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Revealing the microfibrillar arrangement of the cell wall surface and the macromolecular effects of thermochemical pretreatment in sugarcane by atomic force microscopy

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ABSTRACT

The lignocellulosic biomass of plant cell wall is a source of sugar that can be used for second generation of bioethanol. The technology used for converting the fermentable sugar in the cell wall into bioethanol involves a pretreatment step that helps to decrease the recalcitrance of biomass to deconstruction. Atomic force microscopy (AFM), due to its ability to generate high resolution images with negligible sample preparation, is a powerful tool for studying biological samples on a nanometer scale. In this work, we elucidated the microfibrillar arrangement of the sugarcane cell wall and analyzed the effect of a thermochemical pretreatment at the nanoscale level using AFM images. Initially, we isolated the sugarcane cell wall to improve the AFM analysis. The cellulose microfibrils distribution in the sugarcane cell wall was clarified by AFM. Cellulose microfibrils were organized in a polylaminated structure with a distinct orientation. The measured filaments were approximately 20 nm thick, which corresponds to the size of cellulose microfibrils in other crops. Additionally, we investigated the effect of the thermochemical pretreatment on the cell wall structure. The thermochemical-pretreated cell wall had undergone loss of filaments and formation of globular structures, presumably containing lignin. The surface roughness was also dramatically changed. Our results provide insights that may help to understand the sugarcane cell wall organization and the mechanism by which thermochemical pretreatments modify the cell wall structure to improve its digestibility.

1. Introduction

As fossil fuels are now scarce and nonrenewable energy resources are becoming more expensive, there is an increasing demand for an alternative energy source. In terms of energy production, cost and environmental impact, biofuels would be a better alternative than fossil fuels. International research efforts strive to obtain a renewable fuel that is inexpensive and causes less pollution. As such, significant efforts have been made to use vegetal biomass for the production of liquid fuels, including bioethanol, which could be used to power transportation (Goldemberg, 2007).

The conversion of plant biomass (called lignocellulosic biomass), including woody crops, herbaceous plants, grasses, starch, sugar cane, and others, into bioethanol has emerged as potential renewable energy source to replace fossil fuels (Himmel et al., 2007; Jordan et al., 2012). A lignocellulosic biomass consists primarily of plant cell walls (PCW), which are mainly composed of biopolymers, such as cellulose, hemicellulose, and lignin. The PCW involves the entire cells and provides mechanical support, stress protection, and rigidity and support to plant cells (Keegstra, 2010). The PCW is divided into 3 layers: (1) the thinner non-lignified primary cell wall, (2) the thicker highly lignified secondary cell wall, and (3) the middle lamella that provides support to adjacent cells. The general architecture of the cell wall surface contains microfibrils embedded in an amorphous matrix, which results in a rigid and compact mesh-like structure. The microfibril is composed of cellulose and the amorphous material contains hemicellulose and lignin (Kaczkowski, 2003).

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Cellulose, the major constituent of the PCW, is a homopolymer arranged into crystalline and non-crystalline regions that are formed by β-(1,4)-linked D-glucoses (Perez and Mazeau, 2004). The cellulose is arranged into a fibril, which is surrounded by hemicellulose, an amorphous heteropolymer with low molecular weight compounds and a mixture of monosaccharides (glucose, mannose, xylose and arabinose) (Saha, 2003), and lignin, a polymer that provides the stiffness, impermeability, and mechanical strength of the plant tissues (Donaldson, 2001). Lignin is the key molecule responsible for the recalcitrance of the biomass (Grabber, 2005). Cellulose fibrils with a diameter of approximately 20 nm have been found in plant cell walls, as observed using the quick-freeze, deep-etch technique (McCann et al., 1990) and AFM (Kirby et al., 1996; Morris et al., 1997).

The physicochemical traits of the PCW make efficiently converting the biomass into sugars by enzymatic hydrolysis difficult (McCann et al., 1990). It has been postulated that the mechanical and functional properties of the cell wall provided by the molecular architecture cause the recalcitrance of the biomass to deconstruction (Xu et al., 2011). The high degree of compaction and complexity of the lignocellulosic biomass structure makes its conversion into fermentable sugars considerably more difficult. Therefore, producing ethanol from biomass is still costly and inefficient. However, adding a physical, chemical, physicochemical or biological pretreatment step before the enzymatic hydrolysis and sugar fermentation processes can improve the production of bioethanol. These pretreatments make the cellulose more accessible to the enzymes that convert the carbohydrates into fermentable sugars via the solubilization and separation of the lignin–hemicellulose–cellulose complex (Mosier et al., 2005; Agbor et al., 2011). Due to its chemical composition, plant anatomy and the variations in the PCW structure, there is no single pretreatment method employed in the production of biofuels from lignocellulosic biomass. In the PCW, micron-scale traits such as lignin concentration, cell wall thickness, and the cross-linkers between the cell wall polymers, as well as nanoscale traits such as the porosity of the cell wall matrix, the arrangement of the cellulose fibrils and crystallinity, all convey the resistance of the plant cell walls to deconstruction (Sarkar et al., 2009).

