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Facilitated delignification in CAD deficient transgenic poplar studied by confocal Raman spectroscopy imaging

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ABSTRACT

Lignocellulosic biomass represents the only renewable carbon resource which is available in sufficient amounts to be considered as an alternative for our fossil-based carbon economy. However, an efficient biochemical conversion of lignocellulosic feedstocks is hindered by the natural recalcitrance of the biomass as a result of a dense network of cellulose, hemicelluloses, and lignin. These polymeric interconnections make a pretreatment of the biomass necessary in order to enhance the susceptibility of the polysaccharides. Here, we report on a detailed analysis of the favourable influence of genetic engineering for two common delignification protocols for lignocellulosic biomass, namely acidic bleaching and soda pulping, on the example of CAD deficient poplar. The altered lignin structure of the transgenic poplar results in a significantly accelerated and more complete lignin removal at lower temperatures and shorter reaction times compared to wildtype poplar. To monitor the induced chemical and structural alterations at the tissue level, confocal Raman spectroscopy imaging, FT-IR spectroscopy, and X-ray diffraction were used.

1. Introduction

Questions regarding the expected depletion of fossil fuels and the associated increasing energy costs and shortages of the resources which are essential for the production of petrochemical products are of high industrial relevance in our technologized world [1]. Therefore, one of the major challenges of our current society is the development of seminal strategies for a reliable, affordable, and sustainable replacement of these fossil resources [1, 2]. Hence, alternatives based on renewable resources must be established, as potential solutions for the requested energy supply. Further, bio-refinery routes are promising strategies for an efficient production of fuel and could also provide resources for other petroleum-based products [2].

Influenced by the global debate of using agricultural products in biorefinery processes, policies were established to promote the use of lignocellulosic biomass from a broad variety of plants. Nevertheless, the close association of the three main components cellulose, hemicelluloses, and lignin in the cell walls results in difficulties of their separation and represents one of the main drawbacks for an efficient conversion in bio-refinery routes [3–5].

Thus, intense research efforts have been conducted to establish pretreatment technologies that allow for overcoming the intrinsic recalcitrance of the lignocellulosic biomass in order to separate these biopolymers and better access cellulose and hemicelluloses [6–8]. While various different physical, chemical, and biological processes for a separation of lignin and carbohydrate components (cellulose, hemicelluloses, and pectin) have been established by now, their application on the industrial scale is still expensive and energy inefficient, and a high chemical consumption is usually involved. A promising alternative is to profit from the emerging potential of genetic engineering allowing to intrinsically tailor the cell wall composition in perennial plants. A variety of publications report on alterations of the plant cell wall composition in terms of a reduction of the lignin content specifically its macromolecular assembly towards a lower chemical stability and the resulting facilitated removal [9–11].

The downregulation and inhibition of genes transcribing for enzymes, which are crucial for the lignin biosynthesis, were found to be especially effective to alter the lignin structure (e.g. crosslinking density and solubility) and quantity in the plant tissue [9, 12, 13]. These changes result in a lower recalcitrance of the lignocellulosic biomass, improving

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enzymatic digestibility and hydrolysis of lignin, and thereby render delignifying processes towards increased efficiency and lower environmental impact [12, 14–18].

Here we report on the influence of genetic engineering of poplar wood downregulated in CAD (*cinnamyl alcohol dehydrogenase*) activity [16–21] on acidic bleaching and alkaline pulping, which represent two common pretreatment protocols for wood delignification. The CAD enzyme plays a central role in the final steps of the monolignol biosynthesis and regulates content and composition of lignin in the cell wall of plants [12, 21–23]. In particular, an enhanced syringyl propane to guaiacyl propane (S/G) ratio, higher concentrations in aldehyde functionalities and free phenolic groups were determined [22]. Van Acker and coworkers could show for poplar plants with a downregulation of CAD1 by a hairpin-RNA-mediated silencing approach that the sinapaldehyde to coniferaldehyde ratio in lignin was severely changed, due to a strong increase in sinapaldehyde [21]. Such alterations in the molecular structure of the lignin component of plant cell walls potentially lead to enhanced susceptibility for pretreatment processes [7, 12, 21, 24]. The high stability in lignin modulation achieved for transgenic poplar by altered CAD expression and the high abundance of these plants make this species an ideal candidate for bio-refinery processes based on lignocellulosic biomass [10, 16–18, 22].

For a detailed comparative analysis of the changes in the macromolecular assembly of wildtype and CAD deficient poplar as a result of the two different pretreatment processes, confocal Raman spectroscopy imaging was used. X-ray diffraction and FT-IR spectroscopy were further applied to support the Raman microscopy based macromolecular investigation and provide chemical and structural details of the remaining carbohydrate constituents.

The different chemical mechanisms of these two typical pretreatments and the variation in process conditions render a direct comparison of these technologies of special interest.

