan W., Cornet I. Improved sugar yield upon steam explosion and enzymatic saccharification of field-grown poplar downregulated for cinnamyl alcohol dehydrogenase 1. Biofuel Research Journal 50 (2026) 2698-2710. DOI: 10.18331/BRJ2026.13.2.4.

The severity factor was calculated using Riemann integration, taking into account every second that the sample was exposed to temperatures above 100°C during both the heating and incubation phases. The calculation formula is shown in **Equation 2** (Jacquet et al., 2012):

$$S^0 = \log \sum \frac{14.75(t_{n+1}-t_n)}{(T_{n+1}-T_n)} \left(\exp\left(\frac{T_{n+1}-100}{14.75}\right) - \exp\left(\frac{T_n-100}{14.75}\right)\right) \quad \text{Eq. 2}$$

where $(t_{n+1} - t_n)$ is one second and T_n is the temperature [°C] at t_n. 14.75 is a value calculated using the Arrhenius equation, which corresponds to the doubling of the reaction rate of xylan removal with a 10°C increase in temperature (Bhagia et al., 2016). To evaluate the difference in steam explosion severities, a coefficient of dispersion (CoD) was calculated (**Eq. 3**):

$$CoD = \frac{\sum_{i=1}^{n} |x_i - \eta|}{n * \eta} \quad \text{Eq. 3}$$

where x_i is the severity factor of the STEX [–], n is the total number of values, and η is the median severity factor [–].

As a consequence of the STEX pretreatment, the stem chips were disintegrated into fibres, which were subsequently recovered from the expansion vessel together with the STEX hydrolysate. Most of the hydrolysate was removed by sieving through a 38 µm sieve. The wet weight of the remaining wood pulp, which was measured for mass balance analysis, increased 7 to 8.5-fold due to the soaking and the STEX pretreatment. A full process scheme showing the dry and wet weights of each sample after each step in the process is provided in **Figures S5a** and **S5b**. **Table S1** shows the average wet weight per poplar line after soaking and STEX.

2.4 Washing of wood pulp after STEX

After the sieving step, the wood pulp was washed three times with demineralised water to further remove the hydrolysate remaining in the pores of the solid STEX-pretreated wood fibres. Herein, 100 g of the wet wood pulp, which is about one third to one fourth of the total wet mass recovered, was added to 300 mL of demineralised water and shaken vigorously by hand for 1 min. The mixture was then centrifuged for 10 min at 3077 ×g (4000 rpm) using a SIGMA 3-16KL centrifuge (SIGMA, Osterode am Harz, Germany), and 200 mL of the washing water was decanted. Hereafter, another 200 mL of demineralised water was added, and the pulp was washed two more times in the same way. After the last step, the pulp was separated from the washing water using Büchner filtration over a Whatman number 1 filter paper.

2.5 Enzymatic hydrolysis

The dry weight percentage of the washed wood pulp was determined gravimetrically in triplicate by drying approximately 0.5 g of the wet wood pulp in pre-weighed Eppendorf tubes in an oven (Memmert, Schwabach, Germany) at 105°C overnight. Based on this dry weight percentage, a well-determined mass of the wet, filtered, pretreated wood pulp equivalent to 6.5 g of dry wood pulp was brought into 250 mL Schott bottles. Enzymatic hydrolysis was carried out in a 0.05 M citrate buffer (Avantor, Radnor, USA) at pH 4.8. Then, 7.74 mL of 0.5 M citrate buffer, pH 4.6, was added. Per gram of dry wood pulp, 60 µL of a 10 g/L tetracycline (Merck, Darmstadt, Germany) in 70% ethanol solution was added. Cellic Ctec 3 HS cellulase complex, kindly donated by Novonesis (Bagsvaerd, Denmark), was added in different concentrations depending on the experiment at a volume corresponding to 8, 16, 24, or 32 Filter Paper Units (FPUs) per gram of dry wood pulp. The activity of the enzyme stock solution was 216.52 FPU/mL as determined according to the NREL/TP-510-42628 protocol (Adney, 1996).

The volume of the liquid reaction mixture was brought to 77.4 mL by adding demineralised water. If the wood pulp contained more water, less additional water was added to the mixture. This way, a solid-to-liquid loading of 84 g dry wood pulp per litre of reaction mixture was obtained in all cases. This was the highest solid loading that could be achieved, in which all wood pulp was submerged in the hydrolysis mixture. A flowchart of the applied process conditions is provided in **Figure S4**.

The hydrolysis reaction mixtures were incubated at 50°C and shaken linearly at 250 rpm using a reciprocal shaker (Ohaus SHRC0719DG, Parsippany, USA). The glucose concentration of each mixture was followed up in time as follows. After allowing the solids to settle for about 1 min, 0.5 mL of the supernatant was sampled into a 1.5 mL centrifuge tube with a screw cap. The samples were then placed in a boiling water bath for 15 min to denature the enzymes and subsequently cooled in an ice bath for 10 min to precipitate the enzymes. The samples were diluted 10 times with Millipore water and filtered through a 0.22 µm PES syringe filter in an HPLC vial.

The maximum glucose concentration *Gm* [g/L] that can be achieved through the enzymatic hydrolysis of lignocellulosic biomass with a certain cellulose content [m/m%], as determined by compositional analysis, was calculated using **Equation 4**.

$$Gm = \frac{cellulose\ content * m_{dw} * MM_{glucose}}{V_{total} * MM_{cellulose\ unit}} \quad \text{Eq. 4}$$

where m_{dw} [g] is the mass of dry wood introduced into the hydrolysis mixture, i.e., 6.5 g, $MM_{glucose}$ [g/mol] is the molar mass of glucose, i.e., 180 g/mol, V_{total} [L] denotes the total volume of the reaction mixture, i.e., 0.0774 L, and $MM_{cellulose\ unit}$ [g/mol] is the molar mass of one cellulose unit, i.e., 162 g/mol.

The glucose yields Y_G [%] were then calculated using **Equation 5**.

$$Y_G = \frac{G}{Gm} * 100\% \quad \text{Eq. 5}$$

where *G* [g/L] is the glucose concentration determined by HPLC.

This yield was calculated per initial volume of liquid in the reaction mixture. As cellulose in the wood was partially hydrolysed and dissolved during the process, the total liquid volume increased slightly. However, this increase was considered negligible. The applied process conditions of the STEX pretreatment and enzymatic hydrolysis are summarised in **Table 1**.

Table 1.
Process conditions used for STEX and hydrolysis of WT and CAD poplar wood.

Parameter	Value
STEX Severity [-]	3.59 ± 0.04
Enzyme loading [FPUs/g dry stem biomass added]	8
Solids loading enzymatic hydrolysis [g dry stem biomass/mL total liquid volume]	84
Hydrolysis time [h]	96

2.6 Estimation of hydrolysis parameters

For each hydrolysis reaction mixture, the glucose concentration, G [g/L], determined by HPLC as a function of time, t [h], was fitted using a modified Holtzapple model (Holtzapple et al., 1984) implemented with the *lsqnonlin* function in MATLAB. Although the Holtzapple model is generally used to describe reducing sugar concentrations, glucose was considered the platform molecule in the present study. For completeness, the xylose concentrations and yields obtained during enzymatic hydrolysis are shown in **Figure S6**. Other reducing sugars are much less abundant in the hydrolysate than glucose and xylose, and were not considered (Donaldson

Please cite this article as: Geerts J., Croes T., Leemans P.G.C., Richel A., Besserer A., Brosse N., De Meester B., Boerjan W., Cornet I. Improved sugar yield upon steam explosion and enzymatic saccharification of field-grown poplar downregulated for cinnamyl alcohol dehydrogenase 1. Biofuel Research Journal 50 (2026) 2698-2710. DOI: 10.18331/BRJ2026.13.2.4.

et al., 2017). The parameters of the modified Holtzapple model, i.e., maximum glucose concentration G_{max} [g/L] (i.e., the final glucose concentration in case of complete conversion of the available cellulose) and the initial reaction rate r_{0G} [g/L/h], were calculated. The simplified model applied is presented in **Equation 6**.

$$G = (G_{max} * t)/(t + \frac{G_{max}}{r_{0G}}) \quad \text{Eq. 6}$$

Equation 6 was further adapted for application to the calculated enzymatic hydrolysis yields, Y_G (see **Eq. 5**), by accounting for the variable cellulose content of the pretreated wood samples, as expressed in **Equation 7**.

$$Y_G = (Y_{Gm} * t)/(t + \frac{Y_{Gm}}{r_{0Y}}) \quad \text{Eq. 7}$$

where the model parameters Y_{Gm}, representing the maximum glucose yield [m/m%], and r_{0Y}, representing the relative initial reaction rate [m/m% /h], were estimated.

Evaluation of model robustness was performed by calculating the standard error of the model parameters (Akkermans et al., 2018). Model validation was based on visual inspection of the residual plots as presented in **Figure S7**.

2.7 Compositional analysis

The poplar stem biomass composition was determined after grinding the stem chips and freeze-drying them in a lyophilisator (SIGMA, Osterode am Harz, Germany) using the method of Wittner et al. (2023). Briefly, 300 ± 2 mg of ground lyophilised sample was subjected to a two-stage dilute acid hydrolysis with sulfuric acid following the standard NREL/TP-510-42618 protocol (Sluiter et al., 2012). Acid-insoluble lignin (AIL) was determined gravimetrically, while acid-soluble lignin (ASL) was measured spectrophotometrically. Finally, cellulose and hemicellulose percentages were calculated from the concentration of their monomers, glucose and xylose, which were determined by an HPLC system (Agilent, Santa Clara, USA) equipped with a Coregel ORH 801 7.8 × 300 mm column (Altmann Analytik, Munich, Germany) kept at 70°C and a Refractive Index Detector at 55°C. Eight mM sulfuric acid (Avantor, Radnor, USA) was used as the mobile phase at a flow rate of 0.6 mL/min. The injection volume was 20 µL (Broos et al., 2024).

2.8 Glucose and xylose determination

During enzymatic hydrolysis assays, the glucose and xylose concentrations were determined using HPLC, applying the same equipment and methodology as described above.

2.9 Macroscopic image analysis

Three-dimensional stitched images of fibres from the different pretreated wood types and blocks were acquired using a digital microscope (Keyence, Mechelen, Belgium). Each sample was spread in a Petri dish, and nine adjacent locations were selected for imaging. At each location, at least five images were captured at 20× magnification, each focused at a different depth within the sample. The images obtained from each location were subsequently overlaid to generate a single sharp image. Finally, the images from all locations were stitched together to produce a comprehensive high-resolution image capturing the heterogeneity of the STEX-treated samples.

2.10 Separation of fibre aggregates into fractions by size

The STEX-pretreated wood pulp was air-dried. The fibre aggregates were left undisturbed and were sieved for 5 min on a Yellow Line OS 10 orbital shaker at 300 rpm through pore sizes of 5, 2, 0.5, and 0.2 mm. Sieving is a commonly used and easy method to characterise STEX-treated poplar wood (Shu et al., 2020).

2.11 Microscopic analysis of individual fibres

Fibre sizes were measured according to Romero et al. (2026). One gram of dry, pretreated wood fibre aggregates was transferred into a 100 mL Schott bottle. 20 mL of Franklin solution, consisting of 30% H_2O_2 and 99% glacial acetic acid, was added, and the mixture was incubated in an oven (Memmert, Schwabach, Germany) at 60°C for 48 h. The macerated fibres were then separated from the liquid by sieving. The pulp was washed five times in 50 mL of demineralised water, each time shaken manually for 1 min before being sieved over a 75 µm sieve (Endecotts LTD, London, UK). The fibres were then dispersed in water, and one droplet was mounted onto a glass slide and examined under an Olympus CX43 light microscope (Analis, Namur, Belgium). The length of the fibres was measured in Fiji (ImageJ). Five slides were examined for each biological replicate (technical replicates). On each slide, 5 to 12 intact fibres were searched and measured, totalling 33 to 42 measurements per biological replicate.

2.12 Statistical analyses

Throughout the study, CAD 4 and CAD 24 wood were compared with WT wood using unpaired t-tests based on three biological replicates. The figure captions specify whether technical or biological replicates were considered, as well as the significance level applied. The analyses assumed normal data distribution and comparable variance among the three poplar wood types.

3. Results and Discussion

The stems derived from two field-grown, genetically modified *CAD*-deficient poplar lines and their WT control were pretreated by STEX and enzymatically hydrolysed. For both *CAD*-deficient lines and the WT, three biological replicates, each comprising 40 pooled trees collected from different positions in the field, were analysed. The first step in the conversion of wood into fermentable sugars is the pretreatment of the lignocellulosic biomass, consisting of presoaking, STEX, and washing. A presoaking step is applied to improve the uniformity of the heat transfer during the subsequent STEX. The STEX pretreatment aims to increase the porosity, surface area, and permeability of the lignocellulosic biomass to make it more accessible for the subsequent enzymatic conversion. This is achieved by partially depolymerising the hemicellulose fraction, disrupting the lignin-carbohydrate complexes, and removing part of the lignin (Zhang et al., 2021).

3.1 Influence of pretreatment on poplar wood composition and mass balances

The STEX conditions applied were similar for all experiments. The severity factor (**Eq. 2**) was calculated for all STEX performed (**Table S2**). The severity factors ranged between 3.56 and 3.63, corresponding to a dispersion coefficient of 0.6%. This coefficient is sufficiently small to consider the applied STEX severities as equal.

Mass balances of the poplar stem chips, the corresponding wood pulps after STEX, and their components, i.e., cellulose, hemicellulose, and lignin, were calculated for the CAD 4, CAD 24, and WT wood by weighing and performing compositional analyses before and after the different pretreatment steps, i.e., after presoaking, STEX, and washing. This allowed estimating the yields and losses over the entire process. The results are shown as averages of the three biological replicates of the WT, CAD 4, and CAD 24 lines in **Figure 1**. The data for each biological replicate are presented in **Figure S8**. The average wood composition of the WT, CAD 4, and CAD 24, before and after STEX are presented in **Figures S9** and **S10**. **Figure S9** compares the composition of the CAD wood to the WT wood, whereas **Figure S10** compares the effect of the STEX pretreatment on the composition of each wood type. The same data presented in **Figures S9** and **S10**, but shown separately for each biological replicate, are provided in **Figure S11**.

Please cite this article as: Geerts J., Croes T., Leemans P.G.C., Richel A., Besserer A., Brosse N., De Meester B., Boerjan W., Cornet I. Improved sugar yield upon steam explosion and enzymatic saccharification of field-grown poplar downregulated for cinnamyl alcohol dehydrogenase 1. Biofuel Research Journal 50 (2026) 2698-2710. DOI: 10.18331/BRJ2026.13.2.4.