The vast structural diversity of cell walls found in different plants, as well as in the tissues and cells within the same plant that convey the diverse functions of the cell wall, influences the ability of cellulases to penetrate the cell wall to access cellulose (Donohoe et al., 2009). Thus, it is necessary to characterize the organization of the PCW and to determine how this organization relates to the ability of the biomass to be hydrolyzed. Elucidating the cell wall recalcitrance requires a detailed understanding of the cell wall structure, including the arrangement of the cellulose microfibrils. Microscopy techniques, including atomic force microscopy (AFM), have been widely applied to obtain detailed information of the organization and deconstruction of the PCW before and after pretreatments (reviewed by De Souza and Sant'Anna, 2012).

AFM is an important tool for studying biological samples due to its ability to image biological samples under atmospheric conditions, thus allowing measurements to be performed in their native or near-native state (Hansma, 2001; Müller et al., 2011). AFM is based on the physical interaction between the cantilever tip and the sample surface (Binnig et al., 1986). Adhesion forces between the tip and the cell surface molecules are detected during cantilever deflections, which allow one to understand the chemical properties of the sample surface. This technique has been used to provide structural information of the PCW at high resolution, including plant cell wall (Chundawat et al., 2011; Ding et al., 2012).

In this work, AFM was used to investigate the morphology of the cellulose microfibrils in the sugarcane parenchyma cell wall surface. Additionally, we analyzed surface changes after a thermochemical pretreatment using topographic analysis and surface roughness measurements. In addition, we obtained a fraction of the isolated sugarcane cell wall by cell fractionation to improve the AFM analysis.

2. Materials and methods

2.1. Plant material

Stems from the sugarcane cultivar CB45-3 were obtained from plantations at Campos dos Goytacazes, Rio de Janeiro State, Brazil.

2.2. Isolation of the parenchyma cell wall of sugarcane

The sugarcane parenchyma cell wall was isolated according to (McCann et al., 1990). Briefly, 20 g of the central region of sugarcane internodes, where parenchyma cells are predominant, were mixed in 20 mL of homogenization buffer (0.3 M sucrose, 0.1 M HEPES, 2 mM potassium metabisulfide and 10 mM calcium chloride) in a Sorvall Omni-Mixer at 4 °C. The residue was filtered on filter paper, and the paste-like material was transferred to a crucible and frozen in liquid nitrogen. Subsequently, three cycles of freezing and maceration were performed. The sample was transferred to a beaker with 30 mL of maceration buffer (50 mM sodium acetate (pH 5.5), 50 mM NaCl, and 30 mM ascorbic acid) and subjected to shaking for 15 min. The sample was subsequently decanted for 5 min, and the less dense material was re-filtered on filter paper while the thicker fibers were naturally sedimented. The material retained on the filter paper was again subjected to agitation, sedimentation and filtration to achieve a better purification of the sugarcane cell wall. The purity of the fraction was assessed by light microscopy.

2.3. Thermochemical pretreatment

The central region of sugarcane internodes were subjected to cell wall fractionation, as described elsewhere, to obtain purified parenchyma cell wall fragments. The obtained cell wall fragments were washed three times in distilled water and were treated with 2% sulfuric acid at 150 °C for 20 min. After extensive washing in water, the fragments were imaged by AFM as described below.

2.4. AFM image acquisition and analysis

Freshly prepared sugarcane parenchyma cell wall fragments before and after pretreatment were deposited onto freshly cleaved mica sheets. The excess water was removed using a flow of nitrogen to minimize possible artifacts generated by humidity and the air-drying procedure, and the samples were imaged immediately. All images were acquired using a Nanowizard JPK AFM (Berlin, Germany) operating in the dynamic mode under ambient conditions, with 46% relative humidity and using AC240 cantilevers (Veeco, Woodbury, NY) with a spring constant of 2 N/m and a resonance frequency of 70 kHz. The nominal tip radius was 10 nm. AFM image analysis ($n = 98$) was performed using the JPK Image Processing software. The diameter of the sugarcane cellulose microfibrils was measured using the intermittent contact mode. A histogram of the diameter of the cellulose microfibrils was generated, and the standard deviation was calculated.

The roughness measurements (RMS) were performed using the JPK Image Processing software. We used a non-parametric test, the Mann–Whitney test, with a 95% level of significance.

2.5. Ultrastructural cytochemistry

Sugarcane internodes were hand-cut with a razor blade into 2 mm × 2 mm blocks. The blocks were washed in acetone, in