Microscopy as a tool to follow deconstruction of lignocellulosic biomass

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Plant cell walls (PCW) are highly complex structures mainly composed of polysaccharides (cellulose and hemicelluloses) and lignin. Lignocellulosic biomass has been considered a potential source for second generation biofuel. The technology applied to convert fermentable sugars of cell walls into bioethanol involves a pretreatment to improve the digestibility of the biomass. The PCW molecular architecture remains unclear and it has been related to biomass recalcitrance to deconstruction. Microscopy techniques are required to visualize, measure and quantify plant cell wall features as a result of pretreatment. This review provides a brief overview on the feasibility of using confocal laser scanning, atomic force and electron (scanning and transmission) microscopy to follow as well as to evaluate the structural effects of pretreatments on the deconstruction of plant cell walls.

Keywords: Lignocellulosic biomass, Plant Cell wall, Atomic Force Microscopy, Electron Microscopy, Confocal Microscopy

1. Introduction

Microscopy is a powerful tool for understanding the structure and the function of different samples varying from tissues to macromolecules [1]. As such, microscopy approaches have been intensely employed to investigate plant morphology, including cell walls. Recently, microscopy has emerged as a promising tool to understand, through visualization, measurement and quantification, the structural deconstruction of plant cell walls after biological, chemical and/or physical treatments. Here, we will give a brief overview of the recent contributions of Laser Scanning Confocal Microscopy (LSCM), Electron Microscopy (EM) and Atomic Force Microscopy (AFM) to the field of Biotechnology of plant-based biofuels, focusing on second generation bioethanol.

Biofuels are renewable fuels produced from biomass. The sources of biomass, which include woody crops, herbaceous plants, grasses, starch, sugar, among others, are considered a viable energy source as they form a sustainable basis to satisfy socio-economic concerns, providing greater security for energy supply and reducing the environmental impacts associated with fossil fuels [2]. Ethanol is derived from the fermentation of sucrose and simple sugars. These molecules are also found in the lignocellulosic material produced by plants [3]. Therefore, plant cell wall lignocellulosic biomass is considered a potential source of sugars for ethanol production, thus making this process a strong competitor to gasoline fuel.

1.2. Plant cell wall structure, recalcitrance and pretreatment

The plant cell wall is a dynamic rigid structure that surrounds the whole cell surface and is morphologically organized in primary wall, secondary wall (S1, S2 and S3) and the middle lamella [4]. Cell walls are rich-biomaterials, which are highly complex structures, mainly composed of polysaccharides (cellulose and hemicellulose) and lignin. Cellulose fibrils are surrounded by hemicellulose and lignin, forming clusters, which when connected to each other give rise to a highly compact 3D mesh-like structure. Cellulose, the major component of cell walls, is a homopolymer arranged in microfibrils that comprise 50-60% of the biomass, providing rigidity and support to plant cells. This molecule has a very orderly crystalline organization and a less orderly amorphous non-crystalline region. Cellulose is resistant to degradation due to its insolubility in water, and its crystallinity, as well as its combination with lignin and hemicellulose. Hemicellulose is an amorphous water-soluble heteropolymer with low molecular weight compounds and a mixture of monosaccharides, such as glucose, mannose, xylose and arabinose [5]. Lignin is an amorphous polymer associated with cellulose, which plays a role in the stiffness, impermeability, as well as mechanical strength of the plant tissues [6]. Lignin is the main obstacle preventing the conversion of biomass to sugars by the enzymatic processes.

Although the chemical composition of plant cell walls is well-known, the structural information needs to be defined in more detail. Therefore, there is a high demand for microscopy. The structural organization of plant cell wall lignocellulosic biomass has been determined by microscopy approaches and it is fully related to recalcitrance and deconstruction of biomass. Cell wall recalcitrance is determined by multiple macro, micro and nanoscale traits. The limiting factors that affect biomass enzymatic hydrolysis at the macro-scale include plant anatomy, cell type location within the plant tissue and chemical composition. At the micro-scale, factors such as the amount and localization of lignin, the cell wall thickness, and the cellulose, hemicellulose and lignin cross-links contribute to recalcitrance. At the
nanoscale, the limited cell wall matrix porosity, and the length, extent and crystallinity of the cellulose fibrils all impair cellulase penetration and accessibility to the cellulose, thus, contributing to biomass recalcitrance [7-9].