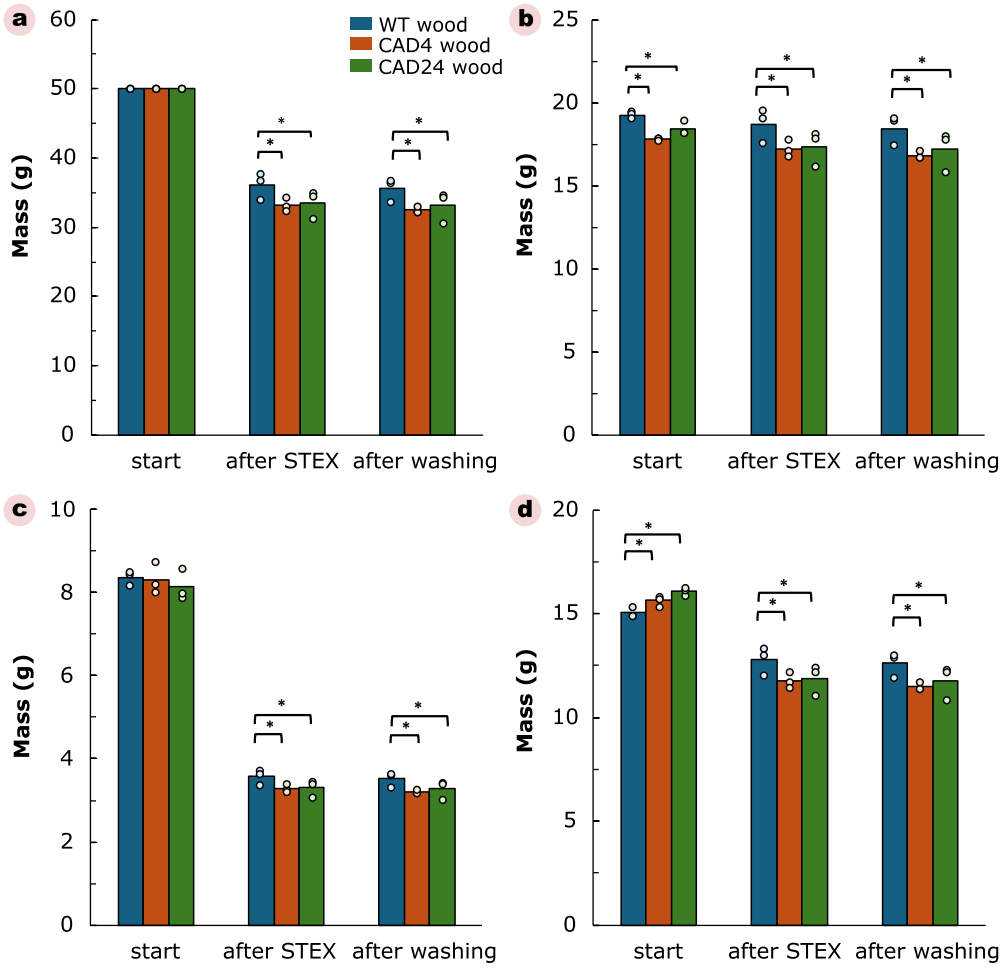


Fig. 1. Mass balances and compositional analysis of **(a)** dry stem biomass, **(b)** cellulose, **(c)** hemicellulose, and **(d)** lignin. The composition of each biological replicate was determined in triplicate. Dots represent the mean values of the three technical replicates for each of the three biological replicates. Significant differences compared to the WT, as determined by an unpaired t-test, are indicated by * ($\alpha = 0.05$).

After presoaking, the soaking liquid obtained after sieving through a steel mesh was centrifuged at 3500 rpm for 5 min to measure possible mass losses, but no solids could be pelletised. Mass losses were detected after STEX (Fig. 1a). No further mass losses were found after washing the STEX pretreated wood pulp (Fig. 1a).

The total lignocellulosic content of each stem biomass sample before STEX (see Fig. 1a) was calculated as the sum of the measured masses of cellulose, hemicellulose, and lignin, presented in Figures 1b, 1c, and 1d, respectively. The average total lignocellulose content was 85.40 ± 2.33% of the dry weight for the WT, 83.54 ± 4.15% for CAD 4, and 85.32 ± 4.40% for CAD 24. Data from literature report total lignocellulose contents of 91% and 92% for WT *Populus alba* and *Populus tremula,* respectively (Rego et al., 2019). The fact that the material analysed here was not debarked may have resulted in a lower total lignocellulose content and higher lignin content in the starting material (Safdari et al., 2011).

Figure 1a shows that significantly more biomass was lost after STEX pretreatment of CAD 4 and CAD 24 compared to the WT. Figure 1b depicts that the cellulose (glucan) mass in the starting material was significantly lower in the CAD 4 and CAD 24 wood than in the WT wood (a difference of 7.6% and 4.4%, respectively). After STEX treatment, there was still significantly less cellulose present in both CAD lines compared to the WT ($p < 0.05$). This is due to a higher loss of stem biomass from the CAD wood, but also to a lower cellulose content after STEX in the CAD 4 wood (see Fig. S9).

As shown in Figures 1c and S8, the hemicellulose (xylan) content before and after STEX indicates that the removal of hemicellulose was large in all samples, and that the loss in hemicellulose was slightly but significantly greater for the CAD lines ($p < 0.05$) than for the WT, even when accounting for the greater loss in biomass in the CAD lines (Figs. S9 and S10). The removal of hemicellulose is one of the key mechanisms by which STEX pretreatment enhances the accessibility of the lignocellulosic matrix to enzymes during enzymatic hydrolysis. However, this process also reduces the maximum theoretical yield of pentoses that can be generated during hydrolysis.

The most pronounced compositional alteration in the wood types resulting from STEX is the change in lignin content (see Fig. 1d). Before STEX, the CAD lines contained significantly more lignin than WT wood, while after STEX, the CAD lines contained significantly less lignin than WT wood. The reduction in lignin content resulting from STEX pretreatment was significantly greater for both the CAD 4 line and the CAD 24 line ($p = 0.03$ and $p = 0.02$, respectively) compared to the WT, i.e., a 25% and 26% reduction for CAD 4 and CAD 24, respectively, compared to 15% for the WT. This is partially explained by the larger wood biomass loss in the CAD lines in general, but also by a comparatively higher loss in lignin.

Please cite this article as: Geerts J., Croes T., Leemans P.G.C., Richel A., Besserer A., Brosse N., De Meester B., Boerjan W., Cornet I. Improved sugar yield upon steam explosion and enzymatic saccharification of field-grown poplar downregulated for cinnamyl alcohol dehydrogenase 1. Biofuel Research Journal 50 (2026) 2698-2710. DOI: 10.18331/BRJ2026.13.2.4.

The cellulose and hemicellulose retention rates, as well as the lignin removal rates, are summarised in **Table S3**. They were calculated using **Equations S1–S3**, respectively. Of these three, only the lignin removal rate was significantly different from that of the WT for both CAD lines, as determined by an unpaired t-test ($p = 0.04$ and $p = 0.01$ for CAD 4 and CAD 24, respectively). The results indicate that the changes in lignin structure of the CAD wood made lignin more easily removable under the applied STEX pretreatment conditions, i.e., 210°C for 90 s. The applied conditions corresponded to a high temperature, short residence time, and moderate severity (Ziegler-Devin et al., 2021). These conditions were chosen not to optimise the STEX process for poplar, but rather to clearly reveal the differences in composition and enzymatic hydrolysis between the two wood types after pretreatment.

STEX is an acid-catalysed pretreatment process in which the catalyst originates from the release of organic acids from the biomass itself, a process called autocatalysis (Deng et al., 2023). The susceptibility of the lignocellulosic matrix of CAD to moderate acid-catalysed pretreatments is supported by a recent article reporting that dilute acid pretreatment results in higher cell wall deformation in CAD wood than in WT wood (du Pasquier et al., 2025). The authors suggested that this deformation is linked to the presence of more hydrophobic and shorter lignin chains and fewer non-covalent interactions between lignin and carbohydrates. Nevertheless, the authors did not observe significant changes in lignin removal after dilute acid pretreatment, unlike after the STEX pretreatment in the present study (see **Fig. 1d**). It is difficult to compare the severity of the two pretreatment methods, as longer times and lower temperatures were applied to the dilute acid pretreatment. The difference could potentially be due to the physical aspect of the explosion phase in the STEX pretreatment. By contrast, previous studies have shown that CAD wood is more susceptible to delignification by alkaline pretreatments than WT wood, primarily due to the increased presence of cinnamaldehydes within its lignin polymer (Van Acker et al., 2017; De Meester et al., 2022a). Apparently, both the acid and alkaline pretreatments exert distinct effects on CAD wood.

3.2 Enzymatic hydrolysis after STEX pretreatment of the different poplar types

Subsequently, the effect of the genetic modification of the wood on the enzymatic hydrolysis after STEX pretreatment was evaluated. First, a screening experiment using different enzyme loadings (i.e., 8, 16, 24, and 32 FPU/g dry wood) was performed on pretreated stem biomass from block B1 of the WT and block B1 of CAD 24 in order to obtain a suitable enzyme concentration for the comparison. We aimed to convert between 50% and 80% of the cellulose for each wood type to ensure that the differences in hydrolysis efficiency between WT and CAD wood were easily revealed. The glucose yields were calculated using **Equation 5** (**Fig. S12**). Initial reaction rates were estimated by the model **Equation 6** and are shown in **Figure S13**. Based on these screening results, an enzyme loading of 8 FPU/g dry wood was selected as the most suitable for our analysis. At this loading, the glucose yields were 64% for the WT and 83% for the CAD 24 wood. Following that, the selected enzyme loading was tested on the three poplar wood types. **Figure 2** shows the average enzymatic hydrolysis results of this experiment based on three biological repeats.

The average glucose concentrations after 96 h of hydrolysis were 28.96 ± 1.63 g/L for the WT wood, 36.65 ± 1.76 g/L for CAD 4 wood (+26%), and 36.46 ± 0.64 g/L (+26%) for CAD 24 wood. These glucose concentrations were significantly higher for both CAD lines than for the WT wood in the applied conditions, with p-values of 0.0051 and 0.0018 for CAD 4 and CAD 24, respectively, as determined by an unpaired t-test. No significant difference was detected between the two CAD lines ($p = 0.87$). The corresponding glucose yields, as calculated using **Equation 5**, were 59.96 ± 3.38% for the WT wood, 75.87 ± 3.63% for CAD 4 (+ 27%), and 75.49 ± 1.33% for CAD 24 (+ 26%). The model estimated the maximum glucose concentrations, $G_{max,}$ to be 35.76 ± 0.88, 46.70 ± 1.62, and 42.87 ± 1.05 g/L, respectively. However, these concentrations were not reached within the experimental timeframe.

Improved glucose yields after the pretreatment of CAD poplar wood in acidic conditions at an average severity were also reported by du Pasquier et al. (2025). Consistent with the results reported here, these authors found that the improved enzymatic hydrolysis was attributable to differences in lignin structure rather than differences in lignin content between the two wood types. du Pasquier et al. (2025) suggest that the increased incorporation of aldehydes in the lignin of CAD wood leads to shorter and more hydrophobic lignin, resulting in fewer noncovalent lignin-carbohydrate interactions and, hence, fewer recalcitrant lignin-carbohydrate complexes, as also discussed by Van Acker et al. (2017). The lignin in CAD wood likely covers the cellulose to a lesser extent, making it more accessible to the enzymes, particularly after the hemicellulose degradation under moderately acidic conditions. Increasing the severity of the pretreatment would lead to a trade-off between higher hemicellulose degradation, reduced cellulose crystallinity and more advanced destruction of the lignocellulosic matrix on the one hand, and cellulose degradation, higher inhibitor concentrations, and potentially overcooking on the other hand (Szadkowski et al., 2023).

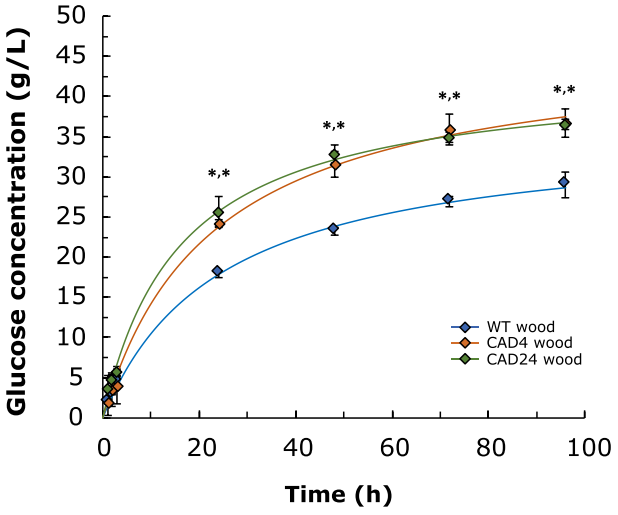


Fig. 2. Glucose production over time during enzymatic hydrolysis of STEX-pretreated WT wood, CAD 4 wood, and CAD 24 wood. ─ represents the fitted curve by applying the Holtzapple model (**Eq. 6**). STEX conditions were 210°C – 90 s in all cases. The enzymatic hydrolysis was performed at 8.4% solid loading and 8 FPU/g wood Cellic Ctec 3 HS. Error bars represent the standard deviation of the experimental results of three biological replicates. *,* indicates that the experimental value of both CAD 4 and CAD 24 is significantly higher compared to the WT wood ($\alpha = 0.05$).

Similar xylose concentrations were obtained for the CAD lines and the WT, despite the significantly higher yields observed for the CAD lines. This indicates enhanced enzymatic digestibility of the hemicellulose fraction in the CAD lines following STEX. This increase is offset by the significantly higher hemicellulose loss in the CAD lines (**Fig. 1c**). The initial hydrolysis reaction rates were estimated using the modified Holtzapple model (**Eq. 6**) as shown in **Figure 3**.

Based on the averages from the three biological replicates, the estimated initial reaction rates were 1.49 ± 0.084 g/L/h, 1.99 ± 0.16 g/L/h (+34%), and 2.68 ± 0.20 g/L/h (+80%) for the WT, CAD 4, and CAD 24 wood, respectively. These unpaired t-test revealed significant differences ($p = 0.0087$ and $p = 0.0007$ for CAD 4 and CAD 24 compared to the WT, respectively), indicating that CAD wood not only achieved a higher glucose concentration in this condition, but also had a higher initial reaction rate.

3.3 Glucose from biomass yields

Next, we calculated the average amount of glucose (based on three biological replicates) that could be produced from 1 kg of the original biomass under the applied conditions by extrapolation and by combining the mass balances (**Figs. 1** and **S5a**) with the achieved glucose concentrations (**Fig. 2**).

Please cite this article as: Geerts J., Croes T., Leemans P.G.C., Richel A., Besserer A., Brosse N., De Meester B., Boerjan W., Cornet I. Improved sugar yield upon steam explosion and enzymatic saccharification of field-grown poplar downregulated for cinnamyl alcohol dehydrogenase 1. Biofuel Research Journal 50 (2026) 2698-2710. DOI: 10.18331/BRJ2026.13.2.4.

