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# Unveiling 3D physicochemical changes of sugarcane bagasse during sequential acid/alkali pretreatments by synchrotron phase-contrast imaging

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ABSTRACT

Several pretreatment strategies focus on the removal of a significant part of lignin from plant cell walls, as it is considered the main barrier to the process of deconstructing biomass to simple sugars by hydrolytic enzymes. The ability to chemically differentiate and spatially locate lignins across cell walls provides an important contribution to the effort to improve these processes. Here, we present a novel approach using synchrotron phase-contrast tomography to probe the physicochemical features of plant cell walls. In addition to the 3D cellular architecture, the distribution of dense packed lignin–carbohydrate complexes (or, simply dense lignin) over larger regions and within cell walls has been successfully provided. We examined in particular the effect of the sequential $H_2SO_4$ and NaOH pretreatment on sugacane bagasse. Our results revealed that aggregates of dense lignin form elongated 3D structures in cell corners (CC) following the orientation of the sclerenchyma fibers; a remarkable result in the light of the conventional perception that lignin particles are discrete and roughly spherical. After the acid pretreatment, a considerable fraction of the dense lignin primarily located in CCs was removed; and it was further dissolved with the subsequent alkali treatment. Unexpectedly, the two-step pretreatment did not contribute to the cellulose accessibility in terms of available surface area and porosity. This study provides new insights into the underlying mechanisms of biomass deconstruction by pretreatments; and the approach established here can be extended to other systems relevant to the bioenergy and biotechnology arenas.

## 1. Introduction

Lignocellulosic biomass is potentially considered to be a renewable and environmentally friendly feedstock for sustainable production of biofuels to meet global concerns on greenhouse gas emissions and depletion of fossil fuels (Himmel et al., 2007; Ragauskas et al., 2006). To convert biomass into liquid fuels, sugars that are stored as high molecular weight polymers in plant cell walls must be deconstructed and released into solution as monosaccharides or short oligosaccharides (Alvira et al., 2010; Chang et al., 1981). Most technology for the conversion of lignocellulosic materials relies on biochemical processing that includes thermal chemical treatments (called pretreatments), enzymatic hydrolysis, and fermentation (Chang et al., 1981).

Plant cell walls are macrometer-sized in nature and their composition and organization vary significantly over the hierarchical structure of plants (tissue, cell-micro and nano-level) (DeMartini et al., 2013). At the macroscopic scale, each plant tissue impacts distinctly on the local transport of catalysts (chemical or enzymes), resulting thus in heterogeneous treatments zones (Viamajala et al., 2010). This problem extends to the micro- and nanometer scale due to the compositional and structural differences between cells types and cell wall layers. This complexity and the 'buried' and intermeshed carbohydrate polymers (mainly, lignin, hemicellulose, and cellulose) are another issue to be addressed during any biomass conversion regime (Simmons et al., 2016; Viamajala et al., 2010).

Among the cell wall polymers, lignin is generally regarded as the

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main barrier to efficient enzymatic saccharification process as it acts as a protective layer over cellulose in plant cell walls, inhibiting the accessibility to enzymes (Donohoe et al., 2008). Besides mass transport issues, lignin poses other processing challenges. It is well documented that the amount of lignin is unevenly distributed among the different plant tissues and cell wall layers (Zeng et al., 2015). Similarly, a recent study showed that the dissolution of lignin occurs at different rates across the various structures found in plant cells during pretreatments (Ji et al., 2016; Wang et al., 2017). For instance, the delignification process was more prominent in secondary walls than in cell corners and compound middle lamellae in eucalyptus (Wang et al., 2017). This phenomenon might be explained by the existence of preferential pathways for the catalyst penetration or variations in the chemical reactivity of lignin in the various subcellular structures (Studer et al., 2011; Wang et al., 2017; Zeng et al., 2015). The reactivity of lignin was previously associated with its concentration in the lignin-carbohydrate complex (Studer et al., 2011; Trajano et al., 2013; Zeng et al., 2015). The loosely packed lignin (or, dilute), localized in secondary walls, was easier to remove during pretreatments; whereas a significant amount of the dense lignin remained in cell corners and compound middle lamellae (Zeng et al., 2015).

Much of the recent progress has been achieved by using novel imaging techniques that can specifically locate polysaccharide and lignin in cell wall layers for tracing the kinetics of the biomass conversion process (Donohoe et al., 2008; Gierlinger, 2014; Gierlinger et al., 2012; Hinkle et al., 2015; Schubert, 2017; Wang et al., 2014; Zhang et al., 2013, 2016). Among these techniques, confocal Raman microscopy emerged as an important tool as molecular vibrations are monitored in context with the cell wall structure, revealing thus the organization of components within the native wall of single cells (Gierlinger et al., 2012). However, further studies on statistical significant part of samples, which includes the spatially heterogeneity in the physicochemical properties of walls at the tissue and cell levels, are crucial.