The high degree of compaction and complexity of the lignocellulosic biomass structure make its transformation into fermentable sugars much more difficult [10]. For this reason, several pretreatment strategies have been developed. The pretreatment, which is essential for ethanol fuel production, solubilizes and separates the cellulose-lignin-hemicellulose complex, making the resulting biomass available for subsequent chemical and biological treatments; in other words, making the cellulose more accessible in a system where enzymatic hydrolysis is efficient [11]. An effective method for pretreatment increases the cellulose accessibility and the complete solubilization of the polymer into sugar monomers without forming products that inhibit degradation and fermentation. The choice of pretreatment has a direct effect on cost and efficiency of hydrolysis and fermentation. A single pretreatment may not be enough due to the diversity of natural biomass. Thus, physical, chemical and biological treatments, or their combination have been developed for efficient hydrolytic processes [12]. The yield is related to the biomass source and the chosen pretreatment.

1.3. Contribution of microscopy to investigate cell wall structures and deconstruction

1.3.1. Confocal Laser Scanning Microscopy (CLSM)

In comparison with conventional widefield fluorescence microscopy, Confocal Laser Scanning Microscopy (CLSM) has the advantage of higher resolution, due to the removal of out-of-focus light, sensitivity, as well as the capability to produce serial optical sections of bulk fluorescent samples, including plant tissues, followed by three-dimensional imaging analysis. Furthermore, reduction of the fluorescence background and improved signal-to-noise are achieved, leading to higher contrast and well-defined structures [13]. In the case of plant tissues, they have the particularity of being composed in many cases of large, thick cells. Hence, CLSM is an excellent method to investigate the internal tissue structures in 3D.

Optical sectioning images of plant tissue succeeded in determining and measuring the plant structure before and after pretreatment [14-16]. Travis and co-workers [14] proposed the confocal microscopy as a valuable method to assess the in situ enzyme degradability of forage cell walls by combining 3-D image reconstruction and quantitative measurements. Tridimensional images of FITC stained samples clearly showed the effect of cellulase treatment on the anatomical features of the plant. Examples of the effects on barley include the disappearance of the vascular bundle phloem and, in maize, include the complete degradation of the epidermal cell wall, decrease of the sclerenchyma cell wall thickness and collapse of parenchyma walls. In addition, a detailed 3D measurement of the perimeter, area and wall thickness from a series of 2D section images of enzyme treated epidermis, parenchyma and sclerenchyma cell walls was performed on the cross-sections of maize internodes. Recently, the maximum intensity projection of confocal microscopy images of corn stover cross-sections treated with ammonia fiber expansion (AFEX) were used to measure cell wall perimeters, areas and isoperimetric quotients [16]. In these works, the differential site-specific degradability of plant biomass structures after enzymatic or thermo-chemical pretreatment was evident.

Fluorophores have been designed to selectively determine the localization of target molecules, such as cellulose and lignin in plants. Calcofluor and acriflavine are examples of fluorescent stains used to investigate the localization of cellulose and lignin, respectively [17, 18]. The intrinsic autofluorescence can also be used, in plant material, to localize lignin, since it has a specific autofluorescence emission at 530 nm [6]. Bond and co-workers [15] reported the localization of cellulose and lignin in different types of wood cell walls through the confocal microscopy images of safranine stained samples. Safranine incubation has the advantage of determining cellulose and lignin signals simultaneously, with no need to counterstain. At 488 nm excitation, safranine produces green and red signals for cellulose and lignin-rich regions, respectively (Fig. 1). The cellulose and lignin signals were quantified using this method. Cell corner and middle lamella were highly lignified, while the cellulose was more concentrated in the secondary cell walls. Furthermore, the degradation profile of the white rot fungus was examined and it was clear that confocal microscopy has sufficient resolution to show that the inner S2 secondary cell wall layer presented the highest level of cellulose degradation. To address chemical changes in sugarcane cell walls as a consequence of the thermo-chemical pretreatment, we imaged the safranine fluorescence signal intensity in cell walls (green signal) and lignin (red signal). In comparison with untreated cell walls (Fig. 1b), the lignin signal appeared to decrease after pretreatment both in middle lamella and cell corner (Fig. 1c). Also, a slighter reduction in the secondary cell wall green signal was found. These findings suggest that lignin was the main target of the thermo-chemical pretreatment.

Biomass pretreatment depends on various factors such as temperature, pH, and pressure, giving rise to a large variability of pretreatment methods. CLSM provides means to accurate image and to extensively quantify cell wall degradability, as a result of pretreatment in site-specific cell wall types in different plant tissues, in a simple and fast way. These characteristics are essential to carry out a large-scale screening of the effectiveness of different pretreatment methods with different plant biomasses.
1.3.2. Scanning and transmission electron microscopy