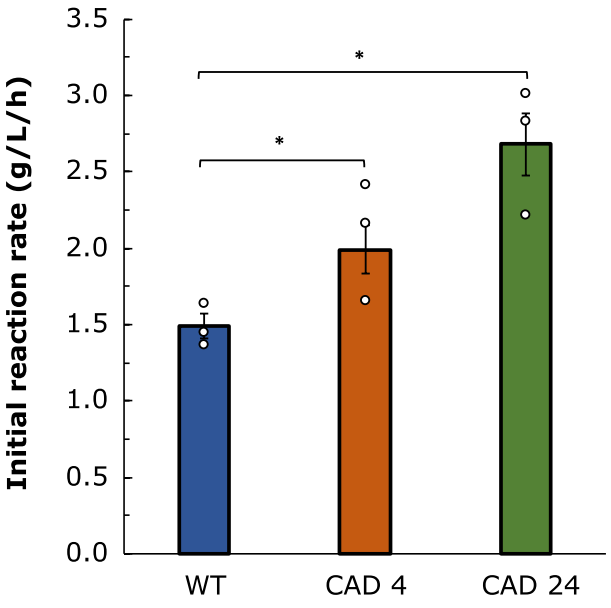


Fig. 3. Initial reaction rates during enzymatic hydrolysis of STEX-pretreated biomass as estimated by the modified Holtzapple model (Eq. 6) based on the average model data for the wood samples. Dots represent model estimations for the data of each of the three individual biological replicates. Error bars represent the standard errors on the estimated parameter values. * indicates significant differences compared to the WT as determined by unpaired t-test ($\alpha = 0.05$).

This amounted to 245.70 ± 25.77 g for the WT, 283.59 ± 17.88 g (+ 15%) for CAD 4, and 295.08 ± 13.92 g (+20%) for CAD 24. Wood from both CAD lines produced significantly more glucose compared to the WT, as determined by unpaired t-tests ($p = 0.0023$ and $p = 0.0363$ for CAD 4 and CAD 24, respectively). From an industrial point of view, enhanced enzymatic hydrolysis yields either require a lower STEX severity or a lower enzyme dose to achieve a similar glucose yield. Pretreatment accounts for 11-27% of the total bioethanol production costs, while enzymes account for 16% (Ceaser et al., 2025). However, it must be noted that, despite the higher glucose yield per kg of CAD wood, CAD trees have a total biomass yield penalty amounting to 32% for CAD24 and 40% for CAD40 when grown in the field (De Meester et al., 2022a). The biomass penalty is due to the strong reduction in *CAD1* gene expression in these lines. This implies that the increase in glucose yield per kg of CAD wood does not outcompete the total biomass yield penalty. Given that the CAD trees are smaller than the WT, one possible approach would be to cultivate the engineered trees at a higher planting density to maintain an overall biomass yield per hectare comparable to that of WT trees. However, a more desirable strategy would be to eliminate the yield penalty altogether. The yield penalty can be overcome by creating lines in which *CAD1* is less severely downregulated. Indeed, genetically modified poplar lines with more modest *CAD1* downregulation had no yield penalty when grown in the field, yet they did exhibit significant improvements in kraft pulping, a highly alkaline process, due to their aldehyde-rich lignin (Pilate et al., 2002). Alternatively, the yield penalty can potentially be avoided by targeting the *CAD1* downregulation to the fibres only (Gui et al., 2020) or by creating weak alleles by CRISPR/Cas9, as shown for *CCR2* in poplar (De Meester et al., 2020).

3.4 Chemical and structural differences between WT and CAD wood

The rapid decompression step of the STEX process, achieved by suddenly opening the explosion valve between the reactor and the expansion vessel, tore the stem chips of all three wood types into fibre-like shreds, thereby producing a wet wood pulp. However, it was visually clear that smaller fibre aggregates were present in the CAD lines than in the WT wood, despite comparable stem chip size before STEX (see Fig. S2) and similar mechanical properties of CAD wood to those of WT before STEX (Özparpucu et al., 2018). These differences in the size of the fibre aggregates were visualised in 3D image stitching pictures of the fibres from the STEX-pretreated wood samples derived from the three different biological replicates of the three poplar wood types (Fig. 4).

These microscopy pictures show that the fibre aggregates of the CAD lines appear smaller and thinner. To confirm this observation, the fibre aggregates were dried and sieved into different fractions without disrupting the aggregates. Additionally, to compare the individual fibres, the STEX-pretreated material of all poplar wood lines and blocks was macerated in Franklin solution, and the separated fibres were examined under a microscope. Their length was determined using ImageJ. The results of the sieving and microscopic analysis are shown in Figures 5a and 5b, respectively.

Figure 5a confirms a difference in fibre aggregate size distribution between the CAD 4 and CAD 24 wood and the WT wood. The data show that the STEX-pretreated CAD lines contained significantly more fibre aggregates in the two smallest size categories than the WT, although slightly more aggregates were also observed in the largest category. However, Figure 5b shows that there is no significant difference in individual fibre size between the treated CAD lines and WT wood when intact fibres are observed. A possible explanation for the increase in smaller fibre aggregates of the CAD wood after STEX is the difference in cell wall structure between the WT wood and the CAD 4 and CAD 24 wood. These differences and, more specifically, the differences in lignin structure, are well documented (Van Acker et al., 2017; De Meester et al., 2022a). The changes in lignin structure of CAD wood caused by the increased incorporation of syringaldehyde and sinapaldehyde, leading to shorter lignin polymer chains with more free phenolic end groups and conjugated carbonyl functions, making the lignin easier to break down under alkaline conditions. Lignin is also more hydrophobic, leading to fewer noncovalent associations between hemicellulose and lignin (Van Acker et al., 2017; Lapierre et al., 2004). Although the effect of STEX pretreatment on the different lignin structures in WT and CAD wood is unknown, the differences in lignin structure could contribute to the smaller fibre aggregates upon STEX. Another notable factor is that CAD wood has been reported to have thinner cell walls compared to WT wood (Nabuqi et al., 2020). In addition, a recent study showed that the wood cell walls collapsed after severe dilute acid pretreatment and that the effect was significantly greater for CAD wood than for WT wood (du Pasquier et al., 2025). This observation suggests that when most hemicellulose is hydrolysed, as is the case in the acid conditions during the incubation phase of the STEX pretreatment, CAD wood cells are less capable of maintaining the cell shape compared to WT wood cells (du Pasquier et al., 2025).

Comparing the enzymatic hydrolysis results obtained for WT poplar wood in this study with published data for poplar wood is not straightforward for several reasons. First, in most cases, wood samples from other poplar species, grown in other environments, were analysed, implying that the starting material was different. Second, the CAD biomass analysed here was chopped biomass from whole stems, including bark and wood. This material was chosen over ground debarked stem samples because chipped whole stems reflect better the type of biomass to be processed in future biorefineries. That said, it is important to note that the hydrolysis yield of wood and bark is different. Van Acker et al. (2014) showed that the saccharification yield of bark samples is only about half that of wood samples from the same poplar genotype as analysed here (*P. tremula x P. alba* cv. 717 1B4). They also showed that downregulation of cinnamoyl CoA reductase (CCR) in poplar, which leads to reduced lignin content, enhanced the saccharification yield in wood but not in bark (Van Acker et al., 2014). Differences in biomass type (ground debarked wood vs. chipped whole stem) and growth conditions (greenhouse-grown vs. field-grown) complicate direct comparisons of the magnitude of improvement in saccharification yield across studies and modified lines.

Third, different pretreatment and enzymatic hydrolysis conditions were applied. The STEX conditions employed in the present study involved high temperatures, short times, and average severities (Wu et al., 1999; Jacquet et al., 2015). The enzymatic hydrolysis conditions we used aimed to achieve average glucose concentrations to investigate whether CAD wood is easier

Please cite this article as: Geerts J., Croes T., Leemans P.G.C., Richel A., Besserer A., Brosse N., De Meester B., Boerjan W., Cornet I. Improved sugar yield upon steam explosion and enzymatic saccharification of field-grown poplar downregulated for cinnamyl alcohol dehydrogenase 1. Biofuel Research Journal 50 (2026) 2698-2710. DOI: 10.18331/BRJ2026.13.2.4.

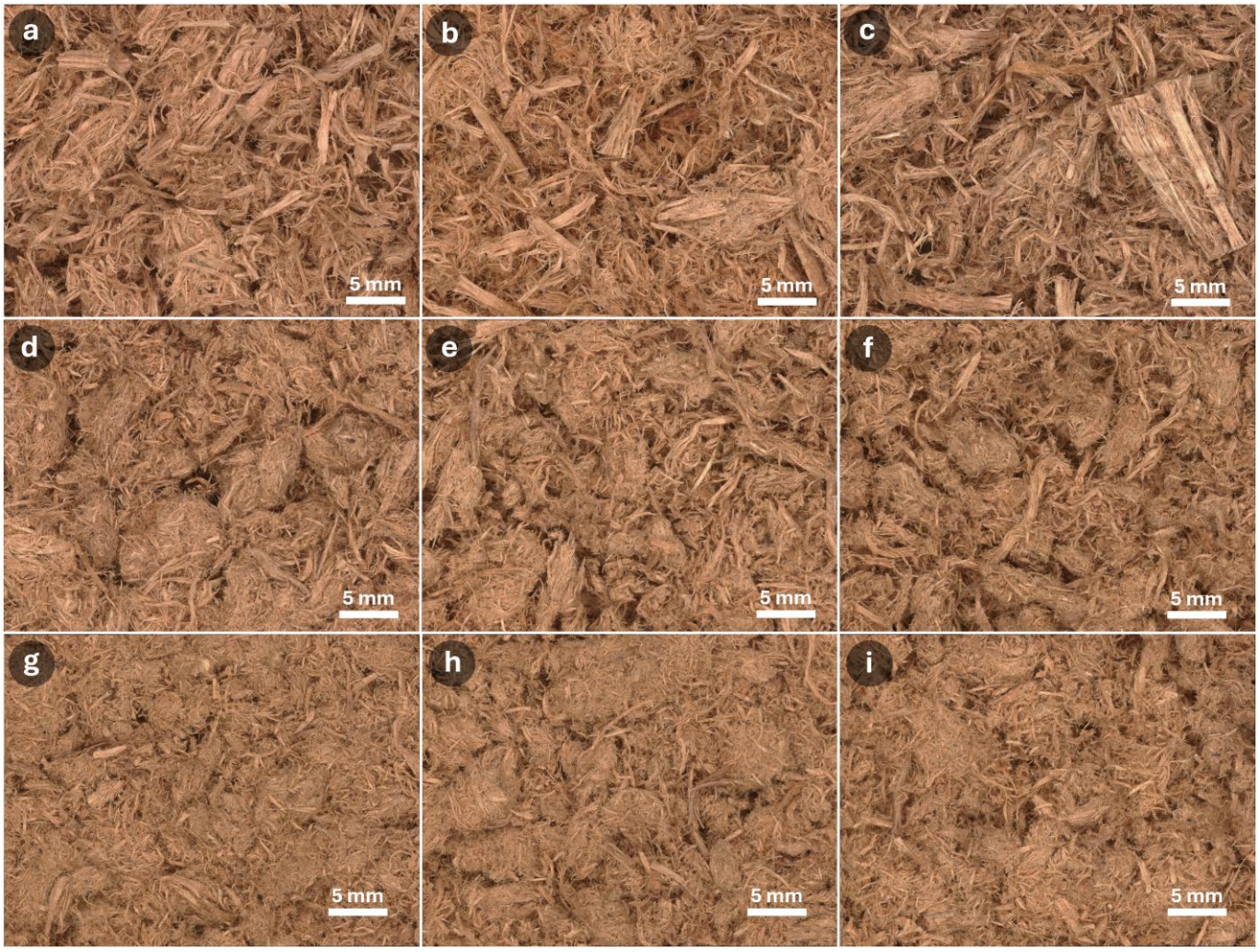


Fig. 4. 3D image stitching of light microscopy pictures at 20 × magnification of STEX-pretreated poplar wood taken using a Keyence VHX-7000 digital microscope. **(a)** WT B1, **(b)** WT B4, **(c)** WT B5, **(d)** CAD 4 B4, **(e)** CAD 4 B5, **(f)** CAD 4 B6, **(g)** CAD 24 B1, **(h)** CAD 24 B2, and **(i)** CAD 24 B3.

to hydrolyse than WT wood upon STEX (Pažitný et al., 2020; Antczak et al., 2022). A single study by Mansfield et al. (2012) reports on the effects of STEX on the enzymatic hydrolysis yield of genetically modified poplar wood, in which either *F5H* was overexpressed (C4H::F5H) or *C3'H* was downregulated (C3'H-RNAi). These poplars had a significant shift in monolignol composition; lignin of the C4H::F5H lines had an increased frequency of S-units, but similar total lignin amounts, whereas the C3'H-RNAi-suppressed poplar lines contained more H-units at the expense of G-units, as well as less total lignin. The authors reported that the reduction in total lignin content in the C3'H-RNAi-suppressed poplar contributed more substantially to the increased enzymatic hydrolysis yield than either the higher S/G ratio in the C4H::F5H lines or the greater abundance of H-units in the C3'H-RNAi-suppressed poplar lines. Enzymatic hydrolysis yields in the applied conditions were 76% for the WT and 85.2% for the C4H::F5H transgenic line. However, solid loadings were very low at 2%, making it difficult to compare the data with our results. Despite the differences in lignin structure in the C4H::F5H and also lignin amount in the C3'H-RNAi-suppressed lines reported by Mansfield et al. (2012), lignin was not more efficiently removed during STEX in these poplar lines. Conversely, our research findings revealed that significantly more lignin was removed in the CAD lines than in the WT. However, the relative lignin contents of the CAD wood were similar to those of the WT after STEX. Despite similar total lignin amounts, the enzymatic hydrolysis was improved.

A key difference in lignin structure between the CAD lines, C4H::F5H lines, C3'H-RNAi lines, and most other lignin-modified lines is that higher amounts of syringaldehyde and sinapaldehyde are incorporated into the lignin polymer. The incorporation of sinapaldehydes in the lignin polymer is expected to result in a more hydrophobic lignin because when sinapaldehyde incorporates into the lignin polymer, aromatisation by elimination of the proton on the C8 position outcompetes nucleophilic addition by water. The more hydrophobic lignin will have fewer non-covalent interactions with hemicelluloses. Likewise, nucleophilic attack by other nucleophiles such as alcohol or carboxylic acid groups from hemicellulose will also be hampered, reducing the creation of benzyl ether or benzyl ester bonds that putatively contribute to recalcitrant lignin-carbohydrate complexes (Mottiar et al., 2016; Van Acker et al., 2017). Such a reduction in lignin-carbohydrate complexes may be the cause of the easier lignin removal during the STEX pretreatment. Likewise, it may increase access to the hydrolysing enzymes.

The conditions applied by Mansfield et al. (2012) were very different from those applied here for both the pretreatment step (addition of SO_2, different severity, and different temperature and time altogether) and the enzymatic hydrolysis step (different solid loading, different enzyme loading, and a different enzyme complex). Nevertheless, both our data and

Please cite this article as: Geerts J., Croes T., Leemans P.G.C., Richel A., Besserer A., Brosse N., De Meester B., Boerjan W., Cornet I. Improved sugar yield upon steam explosion and enzymatic saccharification of field-grown poplar downregulated for cinnamyl alcohol dehydrogenase 1. Biofuel Research Journal 50 (2026) 2698-2710. DOI: 10.18331/BRJ2026.13.2.4.