Accordingly, we monitored the delignification process in different plant tissues and cells types, contributing thus to bridge the gap between the nanometer scale approaches to the overall performance of pretreatments. For that, we applied phase-contrast tomography (PCT) for obtaining *in situ* three-dimensional (3D) information on the physicochemical features at the micrometer scale. This technique allowed not only the visualization of the morphological features, but it was extended here to an interpretation of the chemical contrast in biomass materials. Three-dimensional maps of packing density provided by PCT could reveal changes in the spatial distribution of lignin following pretreatments. The effect of a sequential acid/alkali pretreatment on sugarcane bagasse was investigated. This approach was free of interference from traditional staining, embedding, and processing chemicals that may alter the substrate.

## 2. Material and methods

### 2.1. Biomass source and pretreatments

Sugarcane bagasse was kindly provided by Usina São Francisco located in Sertãozinho, SP, Brazil. Acid hydrolysis was carried out in hydrolysis reactor (30 l) using $H_2SO_4$ (98%) as a catalyst in a ratio of 100 mg of $H_2SO_4$/g of dry material for 20 min at 120 °C.The hydrolysate was separated from solid material via filtration. Solid portion was washed and dried at 45 °C.

The substrate obtained after acid hydrolysis was pretreated by sodium hydroxide. It was carried out in a digestion block (P.H.D). Sodium hydroxide (1% m/v) was used as a catalyst in a ratio of 1/10 between the cellulignin mass and the volume of alkali solution, at 120 °C for 1 h. After the reaction, the solid material was recovered by filtration using muslin cloth. The recovered solid residue was washed and dried at 45 °C.

Enzymatic hydrolysis was performed in a 125 ml Erlenmeyer flask containing 3.0 g d.wt. of the acid–base pretreated sample and the control sample separately, and 40 ml of citrate buffer (50 mM, pH 4.8). The substrate, soaked in citrate buffer was implemented with cellulase loadings (20 FPU/g of the dry substrate from Dyadic) and surfactant (Tween 20) (0.10 g/g substrate). Enzymatic hydrolysis was performed at 50 °C and 150 rpm in an incubator shaker (Innova 4000; New Brunswick Scientific, Enfield, CT, USA) for 48 h. Samples were collected, centrifuged, and analyzed to determine the quantity of sugars released. The glucose yield was determined according to the equation presented by Lu et al. (2012):

$$\text{glucose yield}(\%) = \frac{G \cdot v \cdot 0.9}{m} \times 100 \quad (1)$$

where G is released glucose concentration (g/L); m is mass of sugarcane bagasse (g); v is volume of the solution used in the enzymatic hydrolysis (L); and 0.9 is cellulose to glucose conversion factor.

### 2.2. HPLC analysis

The content of glucose, xylose, arabinose and acetic acid were verified in chromatograph Shimadzu LC- 10AD (Kyoto, Japan) with a column equipped with Aminex HPX-87H (300 × 7.8 mm; Bio-Rad, Hercules, CA, USA), coupled to a refractive index detector (RID-6A). It used 0.01 N sulfuric acid as eluent at a flow rate of 0.6 ml/min, column temperature of 45 °C, and injected volume of 20 µl. Samples were previously filtered through a Sep-Pak C18 filter (Sigma Aldrich, USA). All the samples were filtered in membrane Minisart 0.22 µm (Sartorius, Epsom, UK) before the readings.

### 2.3. Chemical compositional characterization

The chemical characterization (cellulose, hemicellulose, lignin, extractives, and ash) of raw and pretreated biomass samples was carried out according to NREL/TP-510-42618 method (Sluiter et al., 2012). Ash and extractives were determined only for the raw biomass sample. Ethanol extraction was used to determine the extractive composition. This procedure was used, in conjunction with other procedures, to determine the summative mass closure for biomass feedstocks.

To achieve reasonable conclusions, oven drying, which results in significant deformation and collapse of the surfaces, was avoided. Therefore, we used an air-drying method described by Sluiter et al. (2012). This method is suitable for drying materials where ambient humidity allows the sample to air-dry to a moisture content below 10%. The values of this moisture were considered in the calculous for composition determination.

Acid hydrolysis was carried out for all samples by using diluted sulfuric acid (72%) in proportion of 1 g of dry material per 10 ml of solution in water bath for 1 h at 30 °C. After 1 h, hydrolysis was interrupted with adequate volume of distillated water and inserted in autoclave for 1 h at 121 °C (1 atm). Sample was filtered in porous crucible, and an aliquot of the liquid was dilute and analyzed in spectrophotometer (240 nm) for solubility lignin determining, according to Eq. (2):

$$\text{solubility lignin} = \frac{\frac{abs_{240} \times 2}{105}}{\text{dry weight of bagasse}} \times 100 \quad (2)$$

Another aliquot was separated and prepared for HPLC analysis, for cellulose and hemicellulose determination, according to Eqs. (3) and (4), respectively (Lu et al., 2012).

$$\text{cellulose} = \frac{\text{glucose} \times 0.9 \times 0.087}{\text{dry weigth of bagasse}} \times 100 \quad (3)$$