2. Experimental

2.1. Samples

Transgenic and corresponding wildtype juvenile poplar (4 months) samples with an approximate stem diameter of 7 mm were provided by Wout Boerjan, Dept Plant Biotechnol & Bioinformat, University of Ghent, and Gilles Pilate and Annabelle Dejardin, AGPF, INRA, Orléans, who downregulated CAD (cinnamyl alcohol dehydrogenase) activity in the poplar wood by a hairpin-RNA-mediated silencing approach [21]. The stem was debarked, frozen in its green state and kept in this condition until the start of the experiments.

2.2. Sample Preparation

For light microscopy, confocal Raman microscopy, and FT-IR spectroscopy, cross-sections of the stem with a thickness of 20 μm were prepared with a rotary microtome (Leica, RM 2255 Germany). For X-ray diffraction measurements, 5 mm wide stem sections were cut with a Japanese saw and chopped up into a coarse-grained powder after the delignifying treatment.

2.3. Acidic Bleaching (H_2O_2/HAc)

A solution of hydrogen peroxide (30% analytical grade, Merck) and acetic acid (99.8%, Sigma-Aldrich) at a volumetric ratio of 1:1 was prepared prior to the treatment. A volume of 20 mL of solution was used for the treatment of 10 sections. The treatment was conducted for 0.5, 2, and 4 h at 40 °C and 80 °C and for 0.25, 0.75, 1, 1.5, and 3 h at 60 °C under slight stirring (200 rpm). After the reaction, the samples were washed with MilliQ water for a minimum of 7 days, during which the washing water was exchanged several times.

2.4. Alkaline Pretreatment

For the alkaline pretreatment, a stock solution of sodium hydroxide (10 w%) was prepared. A volume of 25 mL was used for the pretreatment of 10 sections. The treatment was conducted for 0.5, 2, and 4 h at 40 °C, 60 °C and 80 °C. After the reaction, the samples were washed with MilliQ water for a minimum of 7 days. The washing water was exchanged several times.

2.5. Raman Analysis

For confocal Raman microscopy, the treated thin sections were sealed in MilliQ water between a glass slide and a cover slip (thickness = 0.17 mm). All measurements were recorded in backscattering configuration with an inVia Raman microscope (Renishaw, UK) equipped with a motorized xyz-stage. A linearly polarized Nd:YAG laser ($\lambda = 532$ nm) and a 100× oil immersion objective with numerical aperture (NA) of 1.3 (Nikon) were used to maximize spatial resolution. The Raman signal was detected by an air-cooled charge coupled device (CCD) camera behind a spectrometer (inVia) with a spectral resolution of approximately 1 cm^{-1}. The mapping was recorded with an integration time of 0.2 s/0.4 s and a laser power of 23 mW in the spectral region between 300 and 1800 cm^{-1} and a step size of 300 nm.

2.6. Data Analysis

Data pretreatment, including cosmic ray removal and baseline correction, was performed in the software Wire 4.1 (Renishaw, UK). For baseline correction, the automatic intelligent background removal was used. The data was further processed in the Matlab based software Cytospec (version 2.00.01). Average spectra of defined areas from the secondary cell walls and the cell corners were extracted and min/max normalized on the cellulose band at 380 cm^{-1} (for Fig. 3).

2.7. FT-IR Measurement

FT-IR measurements were conducted on the same samples used for Raman spectroscopy measurements, utilizing a Bruker Tensor 27 FT-IR spectrometer equipped with a single bounce diamond ATR element (Platinum ATR). On each sample, three spectra were recorded in the range of 350–4000 cm^{-1} and averaged. A baseline correction was applied using the concave rubber band algorithm with 10 iterations and 64 baseline points in OPUS (Bruker, Germany).

2.8. X-ray Diffraction Measurement

X-ray diffraction (XRD) patterns were obtained using a PANalytical Empyrean diffractometer equipped with a PIXcel1D detector in Bragg-Brentano geometry. From a standard X-ray tube, Cu K_α radiation was generated at 45 kV and 40 mA operating conditions. Patterns were recorded in a $2\theta = 8–45°$ range.

3. Results and Discussion

3.1. Raman Analysis of WT and CAD Deficient Poplar Samples

In a first step, a spatially-resolved chemical analysis of the CAD deficient poplar (CAD) in comparison with the corresponding wildtype (WT) samples was performed using Raman spectroscopy imaging. Raman images were obtained by integration of the bands between 1525 and 1700 cm^{-1}, which are characteristic for vibrations of aromatic cell wall components (mainly lignin) (Fig. 1) [25]. The predominant peaks in this spectral region are assigned to the aromatic stretching vibration around 1600 cm^{-1} (1593 cm^{-1} for CAD and 1602 cm^{-1} for WT), and the band at 1656 cm^{-1} mainly attributed to C═C stretching vibrations of coniferyl alcohol and sinapyl derived structures and C═O