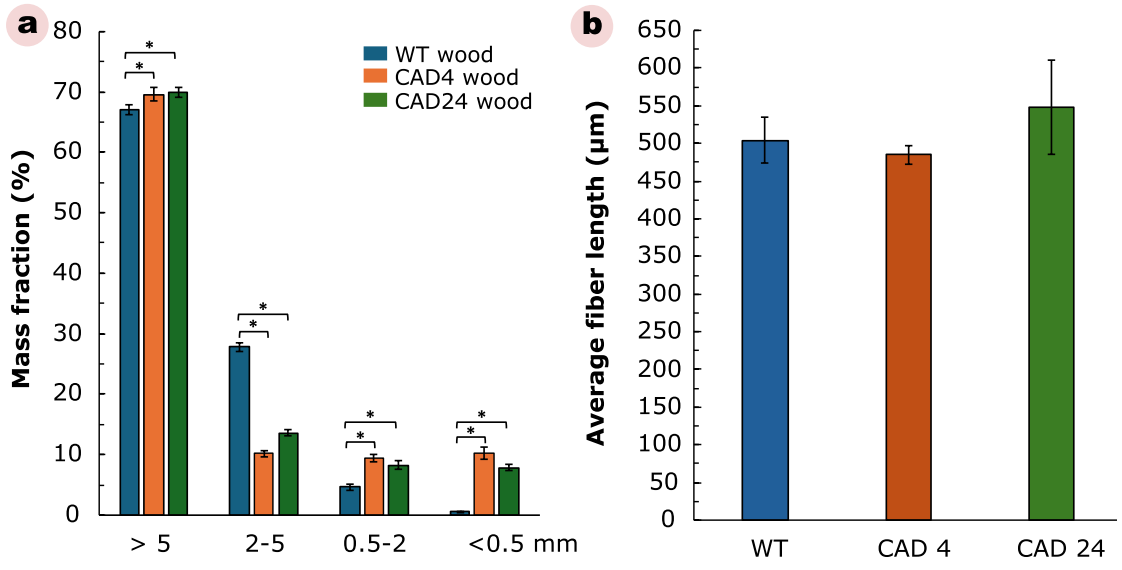


Fig. 5. (a) fibre aggregate size after STEX pretreatment of WT wood, CAD 4, and CAD 24 as determined by sieving through different pore sizes. Error bars represent the standard deviation of three biological replicates. **(b)** individual fibre size as determined microscopically: WT wood, CAD 4, and CAD 24. Error bars represent the standard deviation on three biological repeats. Each biological replicate (block) was determined on 5 different microscopic slides, by measuring 5 to 12 fibres (technical replicates) * indicate significant differences compared to the WT as determined by unpaired t-test ($\alpha = 0.05$).

those of Mansfield et al. (2012) show that lignin can be engineered to improve enzymatic hydrolysis after STEX pretreatment. Our data further motivate the interest in so-called designer lignins, in which foreign lignin monomers are introduced specifically to improve the deconstruction of lignocellulosic biomass (Mottiar et al., 2016).

4. Conclusions and Outlook

Wood from three-year-old, field-grown *CAD*-deficient poplar showed 25% delignification upon STEX compared to 15% for the WT wood, resulting in significantly more small fibre aggregates, and also yielded significantly higher glucose yield upon enzymatic hydrolysis: 76% compared to 60%, respectively. The improved enzymatic hydrolysis results were not caused by different lignin contents after STEX, but likely by the different lignin composition and the associated thinner wood cell walls. Using wood from *CAD*-deficient trees may reduce processing cost in a biorefinery approach and may contribute to the needed transition from a fossil-based to a bio-based economy. It is important to note that the poplar hybrid used for transformation, *Populus tremula x P. alba* cv. 717-1B4 is not a commercial hybrid, but a poplar clone that is easily genetically transformed by *Agrobacterium tumefaciens*. For commercial deployment, elite tree genotypes will need to be transformed, whether from the *Populus* genus or from another wood-producing genus like *Eucalyptus*. When a similar genetic engineering strategy is used as illustrated in this paper, i.e., based on RNA interference, the deployment strategy of the transgenic trees will need to comply with the current regulations concerning the introduction of genetically modified plants into the environment. However, the EU is currently advancing legislation that would exempt certain gene-edited plants lacking foreign DNA from many of the regulatory requirements applied to conventional genetically modified plants, potentially facilitating the commercialisation of CRISPR-edited lines.

Editing strategies that avoid the stable introduction of foreign DNA exist for a variety of species, including poplar (Hoengenaert et al., 2025). As the world faces challenges with climate change and geopolitical instability, alternatives to fossil resources to produce fuels and products are needed. Genetic modification and gene-editing are promising approaches to tailor plants to human needs and are considered similarly safe as conventional breeding strategies (Boerjan and Strauss, 2024). For biofuels, poplar is considered a promising feedstock given its favourable global warming potential compared to fossil resources. The largest contributor to greenhouse gas emissions is the biomass conversion due to the use of chemicals (Morales-Vera et al., 2022). Our lignin engineering approach precisely addresses this hurdle.

Acknowledgements

This study is part of the ADV_BIO project financed by the Energy Transition Fund of the Belgian Federal Public Service of Economy (FOD Economie). The FWO Tournesol grant VS00922N enabled the international collaboration. The authors would like to thank Professor Bartel Vanholme for his help with the microscopy section.

Author Contributions

Jordi Geerts: conceptualisation, design of experiments, methodology, investigation, data collection, visualisation, writing: original draft and review and editing, **Tim Croes:** data collection, review and editing **Paulien Geertje Corlijn Leemans:** visualisation, review and editing, **Aurore Richel:** Funding acquisition, project administration, review and editing, **Arnaud Besserer:** resources, review and editing, **Nicolas Brosse:** methodology, resources, supervision, review and editing, **Barbara De Meester:** Conceptualisation, design of experiments, methodology, data collection, review and editing **Wout Boerjan and Iris Cornet:** conceptualisation, design of experiments, methodology, investigation, preparation, writing – review and editing, supervision, funding acquisition, resources.

Conflict of Interest

The authors declare that they have no conflicts of interest to disclose.

References

[1] Adney, B., Baker, J., 1996. Measurement of Cellulase Activities Laboratory Analytical Procedure (LAP). Golden, Colo, NREL.

[2] Akkermans, S., Nimmegeers, P., Van Impe, J.F., 2018. A tutorial on uncertainty propagation techniques for predictive microbiology models: A critical analysis of state-of-the-art techniques. Int. J. Food Microbiol. 282, 1-8.

Please cite this article as: Geerts J., Croes T., Leemans P.G.C., Richel A., Besserer A., Brosse N., De Meester B., Boerjan W., Cornet I. Improved sugar yield upon steam explosion and enzymatic saccharification of field-grown poplar downregulated for cinnamyl alcohol dehydrogenase 1. Biofuel Research Journal 50 (2026) 2698-2710. DOI: 10.18331/BRJ2026.13.2.4.

[3] Antczak, A., Szadkowski, J., Szadkowska, D., Zawadzki, J., 2022. Assessment of the effectiveness of liquid hot water and steam explosion pretreatments of fast-growing poplar (Populus trichocarpa) wood. Wood Sci. Technol. 56, 1-23.
[4] Bhagia, S., Muchero, W., Kumar, R., Tuskan, G.A., Wyman, C.E., 2016. Natural genetic variability reduces recalcitrance in poplar. Biotechnol. Biofuels Bioprod. 9(1).
[5] Boerjan, W., Strauss, S.H., 2024. Social and biological innovations are essential to deliver transformative forest biotechnologies. New Phytol. 243(2), 526-536.
[6] Broos, W., Wittner, N., Dries, J., Vlaeminck, S.E., Gunde-Cimerman, N., Cornet, I., 2024. Rhodotorula kratochvilovae outperforms Cutaneotrichosporon oleaginosum in the valorisation of lignocellulosic wastewater to microbial oil. Process Biochem. 137, 229-238.
[7] Bryant, N.D., Pu, Y., Tschaplinski, T.J., Tuskan, G.A., Muchero, W., Kalluri, U.C., Yoo, C.G., Ragauskas, A.J., 2020. Transgenic poplar designed for biofuels. Trends Plant Sci. 25(9), 881-896.
[8] Ceaser, R., Montané, D., Constantí, M., Medina, F., 2025. Current progress on lignocellulosic bioethanol, including a technological and economical perspective. Environ. Dev. Sustain. 27(11).
[9] De Meester, B., Madariaga Calderón, B., de Vries, L., Pollier, J., Goeminne, G., Van Doorsselaere, J., Chen, M., Ralph, J., Vanholme, R., Boerjan, W., 2020. Tailoring poplar lignin without yield penalty by combining a null and haploinsufficient CINNAMOYL-CoA REDUCTASE2 allele. Nat. Commun. 11(1).
[10] De Meester, B., Van Acker, R., Wouters, M., Traversari, S., Steenackers, M., Neukermans, J., Van Breusegem, F., Déjardin, A., Pilate, G., Boerjan, W., 2022a. Field and saccharification performances of poplars severely downregulated in CAD1. New Phytol. 236(6), 2075-2090.
[11] De Meester, B., Vanholme, R., Mota, T., Boerjan, W., 2022b. Lignin engineering in forest trees: From gene discovery to field trials. Plant Commun. 3(6), 100465.
[12] Deng, W., Feng, Y., Fu, J., Guo, H., Guo, Y., Han, B., Jiang, Z., Kong, L., Li, C., Liu, H., Nguyen, P.T.T., Ren, P., Wang, F., Wang, S., Wang, Y., Wang, Y., Wong, S.S., Yan, K., Yan, N., Yang, X., Zhang, Y., Zhang, Z., Zeng, X., Zhou, H., 2023. Catalytic conversion of lignocellulosic biomass into chemicals and fuels. Green Energy Environ. 8(1), 10-114.
[13] Donaldson, L., Nanayakkara, B., Harrington, J., 2017. Wood Growth and Development, in: Thomas, B., Murray, B.G., Murphy, D.J. (Eds.), Encycl. Appl. Plant Sci. (Second Edition). Academic Press, Oxford, pp. 203-210.
[14] du Pasquier, J., Zoghlami, A., Naudin, Y., Déjardin, A., Pilate, G., Paës, G., Perré, P., 2025. Cinnamyl alcohol dehydrogenase downregulation in poplar wood increases saccharification after dilute acid pretreatment: a key role for lignin revealed by a multimodal investigation. Biotechnol. Biofuels Bioprod. 18(1).
[15] Fang, L., Lai, C., Hua, Q., Wang, P., Huang, C., Ling, Z., Yong, Q., 2025. Lignin-associated factors impede enzymatic hydrolysis of hydrothermally pretreated birch and poplar wood. Ind. Crops Prod. 226, 120704.
[16] Gautam, A., Pant, M., Pant, G., Kumar, G., 2024. Second-Generation Biofuels: Concepts, Applications, and Challenges, in: Karnwal, A., Mohammad Said Al-Tawaha, A.R. (Eds.), Microbial Applications for Environmental Sustainability. Springer Nature Singapore, Singapore, pp. 277-304.
[17] Gui, J., Lam, P.Y., Tobimatsu, Y., Sun, J., Huang, C., Cao, S., Zhong, Y., Umezawa, T., Li, L., 2020. Fibre-specific regulation of lignin biosynthesis improves biomass quality in Populus. New Phytol. 226(4), 1074-1087.
[18] Hemansi, Gupta, R., Yadav, G., Kumar, G., Yadav, A., Saini, J., Kuhad, R., 2019. Second Generation Bioethanol Production: The State of Art: From Current Status to Practical Implementation, pp. 121-146.
[19] Hoang, A.T., Nguyen, X.P., Duong, X.Q., Ağbulut, Ü., Len, C., Nguyen, P.Q.P., Kchaou, M., Chen, W.-H., 2023. Steam explosion as sustainable biomass pretreatment technique for biofuel production: Characteristics and challenges. Bioresour. Technol. 385, 129398.
[20] Hoengenaert, L., Anders, C., Van Doorsselaere, J., Vanholme, R., Boerjan, W., 2025. Transgene-free genome editing in poplar. New Phytol. 247(1), 224-232.
[21] Holtzapple, M.T., Caram, H.S., Humphrey, A.E., 1984. A comparison of two empirical models for the enzymatic hydrolysis of pretreated poplar wood. Biotechnol. Bioeng. 26(8), 936-941.
[22] Jacquet, N., Maniet, G., Vanderghem, C., Delvigne, F., Richel, A., 2015. Application of Steam Explosion as Pretreatment on Lignocellulosic Material: A Review. Ind. Eng. Chem. Res. 54(10), 2593-2598.
[23] Jacquet, N., Vanderghem, C., Danthine, S., Quiévy, N., Blecker, C., Devaux, J., Paquot, M., 2012. Influence of steam explosion on physicochemical properties and hydrolysis rate of pure cellulose fibers. Bioresour. Technol. 121, 221-227.
[24] Lapierre, C., Pilate, G., Pollet, B., Mila, I., Leplé, J.-C., Jouanin, L., Kim, H., Ralph, J., 2004. Signatures of cinnamyl alcohol dehydrogenase deficiency in poplar lignins. Phytochemistry 65(3), 313-321.
[25] Lapierre, C., Pollet, B., Petit-Conil, M., Toval, G., Romero, J., Pilate, G., Leple, J.C., Boerjan, W., Ferret, V.V., De Nadai, V., Jouanin, L., 1999. Structural alterations of lignins in transgenic poplars with depressed cinnamyl alcohol dehydrogenase or caffeic acid O-methyltransferase activity have an opposite impact on the efficiency of industrial kraft pulping. Plant Physiol. 119(1), 153-164.
[26] Li, W., Lin, Y.-C.J., Chen, Y.-L., Zhou, C., Li, S., De Ridder, N., Oliveira, D.M., Zhang, L., Zhang, B., Wang, J.P., Xu, C., Fu, X., Luo, K., Wu, A.-M., Demura, T., Lu, M.-Z., Zhou, Y., Li, L., Umezawa, T., Boerjan, W., Chiang, V.L., 2024. Woody plant cell walls: Fundamentals and utilization. Mol. Plant. 17(1), 112-140.
[27] Ma, S., Wang, L.-J., Jiang, J., Zhao, Y.-G., 2023. Direct and indirect effects of agricultural expansion and landscape fragmentation processes on natural habitats. Agric. Ecosyst. Environ. 353, 108555.
[28] Mansfield, S.D., Kang, K.-Y., Chapple, C., 2012. Designed for deconstruction – poplar trees altered in cell wall lignification improve the efficacy of bioethanol production. New Phytol. 194(1), 91-101.
[29] Morales-Vera, R., Vásquez-Ibarra, L., Scott, F., Puettmann, M., Gustafson, R., 2022. Life Cycle Assessment of Bioethanol Production: A Case Study from Poplar Biomass Growth in the U.S. Pacific Northwest. Fermentation 8(12), 734.
[30] Mottiar, Y., Vanholme, R., Boerjan, W., Ralph, J., Mansfield, S.D., 2016. Designer lignins: harnessing the plasticity of lignification. Curr. Opin. Biotechnol. 37, 190-200.
[31] Nabuqi, Nuoendagula, Wu, S., Takata, N., Sakamoto, S., Yamamoto, M., Uesugi, M., Déjardin, A., Pilate, G., Taniguchi, T., Mitsuda, N., Kajita, S., 2020. Simultaneous manipulation of lignin structure and secondary cell wall formation in transgenic poplar. J. Wood Sci. 66(1), 56.
[32] NNFCC, 2010. Short rotation coppice willow - crop fact sheet. (Accessed 21/06/2024 2024)
[33] Oliveira, F.M.V., Pinheiro, I.O., Souto-Maior, A.M., Martin, C., Gonçalves, A.R., Rocha, G.J.M., 2013. Industrial-scale steam explosion pretreatment of sugarcane straw for enzymatic hydrolysis of cellulose for production of second generation ethanol and value-added products. Bioresour. Technol. 130, 168-173.
[34] Özparpucu, M., Gierlinger, N., Burgert, I., Van Acker, R., Vanholme, R., Boerjan, W., Pilate, G., Déjardin, A., Rüggeberg, M., 2018. The effect of altered lignin composition on mechanical properties of CINNAMYL ALCOHOL DEHYDROGENASE (CAD) deficient poplars. Planta 247(4), 887-897.
[35] Pažitný, A., Russ, A., Boháček, Š., Stankovska, M., Ihnát, V., Sutý, S., 2020. Effect of steam explosion on enzymatic hydrolysis of various parts of poplar tree. Wood Res. 65, 579-590.
[36] Pilate, G., Guiney, E., Holt, K., Petit-Conil, M., Lapierre, C., Leplé, J.-C., Pollet, B., Mila, I., Webster, E.A., Marstorp, H.G., Hopkins, D.W., Jouanin, L., Boerjan, W., Schuch, W., Cornu, D., Halpin, C., 2002. Field and pulping performances of transgenic trees with altered lignification. Nat. Biotechnol. 20(6), 607-612.
[37] Polaris Market Research, 2023. Global Cellulosic Ethanol Market Size/Demand Envisaged to Reach USD 23,738.98 Million By 2032,