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) have been extensively used to follow, at high resolution, the structural changes in cell walls after biomass pretreatment. SEM is the method of choice to describe anatomical features and degradation at cellular and nano-resolution of biomass surfaces. SEM was used to investigate the alkaline peroxide pretreatment effect on corn stover cell walls [19]. This pretreatment decreased the lignin content in the cell wall that, in turn, changed the surface texture, which became rougher than non-treated samples. Rezende et al. [20] used SEM to examine the effects of acid and alkaline pretreatment of sugarcane bagasse. This approach readily showed a loss of pith and exposure of fiber strips in the acid treated samples. In NaOH treated bagasse, detachment of fibers, cell wall collapse and porous formation on cell wall surfaces were seen. In this later treatment, the morphological abnormalities were more evident at higher concentrations and may be a consequence of lignin removal. When SO2 or CO2 were used as impregnating agents [21], the disorganization in sugarcane bagasse was also visualized and in this case the most pronounced effect was the exposure of the fiber [22]. At the nano-scale, SEM images showed several lignin-rich particles on the surface of corn stover cell walls, as a result of diluted acid treatment [23]. These particles were rounded with smooth surfaces and had diameters varying from 5 nm to 10µm. Our group found similar results using the same pretreatment with sugarcane (Figs. 2).

A wide range of TEM techniques have also been applied to investigate the ultrastructural properties of plant cell walls, such as conventional ultra-thin sections [24, 25], rapid-freezing followed by deep etching [26], ultrastructural cytochemistry [27], immunogold [9] and electron tomography [28]. TEM images of ultra-thin sections of untreated biomass readily distinguished the typical cell wall layers: primary cell wall (PCW), secondary cell wall (SCW) and middle lamella (ML). These structures are bonded strongly together, giving rise to the typical dense architecture of cell walls. Potassium permanganate staining has proved to be a reliable technique to track lignin in different cell wall types [23, 29, 30]. This technique has been applied especially to investigate lignification during cell wall differentiation [30] and in the delignification process as a function of pretreatment [16, 23]. In sugarcane, KMnO4 staining in ultra-thin sections provided the clue of the lignin in the extruding droplets from the cell wall, after thermo-chemical treatment.
observed in maize [23] and in sugarcane (Fig 3a, b). Chemical and EM analyses (KMnO4 staining and Immuno-SEM) revealed that these droplets in maize are lignin-rich structures appearing after lignin coalescence in the inner region of the cell wall. These particles are believed to move within the cell wall and part of them redeposit onto the cell wall surface, a factor that increases the enzymatic digestibility [23]. TEM images of sugarcane bagasse showed that CO2 and SO2 partially solubilized the cell wall matrix components (hemicelluloses and lignin) leading to increased cell wall porosity due to the formation of irregular pores in the outer secondary cell wall region (Fig. 3c). A similar ultrastructural effect on the cell wall was recently reported by Chundawat and co-workers [16] in corn stover after ammonia fiber expansion (AFEX) treatment. This strategy showed that the cellulose hydrolysis increased about 5-fold, if compared with untreated samples. The three-dimensional architecture of plant cell wall has been determined by means of dual-axis electron tomography in resin embedded samples. This advanced approach provided a resolution of about 2 nm through topographic slices and the individual cellulose fibril diameter (3.2 nm), in the inner region of the S2 layer of Radiata pine secondary cell wall, could be seen [28]. One highlight of this study was the definition of the 3-D conformation of the main cell wall components (cellulose, hemicelluloses and lignin), which have a core of cellulose microfibrils surrounded by amorphous hemicelluloses and peripheral lignin. The remarkable resolution of tomography-EM slice was used to minutely describe the lignin droplet morphology in KMnO4 stained sections of thermo-chemical treated corn stover [23]. Dark staining showed the round and disc-like shaped lignin structures in the cell wall region called “delamination zone”, which is formed after treatment. The pioneering study using dual-axis electron tomography readily showed the 3D ultrastructural modification of the maize cell wall, after AFEX treatment [16], which may enhance cellulose conversion by cellulases. A 3D pore network formed after pretreatment was observed for the first time within the cell wall of the plant.

The use of cellulase producing microorganisms is considered an attractive model for the polysaccharide bioconversion to ethanol [31]. TEM images have been used to follow the biological degradability of plant cell walls by rumen microorganisms, especially bacteria [32]. The bacteria were seen attached to the cell wall and were able to degrade the cell wall by cellulase attack. Plant cell wall degrading enzymes are cell-free exocellulase, endocellulase, and β-glucosidase which show biochemical synergism on cellulose, as well as, the macromolecular machine named cellulosome (reviewed by [33]). Cellulosomes are multi-enzyme complexes, mostly described in cellulolytic anaerobic bacteria and in fungi that enzymatically breakdown cellulose into cellobiose and glucose [34, 35]. Cellulosome morphology has been intensely examined by TEM [36]. The cellulosomes are seen on the cell surface as a multi-enzyme protruberant structure, bound together via fibrous anchoring proteins. After detaching, celluloses are seen bound to the target cell walls. Microbial secreted free cellulase penetration into the cell wall, at the nano-scale, is limited by the wall matrix porosity and the cellulose cross-link with hemicellulose and lignin. Donohoe et al. [9] demonstrated by immune-TEM in thermo-chemical pretreated corn stover, the mechanism of cellulose accessibility by cellulolytic enzymes. According to the authors, TEM images revealed three structural changes as a consequence of the treatment: (i) cell lumen collapse, (ii) de-lamination of secondary cell wall and (iii) loss of density. Imaging the cellulose penetration after different acid treatments showed that the more dramatic treatment, more severe are the cell wall architectural modifications and, hence, the enzymes penetrate deeper in the cell wall matrix.