Please cite this article as: Geerts J., Croes T., Leemans P.G.C., Richel A., Besserer A., Brosse N., De Meester B., Boerjan W., Cornet I. Improved sugar yield upon steam explosion and enzymatic saccharification of field-grown poplar downregulated for cinnamyl alcohol dehydrogenase 1. Biofuel Research Journal 50 (2026) 2698-2710. DOI: 10.18331/BRJ2026.13.2.4.

at 39.0% CAGR Growth: Polaris Market Research. (Accessed 13/06/2023 2023)
[38] Rego, F., Soares Dias, A.P., Casquilho, M., Rosa, F.C., Rodrigues, A., 2019. Fast determination of lignocellulosic composition of poplar biomass by thermogravimetry. Biomass Bioenergy 122, 375-380.
[39] Ritchie, H., Rosado, P., Roser, M., 2025. Stat. Rev. World Energy.
[40] Romero, E., Olson, M., Segovia-Rivas, A., Pittermann, J., Jupa, R., 2026. Providing maceration protocols for xylem and phloem research. Front. Plant Sci. 16:1740174.
[41] Safdari, V., Khodadadi, H., Hosseinihashemi, S.K., Ganjian, E., 2011. The Effects of Poplar Bark and Wood Content on the Mechanical Properties of Wood-Polypropylene Composites. BioResources 6, 5180-5192.
[42] Sandford, C., Malins, C., Phillips, J., 2024. Diverted Harvest - Environmental Risk from Growth in International Biofuel Demand. Cerulogy, p. 84.
[43] Segmehl, J., Keplinger, T., Krasnobaev, A., Berg, J., Willa, C., Burgert, I., 2018. Facilitated delignification in CAD deficient transgenic poplar studied by confocal Raman spectroscopy imaging. Spectrochim. Acta A Mol. Biomol. Spectrosc. 206.
[44] Shrotri, A., Kobayashi, H., Fukuoka, A., 2017. Chapter Two - Catalytic Conversion of Structural Carbohydrates and Lignin to Chemicals, in: Song, C. (Ed.) Advances in Catalysis. Academic Press, pp. 59-123.
[45] Shu, B., Ren, Q., Hong, L., Xiao, Z., Lu, X., Wang, W., Yu, J., Fu, N., Gu, Y., Zheng, J., 2020. Effect of Steam Explosion Technology Main Parameters on Moso Bamboo and Poplar Fiber. J. Renew. Mater. 9(3), 585-597.
[46] Sluiter, Hames, Ruiz, Scarlata, Sluiter, Templeton, Crocker, 2012. Determination of Structural Carbohydrates and Lignin in Biomass. NREL.
[47] Statista, 2024. Market value of bioethanol worldwide in 2023 with a forecast for 2029. (Accessed 15/10/2024 2024).
[48] Studer, M.H., DeMartini, J.D., Davis, M.F., Sykes, R.W., Davison, B., Keller, M., Tuskan, G.A., Wyman, C.E., 2011. Lignin content in natural Populus variants affects sugar release. Proc. Natl. Acad. Sci. USA 108(15), 6300-6305.
[49] Sulis, D.B., Jiang, X., Yang, C., Marques, B.M., Matthews, M.L., Miller, Z., Lan, K., Cofre-Vega, C., Liu, B., Sun, R., Sederoff, H., Bing, R.G., Sun, X., Williams, C.M., Jameel, H., Phillips, R., Chang, H.-m., Peszlen, I., Huang, Y.-Y., Li, W., Kelly, R.M., Sederoff, R.R., Chiang, V.L., Barrangou, R., Meester, J.P., 2023. Multiplex CRISPR editing of wood for sustainable fiber production. Science 381(6654), 216-221.
[50] Szadkowski, J., Vybohova, E., Kučerová, V., Čabalová, I., Antczak, A., Szadkowska, D., Drożdżek, M., Zawadzki, J., 2023. Effect of steam explosion pretreatment of fast-growing poplar (*Populus deltoides* x *maximowiczii*) wood on selected properties of structural substances.
[51] Van Acker, R., Déjardin, A., Desmet, S., Hoengenaert, L., Vanholme, R., Morreel, K., Laurans, F., Kim, H., Santoro, N., Foster, C., Goeminne, G., Légée, F., Lapierre, C., Pilate, G., Ralph, J., Boerjan, W., 2017. Different Routes for Conifer- and Sinapaldehyde and Higher Saccharification upon Deficiency in the Dehydrogenase CAD1. Plant Physiol. 175(3), 1018-1039.
[52] Vanhevel, Y., De Moor, A., Muylle, H., Vanholme, R., Boerjan, W., 2024. Breeding for improved digestibility and processing of lignocellulosic biomass in Zea mays. Front. Plant Sci. 15: 1419796.
[53] Wang, N., Takada, M., Sakamoto, S., Vanholme, R., Goeminne, G., Kim, H., Nagano, S., Takata, N., Kamimura, N., Uesugi, M., Izumi-Nakagawa, A., Masai, E., Mitsuda, N., Boerjan, W., Ralph, J., Kajita, S., 2025. Effects of feruloyl-CoA 6′-hydroxylase 1 overexpression on lignin and cell wall characteristics in transgenic hybrid aspen. Front. Plant Sci. 16:1543168.
[54] Wittner, N., Slezsák, J., Broos, W., Geerts, J., Gergely, S., Vlaeminck, S.E., Cornet, I., 2023. Rapid lignin quantification for fungal wood pretreatment by ATR-FTIR spectroscopy. Spectrochim. Acta A Mol. Biomol. Spectrosc. 285, 121912.
[55] Wu, M.M., Chang, K., Gregg, D.J., Boussaid, A., Beatson, R.P., Saddler, J.N., 1999. Optimization of steam explosion to enhance hemicellulose recovery and enzymatic hydrolysis of cellulose in softwoods. Appl. Biochem. Biotechnol. 77(1), 47-54.
[56] Zhang, H., Han, L., Dong, H., 2021. An insight to pretreatment, enzyme adsorption and enzymatic hydrolysis of lignocellulosic biomass: Experimental and modeling studies. Renew. Sustain. Energy Rev. Reviews 140, 110758.
[57] Ziegler-Devin, I., Chrusciel, L., Brosse, N., 2021. Steam explosion pretreatment of lignocellulosic biomass: a mini-review of theorical and experimental approaches. Front. Chem. 9.

Jordi Geerts, Ing., is a PhD candidate at the Faculty of Applied Engineering at the University of Antwerp. He obtained his master's degree in Industrial Sciences, Biochemistry, in 2020. His current research within the Biochemical Wastewater Valorisation and Engineering research group focuses on industrial biotechnology, including waste valorisation, biofuel production, and lignocellulosic pretreatment, with a particular interest in steam explosion pretreatment.

Paulien Leemans, Ir., is a PhD researcher and teaching assistant at the Faculty of Applied Engineering at the University of Antwerp. She obtained her Master's degree in Bioscience Engineering, with a specialisation in Cell and Gene Technology, from Ghent University in 2021. Her current research focuses on industrial biotechnology, microbiology, synthetic biology, and process optimisation, with a particular interest in microbial production platforms and their application in sustainable bioprocesses.

Tim Croes, PhD, is a researcher and lecturer in biochemical and chemical engineering at the University of Antwerp. Dr. Croes obtained a PhD from KU Leuven, with research focused on lignocellulosic biomass valorisation and membrane-based separation of lignin oil. Their research interests include sustainable chemical processes, particularly biomass and waste valorisation, membrane-based process intensification, downstream processing, and life cycle assessment. These research activities are combined with teaching efforts aimed at advancing circular and sustainable chemical engineering.

Arnaud Besserer is an Associate Professor at the Wood Research Laboratory (LERMAB) at the University of Lorraine. He earned his PhD in Plant Physiology in 2008 and completed his research habilitation in Wood Sciences in 2025. He has authored 29 scientific publications and has an h-index of 16, with more than 2,400 citations. His research focuses on wood recycling, wood–fungi interactions, mycelium-based materials, post-consumer wood modification and detoxification, and steam explosion processes applied to lignocellulosic biomass. Google Scholar profile: https://scholar.google.fr/citations?user=IF1iiq0AAAAJ

Please cite this article as: Geerts J., Croes T., Leemans P.G.C., Richel A., Besserer A., Brosse N., De Meester B., Boerjan W., Cornet I. Improved sugar yield upon steam explosion and enzymatic saccharification of field-grown poplar downregulated for cinnamyl alcohol dehydrogenase 1. Biofuel Research Journal 50 (2026) 2698-2710. DOI: 10.18331/BRJ2026.13.2.4.

Nicolas Brosse is a Full Professor of Chemistry at the University of Lorraine and group leader at LERMAB. His research focuses on the fractionation and valorisation of lignocellulosic biomass, including wood, plant fibres, and algae, through green chemistry and biorefinery approaches. He has authored more than 200 scientific publications on biomass pretreatment, lignin chemistry, and bio-based materials. He is an elected member of the International Academy of Wood Science.

Aurore Richel is a Full Professor at the University of Liège, where she is involved in research and education in the chemistry of renewable resources and associated technologies. Dr. Richel is actively engaged in more than 15 national and international projects focused on bioenergy and bioproduct production. Her work encompasses innovative pretreatment protocols for lignocellulosic materials, extraction of platform chemicals for the polymer sector, formulation of novel biomass-to-energy solutions, and the valorisation of lignin for high-value applications. Aurore Richel has published more than 220 articles in peer-reviewed international journals.

Barbara De Meester currently serves as an Inspector at the Belgian Federal Agency for the Safety of the Food Chain. Prior to this role, she spent a decade conducting research in lignin engineering in the Boerjan Lab at the VIB Center for Plant Systems Biology and the Department of Plant Biotechnology and Bioinformatics at Ghent University. Her work focused on improving the cost-efficiency of converting plant biomass into fermentable sugars and aromatic building blocks without compromising plant yield. This research was conducted across multiple model and crop species, including Arabidopsis, poplar, and maize, under both greenhouse and field conditions. She obtained her PhD from Ghent University in 2018.

Iris Cornet holds a Master's degree in Bioscience Engineering from the Free University of Brussels and obtained her PhD in Engineering from KU Leuven in 2013. She is an Associate Professor in the Department of Biochemical and Chemical Engineering at the University of Antwerp, where she teaches biochemical engineering and industrial biotechnology. Her research is situated within industrial biotechnology and focuses on the valorisation of lignocellulosic biomass and industrial side streams into biofuels and value-added chemicals using microbial and enzymatic processes. Her work integrates biorefinery concepts with process optimisation, including the development of advanced pretreatment strategies and fermentation technologies, and extends to applications in the food sector. She has represented Belgium on the Chemistry Europe board and was nominated as a Chemistry Europe Fellow for 2018–2019.

Wout Boerjan is a Full Professor at Ghent University and group leader at the VIB Center for Plant Systems Biology, where he leads the Bioenergy and Bio-aromatics Group. His team investigates the biosynthesis and structure of lignin to generate the fundamental knowledge needed to improve the processing of biomass into pulp, fermentable sugars, and building blocks for the chemical industry. The group employs genetic engineering and gene editing and translates discoveries from the laboratory to experimental field trials. Metabolomics plays a central role in elucidating the functions of newly discovered genes involved in aromatic metabolism. Highlights of his career include being named "Forest Biotechnologist of the Year" by the Institute for Forest Biotechnology (2009), being listed among Clarivate Analytics' Highly Cited Researchers annually since 2014, receiving the Dr. A. De Leeuw-Damry-Bourlart FWO Excellence Prize in Applied Sciences (2020), being elected as an EMBO member (2019), and receiving the Marcus Wallenberg Prize in 2024. His Google Scholar h-index is 101. Research profile: https://www.boerjanlab.be/research

Please cite this article as: Geerts J., Croes T., Leemans P.G.C., Richel A., Besserer A., Brosse N., De Meester B., Boerjan W., Cornet I. Improved sugar yield upon steam explosion and enzymatic saccharification of field-grown poplar downregulated for cinnamyl alcohol dehydrogenase 1. Biofuel Research Journal 50 (2026) 2698-2710. DOI: 10.18331/BRJ2026.13.2.4.

Supplementary Information

Table S1.
Mass of the wood at the start of the experiment (dry), after soaking (wet), and after STEX (wet). Values represent the averages and standard deviations of three biological replicates. Underlined values indicate a significant difference compared to the wild type at α = 0.05 as determined by an unpaired T-test.

Parameter	WT	CAD 4	CAD 24
Mass start (g)	50.00 ± 0.00	50.00 ± 0.00	50.00 ± 0.00
Mass after soaking (g)	119.99 ± 4.53	126.44 ± 7.13	132.39 ± 1.64
Mass after STEX (g)	354.42 ± 30.15	424.60 ± 12.38	428.23 ± 14.67

Table S2.
Severity factor (S^0) [-] per steam explosion* applied as calculated using Equation 2.

Sample	Severity factor (S^0)
WT wood, B1 (Fig. S9)	3.59
WT wood, B1	3.59
WT wood, B4	3.63
WT wood, B5	3.62
CAD 4 wood, B4	3.62
CAD 4 wood, B5	3.56
CAD 4 wood, B6	3.56
CAD 24 wood, B1 (Fig. S9)	3.57
CAD 24 wood, B1	3.56
CAD 24 wood, B2	3.59
CAD 24 wood, B3	3.58

* The temperature-over-time profile for each steam explosion is available upon request.

Table S3.
Cellulose and hemicellulose retention rate and lignin removal rate during STEX of the different poplar wood types, as calculated by Equations 1-3, respectively. Values represent the averages and standard deviations of three biological replicates. The compositional analysis was performed in threefold for each biological repeat (three technical repeats). Underlined values indicate a significant difference compared to the wild type at α = 0.05 as determined by an unpaired T-test.

Parameter	WT	CAD 4	CAD 24
Cellulose retention rate (%)	97.07 ± 3.77	96.68 ± 6.97	94.19 ± 3.88
Hemicellulose retention rate (%)	42.64 ± 2.45	39.55 ± 3.10	40.65 ± 1.78
Lignin removal rate (%)	14.98 ± 2.87	24.63 ± 4.80	26.10 ± 3.17

Equations:

$$Cellulose\ retention\ rate\ (\%) = \frac{m_{cellulose\ after\ STEX}\ (g)}{m_{\ cellulose\ before\ STEX}\ (g)} \times 100\% \qquad \text{Eq. S1}$$

$$Hemicellulose\ retention\ rate\ (\%) = \frac{m_{hemicellulose\ after\ STEX}\ (g)}{m_{hemicellulose\ before\ STEX}\ (g)} \times 100\% \qquad \text{Eq. S2}$$

$$Lignin\ removal\ rate\ (\%) = \frac{m_{lignin\ before\ STEX}\ (g) - m_{lignin\ after\ STEX}\ (g)}{m_{lignin\ before\ STEX}\ (g)} \times 100\% \qquad \text{Eq. S3}$$

Please cite this article as: Geerts J., Croes T., Leemans P.G.C., Richel A., Besserer A., Brosse N., De Meester B., Boerjan W., Cornet I. Improved sugar yield upon steam explosion and enzymatic saccharification of field-grown poplar downregulated for cinnamyl alcohol dehydrogenase 1. Biofuel Research Journal 50 (2026) 2698-2710. DOI: 10.18331/BRJ2026.13.2.4.

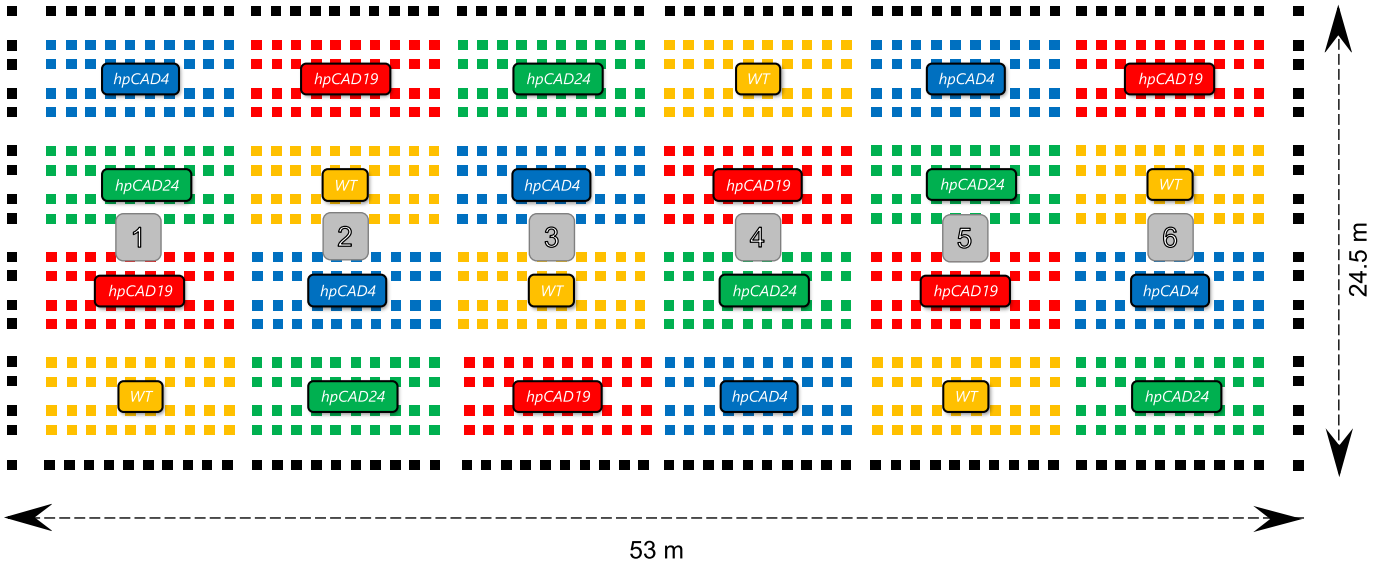


Fig. S1. Plan of the field trial consisting of six randomized blocks, each block containing 40 clonal replicates per line (WT, yellow; *hpCAD4*, blue; *hpCAD19*, red; *hpCAD24*, green). A border line of WT trees is indicated in black and republished from New Phytologist.

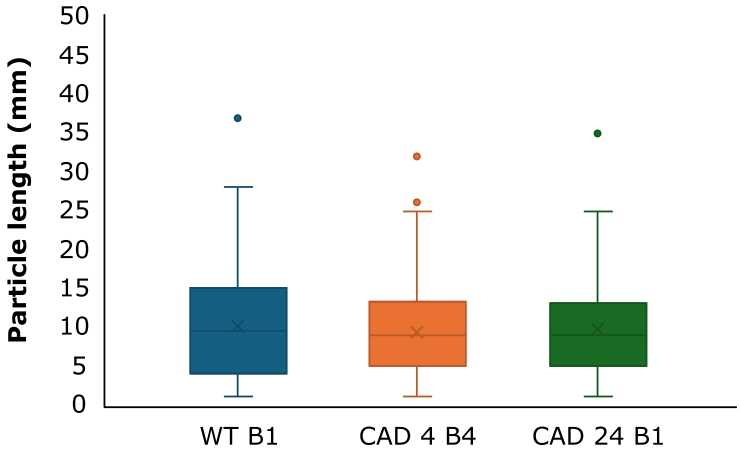


Fig. S2. Distribution of the stem biomass chip length. The length of all stem biomass chips longer than or equal to 1 mm in 10 g of the shown materials was measured (n = 186 for WT, n = 210 for CAD 4 B4, and n = 174 for CAD 24 B1).

Please cite this article as: Geerts J., Croes T., Leemans P.G.C., Richel A., Besserer A., Brosse N., De Meester B., Boerjan W., Cornet I. Improved sugar yield upon steam explosion and enzymatic saccharification of field-grown poplar downregulated for cinnamyl alcohol dehydrogenase 1. Biofuel Research Journal 50 (2026) 2698-2710. DOI: 10.18331/BRJ2026.13.2.4.

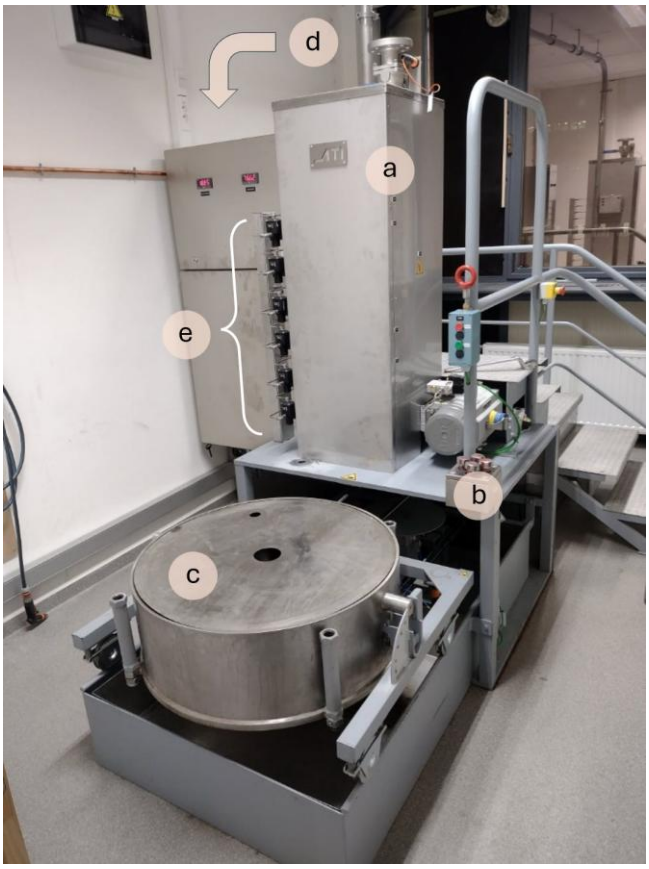


Fig. S3. Steam explosion equipment applied in this article: **(a)** Vertical reactor, covered by insulation, an electrical heating mantle, and the steam introduction equipment; **(b)** Explosion valve; **(c)** Retractable expansion vessel; **(d)** Steam generator (at the back, not visible), and **(e)** Steam introduction equipment.

Please cite this article as: Geerts J., Croes T., Leemans P.G.C., Richel A., Besserer A., Brosse N., De Meester B., Boerjan W., Cornet I. Improved sugar yield upon steam explosion and enzymatic saccharification of field-grown poplar downregulated for cinnamyl alcohol dehydrogenase 1. Biofuel Research Journal 50 (2026) 2698-2710. DOI: 10.18331/BRJ2026.13.2.4.

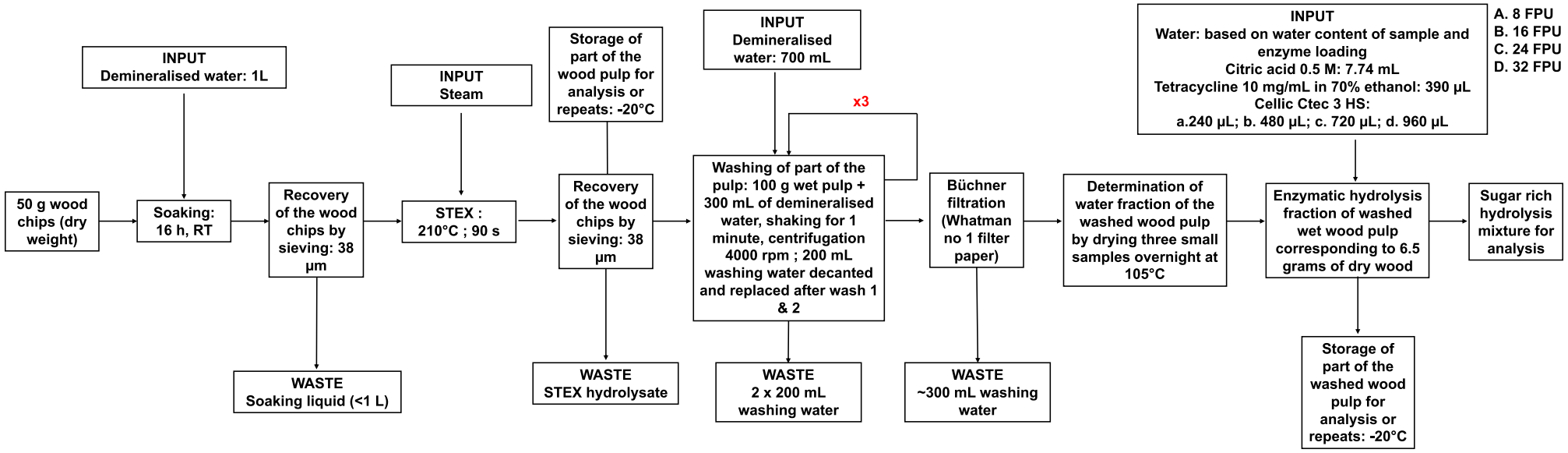


Fig. S4. Flowchart of the applied process conditions in the soaking – STEX – washing – enzymatic hydrolysis process.

Please cite this article as: Geerts J., Croes T., Leemans P.G.C., Richel A., Besserer A., Brosse N., De Meester B., Boerjan W., Cornet I. Improved sugar yield upon steam explosion and enzymatic saccharification of field-grown poplar downregulated for cinnamyl alcohol dehydrogenase 1. Biofuel Research Journal 50 (2026) 2698-2710. DOI: 10.18331/BRJ2026.13.2.4.

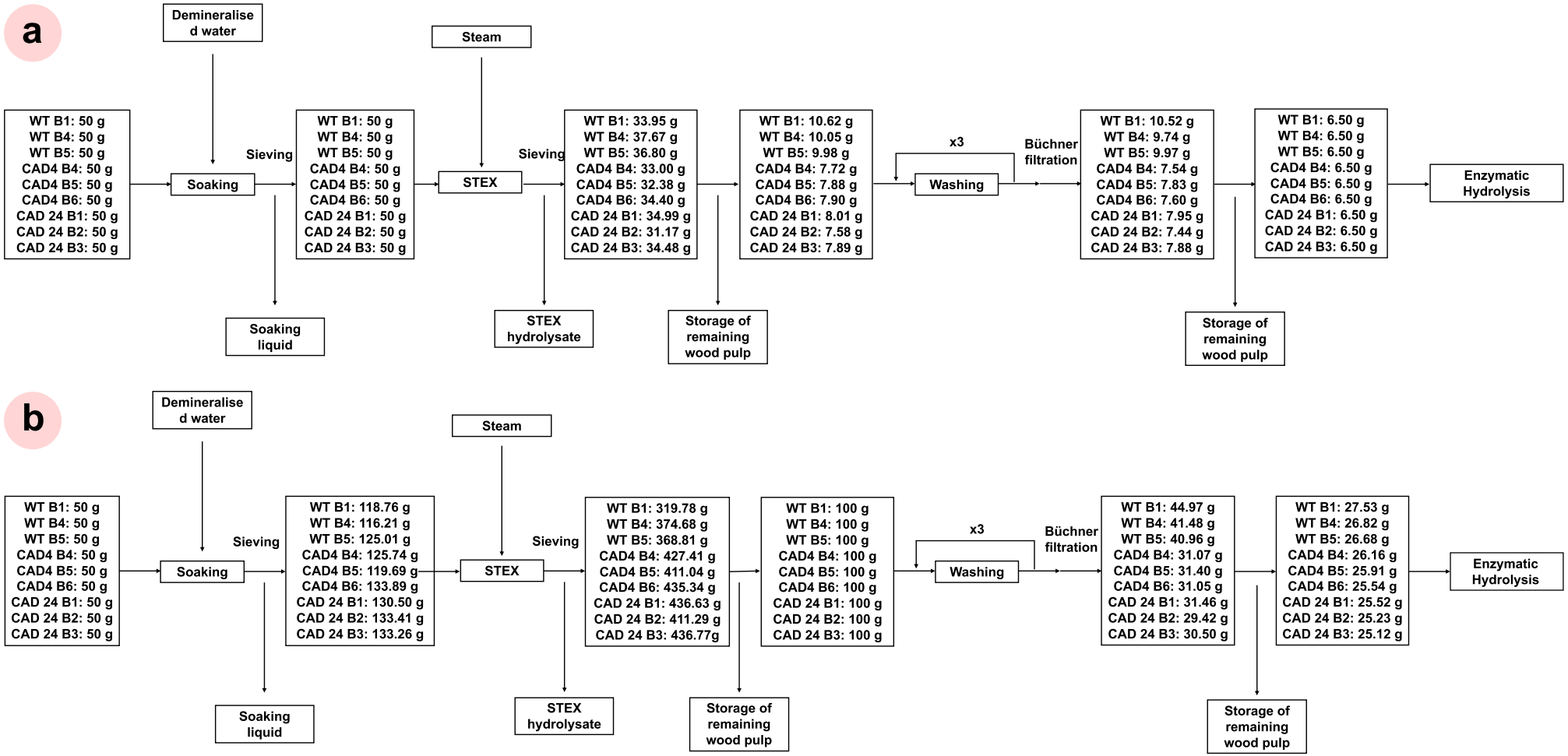


Fig. S5. Simplified process flowchart indicating the mass of each sample separately after every process step. **(a)** dry weight and **(b)** wet weight.