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Fig. 3 – Fig. 3a – TEM image of untreated sugarcane bagasse; Fig. 3b – thermo-chemical treated bagasse stained with KMnO4 to determine the localization of lignin. Several dark stained lignin droplets are observed extruding from cell wall (arrows). Fig. 3c – Pore formation in sugarcane bagasse after CO2 treatment (asterisk).
1.3.3. Atomic Force Microscopy

Filament organization of cell walls in native biomass has often been imaged by the fast-freeze deep-etch technique [26], field-emission scanning electron microscopy (FESEM) [37], and atomic force microscopy (AFM). AFM is a versatile powerful tool to study topographic, physical and chemical properties of biological samples at nanometer scale near to the native state [38]. Furthermore, AFM is able to generate high resolution 3D images with negligible sample preparation, avoiding fixation, dehydration or metal coating, as the electron microscopy methods do. Kirby et al [39] compared the arrangement of the individual cellulose fibrils in parenchyma cell walls of different crops (apple, water chestnut, potato and carrot) by AFM. Our group imaged sugarcane cell wall from parenchymal tissue (Fig. 4a). Topography and error signal AFM images clearly resolved single cellulose chains with thickness around 25 nm. Clear evidence of a multi-layered structure with different patterns of fiber organizations was acquired. Comprehensive analysis of AFM images evidenced that cellulose fiber orientation differs according to the plant cell wall investigated. For more detailed information about the use of AFM for cell wall morphology see [40]. Combined chemical extraction of cell wall molecules associated with AFM imaging has contributed to identify the role and the localization of the wall components [41].

The crossed structure of cellulose filaments provides high axial stiffness and, therefore, directly contributes to cell wall integrity related to its level of degradability. AFM phase images has the ability to map hydrophilic and hydrophobic regions, due to the physical property of the AFM tip, which adheres more strongly in hydrophilic regions. By using this approach, Chundawat et al., [16] demonstrated that native corn stover cell walls are essentially hydrophobic and, after AFEX treatment, amorphous hydrophilic deposits were found, most likely through removing the cell wall extractables. Furthermore, another change on the surface of the cell wall after AFEX treatment was the increased roughness of the cell wall, as calculated by RMS roughness factors from AFM amplitude images.

We took advantage of AFM to visualize the morphological changes that took place in sugarcane cell wall cellulose filaments after thermo-chemical pretreatment. Following the protocol established by McCann et al. [26], we produced cell wall fragments in order to improve the AFM analysis. Thermo-chemical pretreated cell walls underwent considerable loss of filaments, affecting the cell wall integrity by disruption of all linkages among cellulose, hemicellulose, and lignin (Fig. 4b). Striking deconstruction of cell wall components was also observed in corn stover after phosphoric acid treatment [42]. The authors showed that after pretreatment, no fibril structures were observed.

![AFM image of cell wall filament of untreated (Fig. 4a) and thermo-chemical treated (Fig. 4b) sugarcane cell wall. After treatment the severe loss of filament organization is observed.](image)

**Conclusions**

While the chemical composition of biomass is well-known the cell wall organization, assembly, and interactions of their macromolecules need to be better defined. In the field of technological research, efforts have been made to search for optimal methods to identify, evaluate, and demonstrate the efficiency of enzymatic hydrolysis processes of biomass after pretreatment. An effective method of pretreatment is one that increases the accessibility to cellulose and increases the solubility of the polymers, in order to deliver sugar monomers without forming products that inhibit fermentation and degradation. The choice of a pretreatment has a direct effect on cost and efficiency of hydrolysis and fermentation. While the pretreatment effect on the biomass cell wall has been widely investigated by chemical analysis, the effect on...
the molecular organization and the influence of the cell wall structure on these processes are still missing. A wide range of techniques are currently employed to accurately determine the cellular and molecular level effects of a pretreatment on the biomass plant cell wall. These include X-ray diffraction, NMR spectroscopy and microscopy. Combining different microscopy approaches, such as CLSM, TEM, SEM and AFM, is required to understanding, in detail, how structural changes in the cell wall, caused by biomass pretreatment, enhance the cellulose accessibility, which is crucial in terms of research and technology for biofuel production.

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