Please cite this article as: Geerts J., Croes T., Leemans P.G.C., Richel A., Besserer A., Brosse N., De Meester B., Boerjan W., Cornet I. Improved sugar yield upon steam explosion and enzymatic saccharification of field-grown poplar downregulated for cinnamyl alcohol dehydrogenase 1. Biofuel Research Journal 50 (2026) 2698-2710. DOI: 10.18331/BRJ2026.13.2.4.

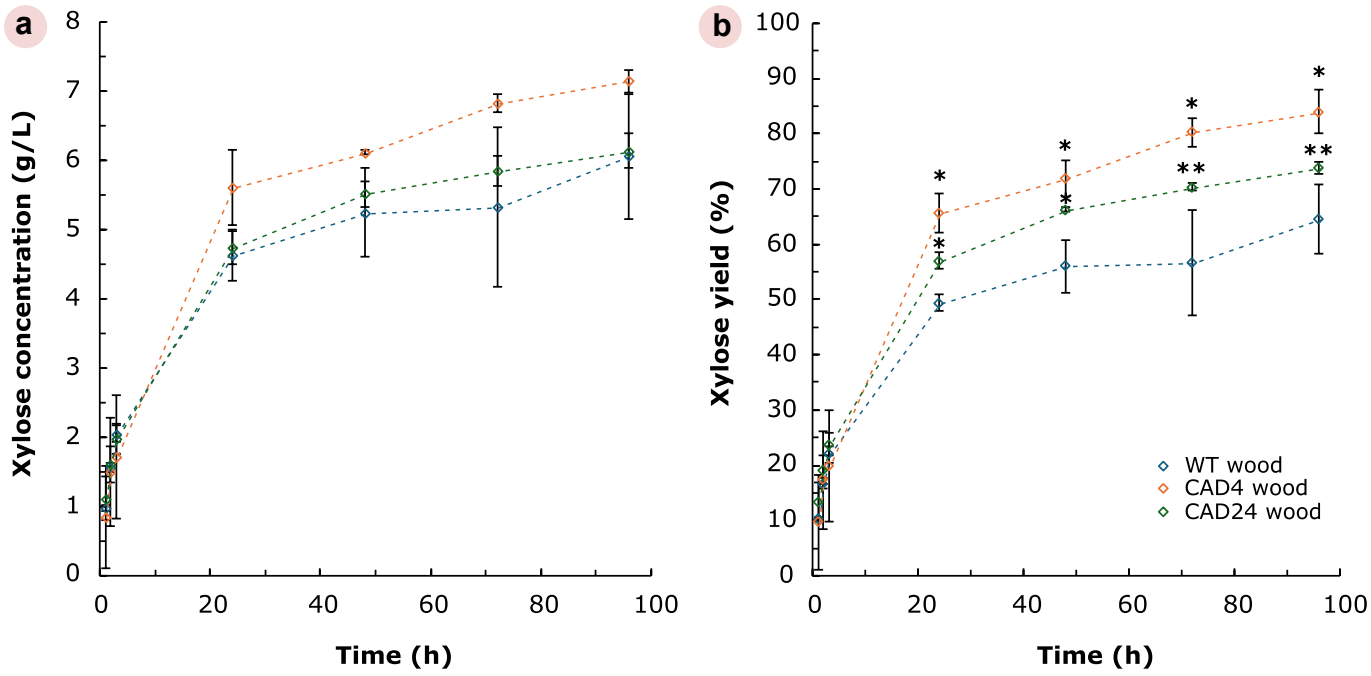


Fig. S6. Supplementary figure to Figure 2. **(a)** xylose concentration and **(b)** xylose yield during enzymatic hydrolysis of WT wood, CAD 4 wood, and CAD 24 wood. Error bars represent the standard deviation on three biological replicates. * represents a significant difference compared to the WT at α = 0.05. ** represents a significant difference at α = 0.10 as determined by an unpaired t-test. Cellic Ctec 3 HS was applied as the cellulase complex. The enzymatic hydrolysis was performed at 8.4% solid loading. The enzyme loading was 8 FPU/g (cellulase activity).

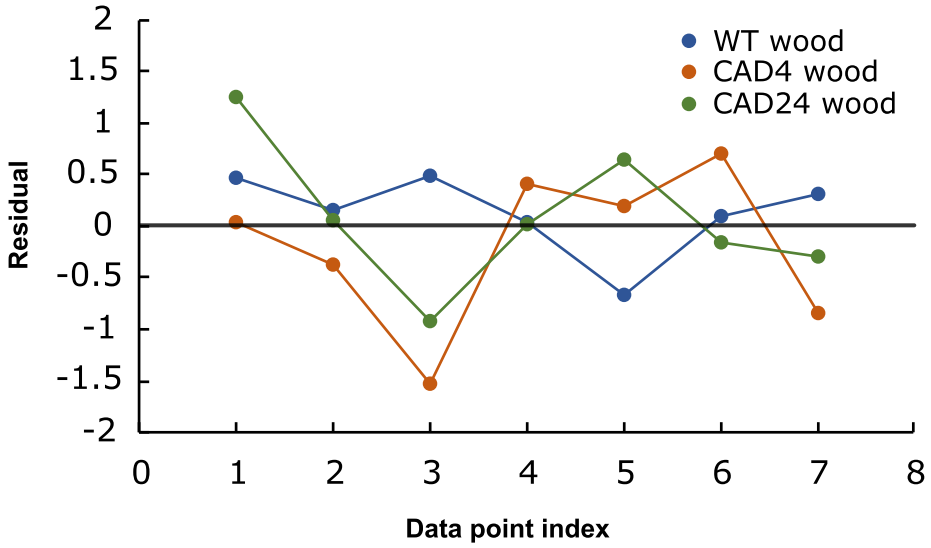


Fig. S7. Residual plot presenting the difference between experimental data and model data for the modified Holtzapple model as applied in Figures 2 and 3 in the main text. WT, CAD 4, and CAD 24. Data points represent the difference between the model values and experimental values (averages of three biological replicates) at 1, 2, 3, 24, 48, 72, and 96 h of hydrolysis, respectively.

Please cite this article as: Geerts J., Croes T., Leemans P.G.C., Richel A., Besserer A., Brosse N., De Meester B., Boerjan W., Cornet I. Improved sugar yield upon steam explosion and enzymatic saccharification of field-grown poplar downregulated for cinnamyl alcohol dehydrogenase 1. Biofuel Research Journal 50 (2026) 2698-2710. DOI: 10.18331/BRJ2026.13.2.4.

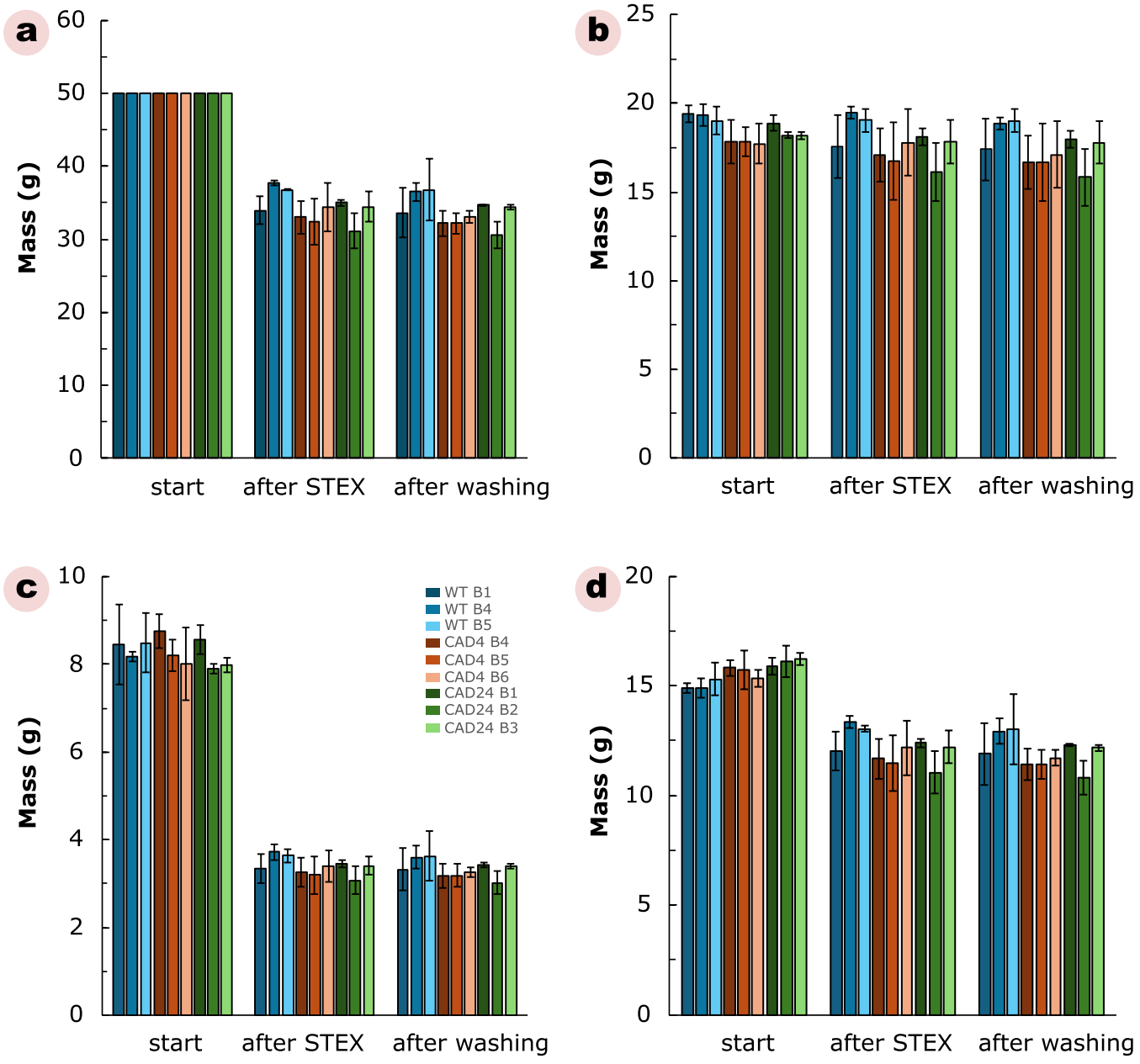


Fig. S8. Mass balances and composition analysis of **(a)** dry stem biomass, **(b)** cellulose, **(c)** hemicellulose, and **(d)** lignin. Error bars represent the standard deviation on three technical replicates. These are the same data as in Figure 1, but here the values of the individual biological replicates, corresponding to the different blocks in the field, are presented.

Please cite this article as: Geerts J., Croes T., Leemans P.G.C., Richel A., Besserer A., Brosse N., De Meester B., Boerjan W., Cornet I. Improved sugar yield upon steam explosion and enzymatic saccharification of field-grown poplar downregulated for cinnamyl alcohol dehydrogenase 1. Biofuel Research Journal 50 (2026) 2698-2710. DOI: 10.18331/BRJ2026.13.2.4.

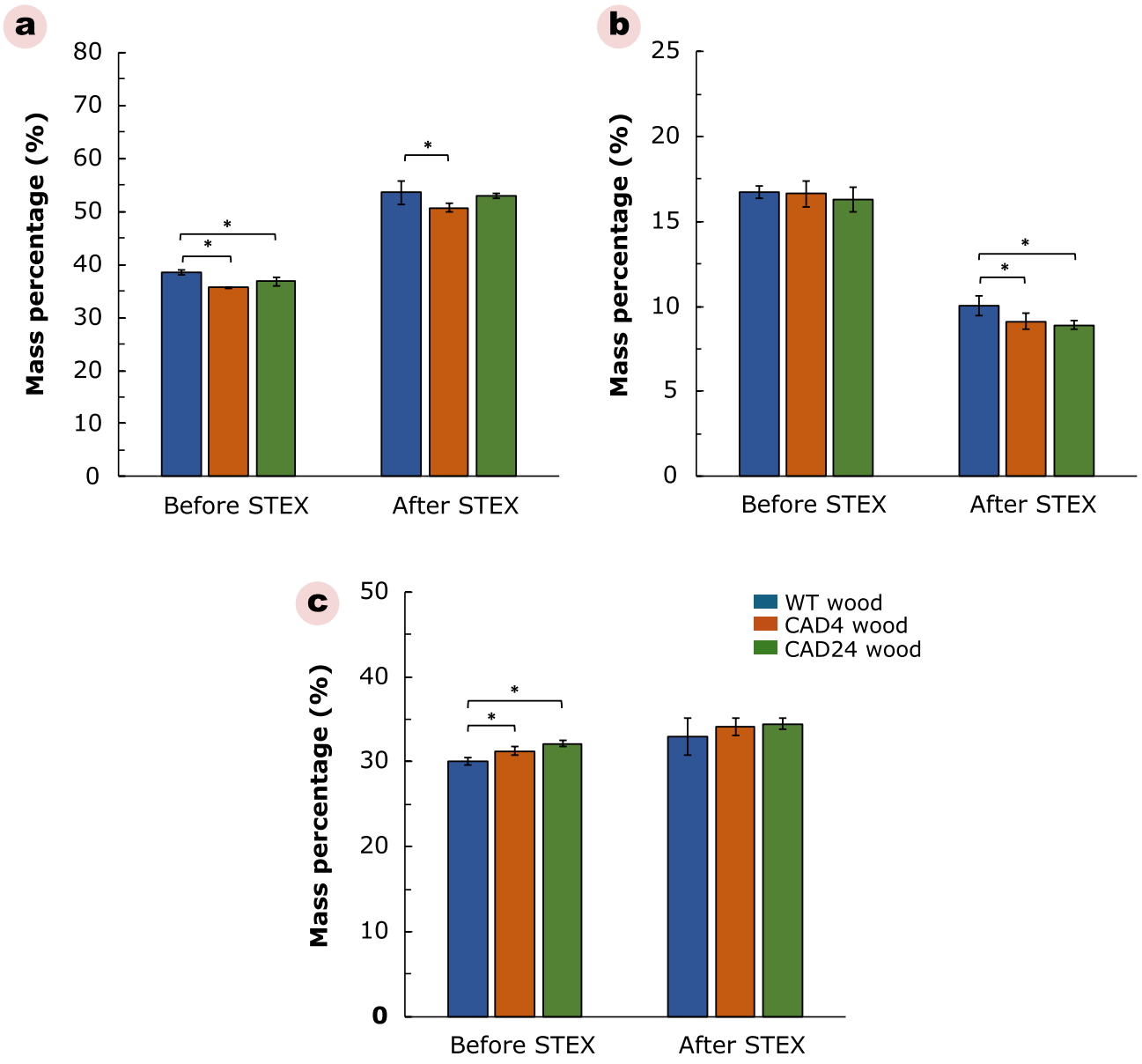


Fig. S9. Comparison of the composition of the stem biomasses before and after STEX. **(a)** cellulose, **(b)** hemicellulose, and **(c)** lignin. Error bars represent the standard deviation of the averaged data of three biological replicates. * indicates a significant difference as determined by unpaired T-test (α = 0.05). These are the same data as in Figure S10, but here the CAD stem biomasses are compared to the WT stem biomass.

Please cite this article as: Geerts J., Croes T., Leemans P.G.C., Richel A., Besserer A., Brosse N., De Meester B., Boerjan W., Cornet I. Improved sugar yield upon steam explosion and enzymatic saccharification of field-grown poplar downregulated for cinnamyl alcohol dehydrogenase 1. Biofuel Research Journal 50 (2026) 2698-2710. DOI: 10.18331/BRJ2026.13.2.4.

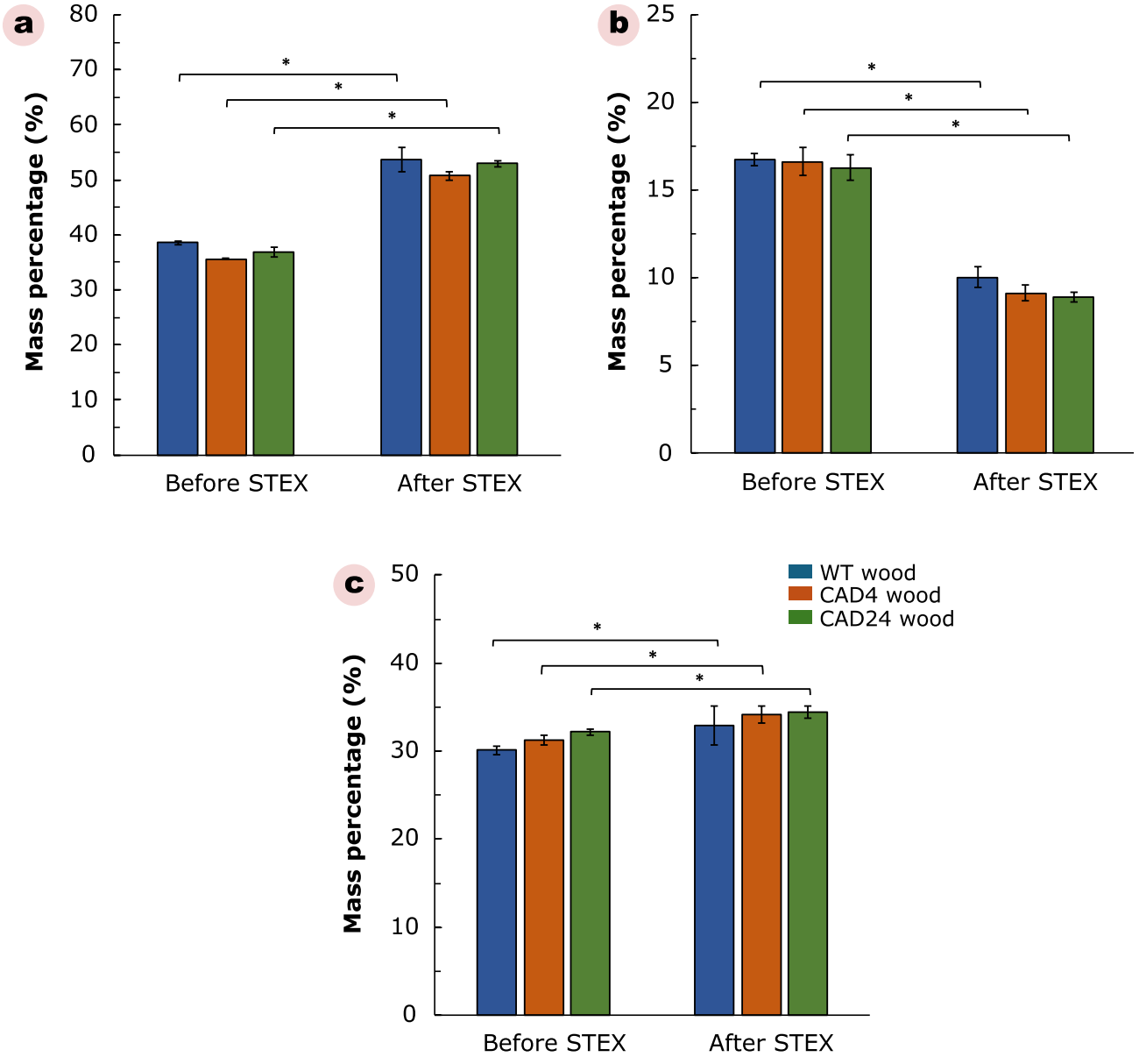


Fig. S10. Comparison of the composition of the stem biomasses before and after STEX. **(a)** cellulose, **(b)** hemicellulose, and **(c)** lignin. Error bars represent the standard deviation of the averaged data of three biological replicates. * indicates a significant difference as determined by unpaired T-test ($\alpha = 0.05$). These are the same data as in Figure S9, but here the effect of STEX pretreatment is compared for the corresponding stem biomasses.

Please cite this article as: Geerts J., Croes T., Leemans P.G.C., Richel A., Besserer A., Brosse N., De Meester B., Boerjan W., Cornet I. Improved sugar yield upon steam explosion and enzymatic saccharification of field-grown poplar downregulated for cinnamyl alcohol dehydrogenase 1. Biofuel Research Journal 50 (2026) 2698-2710. DOI: 10.18331/BRJ2026.13.2.4.

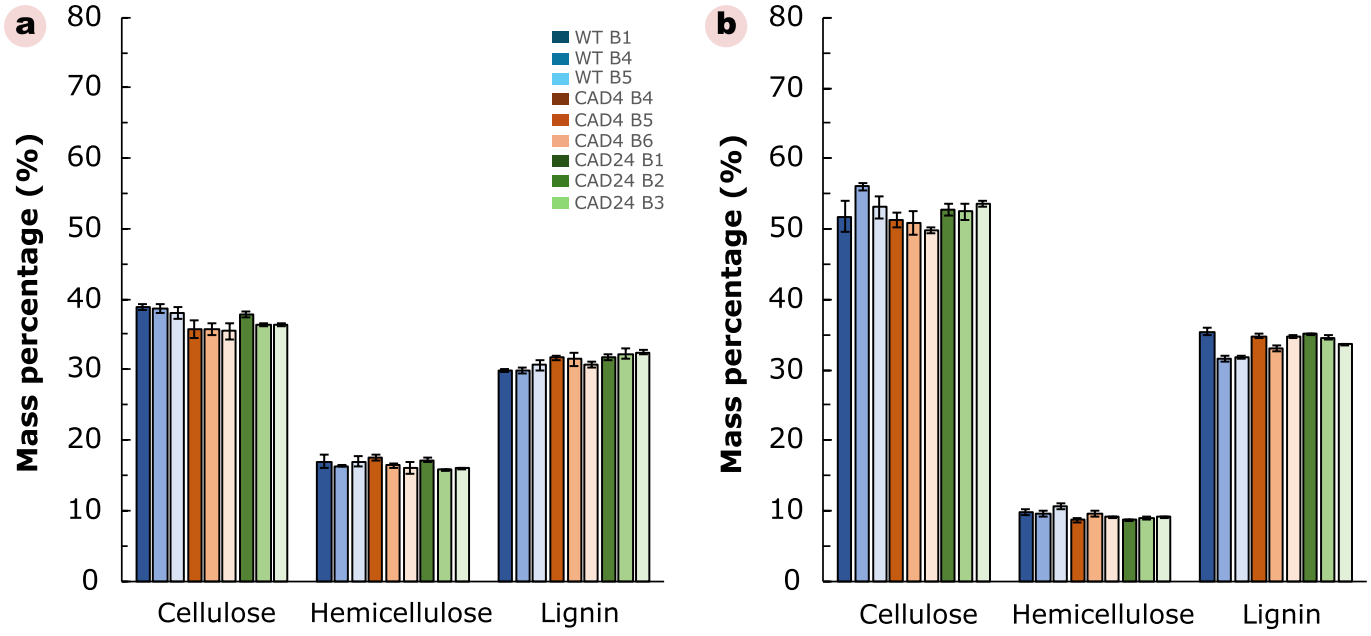
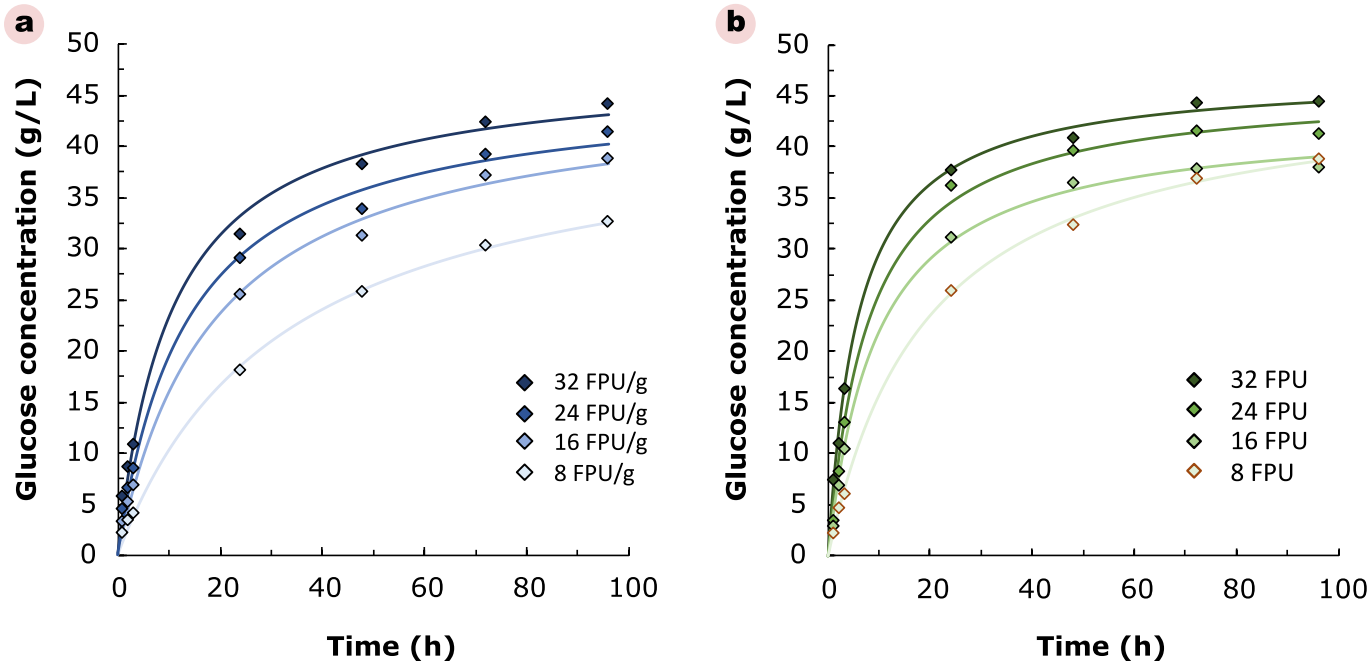


Fig. S11. Comparison of the composition of stem biomasses for each biological replicate. **(a)** Before STEX, and **(b)** after STEX. Error bars represent the standard deviation on three technical replicates. These are the same data as in Figures S8 and S9, but here the values of the individual biological replicates, corresponding to the different blocks in the field, are presented.



Fige. S12. Enzymatic hydrolysis of poplar wood; screening experiment. Glucose production over time during EH of STEX treated **(a)** WT wood, B1; and **(b)** CAD-line 24, B1. The fitted curve obtained by applying the Holtzapple model, as described in Equation 5, is presented. STEX conditions were 210°C – 90 s in all cases. Cellic Ctec 3 HS was applied as the cellulase complex. The enzymatic hydrolysis was performed at 8.4% solid loading.

Please cite this article as: Geerts J., Croes T., Leemans P.G.C., Richel A., Besserer A., Brosse N., De Meester B., Boerjan W., Cornet I. Improved sugar yield upon steam explosion and enzymatic saccharification of field-grown poplar downregulated for cinnamyl alcohol dehydrogenase 1. Biofuel Research Journal 50 (2026) 2698-2710. DOI: 10.18331/BRJ2026.13.2.4.

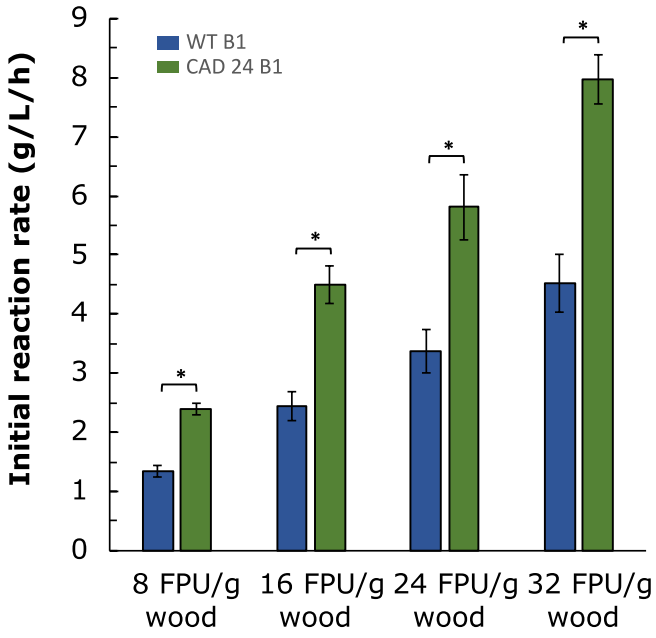


Fig. S13. Initial reaction rates during enzymatic hydrolysis of STEX-treated poplar wood in function of applied enzyme concentration as estimated by the modified Holtzapple model (**Eq. 5**): WT wood, B1; CAD-line 24, B1. Error bars represent the standard errors on the estimated parameter values. * indicate significant differences compared to the WT as determined by unpaired t-test (α = 0.05).

Please cite this article as: Geerts J., Croes T., Leemans P.G.C., Richel A., Besserer A., Brosse N., De Meester B., Boerjan W., Cornet I. Improved sugar yield upon steam explosion and enzymatic saccharification of field-grown poplar downregulated for cinnamyl alcohol dehydrogenase 1. Biofuel Research Journal 50 (2026) 2698-2710. DOI: 10.18331/BRJ2026.13.2.4.