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Fourier transform infrared imaging and microscopy studies of *Pinus radiata* pulps regarding the simultaneous saccharification and fermentation process

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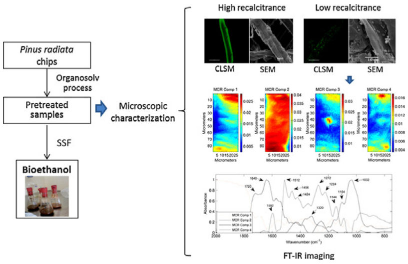
GRAPHICAL ABSTRACT



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ABSTRACT

The distribution and chemical patterns of lignocellulosic components at microscopic scale and their effect on the simultaneous saccharification and fermentation process (SSF) in the production of bioethanol from *Pinus radiata* pulps were analyzed by the application of diverse microscopical techniques, including scanning electronic microscopy (SEM), confocal laser scanning microscopy (CLSM) and attenuated total reflectance (ATR) – Fourier transform infrared microspectroscopy. This last technique was accompanied with multivariate methods, including principal component analysis (PCA) and multivariate curve resolution with alternating least squares (MCR-ALS) to evaluate the distribution patterns and to generate pure spectra of the lignocellulosic components of fibers. The results indicate that the information obtained by the techniques is complementary (ultrastructure, confocality and chemical characterization) and that the distribution of components affects the SSF yield, identifying lignin coalescence droplets as a characteristic factor to increase the SSF yield. Therefore, multivariate analysis of the infrared spectra enabled the *in situ* identification of the cellulose, lignin and lignin-carbohydrates arrangements. These techniques could be used to investigate the lignocellulosic components distribution and consequently their recalcitrance in many applications where minimal sample manipulation and microscale chemical information is required.

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1. Introduction

Lignocellulosic biomass has been investigated as sustainable raw materials for the production of different biomaterials and biofuels. Wood has a complex arrangement formed principally by cellulose, lignin and hemicelluloses. Organosolv process is a chemical pretreatment based on delignification with varying degrees of hemicellulose removal, used to improve enzymatic hydrolysis (EH) and the simultaneous saccharification and fermentation process (SSF) of wood substrates for bioethanol production [1]. EH and SSF are affected by chemical and physical properties of substrates including lignin, hemicelluloses and acetyl groups content, as well as cellulose crystallinity [2], polymerization degree, specific surface area [3,4], pore volume [5], particle size [6,7], among others. Several studies show that the lignin content negatively affects the enzymatic digestibility [4,8–12], possibly acting as a physical barrier to restrict the access of cellulases to cellulose [5,10,13]. However, the way in which lignin protects the polysaccharides from enzymatic attack is not clear to date. For example, some studies indicate that the nearly complete lignin removal (below 5%) in corn stover after dilute acid pretreatment reduced cellulose conversion, possibly due to aggregation of adjacent cellulose microfibrils, which was caused by the elimination of the lignin spacer [14,15]. Laureano-Perez et al. [13] reported that the degree and type of cross-linkage of lignin to polysaccharides, the diversity of structures found in the lignin composition, and the distribution of phenolic polymers through the cell wall are important factors for this protection, i.e., the structure of the lignin plays a significant role in determining the magnitude of the cellulose hydrolysis.

Several analytical techniques are used to investigate the chemical, physical and spatial characteristics of the biomass affecting its recalcitrance. Among them, microscopy and spectroscopy are powerful tools to evaluate these characteristics at the nano and microscale. Some methods are atomic force microscopy (AFM), scanning electron microscopy (SEM), scanning transmission X-ray microscopy (STXM), transmission electron microscopy (TEM), and confocal laser scanning microscopy (CLSM). SEM was used to study the effect of pretreatments on corn stover, maize [16,17], sugarcane bagasse [18], as well spruce and beech wood [19]. Several lignin-rich particles with sizes from 5 nm to 10 μm on the surface of maize cell walls [16] and sugarcane [20] after diluted acid treatment were observed by this technique. Li etal. [21] used SEM to evaluate a mixture of Avicel cellulose and poplar wood after hydrothermal pretreatment. They found that lignin droplets from poplar wood relocated onto the Avicel surface and confirmed that blockage of the cellulose surface by lignin droplets was the main cause of cellulase inhibition. CLSM has been used to evaluate the enzyme degradability on forage cell walls [22], as well as lignin distribution in coppice poplar, linseed and wheat straw [23]. Coletta etal. [24] used confocal and fluorescence lifetime imaging microscopy to map the lignin distribution in pretreated sugarcane bagasse after acid and alkali pretreatments and a study of different types of wood cell walls to localize cellulose and lignin was performed by Bond et al. [25].

Even though microscopy can describe the localization and distribution of the lignocellulosic components, characterization of these components *in situ*, especially lignin, without isolation and consequently without chemical modification, is a challenge. A very promising technique to evaluate the chemical characteristics of lignocellulosic components and their distribution simultaneously is mid FT-IR microspectroscopy. With this technique, chemical and microstructural information at cellular level, to a certain extent, on the subcellular level can be gained [26]. This technique has been used by some authors to describe the wood molecular structure, including lignin and polysaccharides within cross-sections of beech and poplar wood, and the spatial distribution of G/S lignin [27,28]. Fackler et al. [29] used multivariate analysis of infrared images (FT-IR imaging) from wood in a study of the biodegradation of spruce wood. Gorzsas et al. [30] visualized, identified and characterized secondary xylem cell types of *Populus tremula* applying unsupervised and supervised chemometrics techniques on infrared spectra. In these works, it is clear that the distribution images based on multivariate analysis overcome the results obtained by univariate analysis to describe the real distribution of the samples components. Budevska et al. [31] evaluated the application of multivariate curve resolution with alternating least squares (MCR-ALS) for the analysis of FT-IR images of *in situ* plant tissue. They concluded that FT-IR imaging analysis by MCR-ALS could be a complementary technique to the staining procedures in histochemical analysis.

Although FT-IR microspectroscopy has been used on plant tissues [27,28,32,33], its full potential in combination with multivariate image analysis for wood analysis has only been recently explored. Besides, the evaluation of the parameters affecting the biomass recalcitrance to pretreatments and SSF process requires the application of diverse analytical methodologies, in most of cases consisting of destructive, laborious and chemical-dependent methods. In this work, we propose the evaluation of the distribution and the chemical characterization of lignocellulosic components of substrates of *P. radiata* wood pretreated by Organosolv process using FT-IR imaging and the microscopical techniques SEM and CLSM to recognize, evaluate and understand, at the microscale level, the chemical and physical characteristics of pretreated samples and to evaluate their influence on the SSF process.

2. Theory

A spectroscopic image can be displayed as a data cube with two dimensions related to the *x*,*y* coordinates of the scanned surface and a third spectral dimension. However, the measurement variation in an image data set follows a bilinear model, where the mixed signal recorded in each pixel is described by the concentration-weighted sum of the pure signals of the chemical compounds [34]. As a first step an unfolding of the original image cube into a matrix of pixel spectra is required. Then, bilinear models can be performed followed by a refolding of the elements according to the original spatial structure of the image. MCR-ALS is a bilinear method of resolution based in the mathematical decomposition of a global mixed instrumental response into the pure contributions due to each of the components in the system [34–36]. This global response is organized in a matrix **D** which is decomposed according the equation:

$$\mathbf{D} = \mathbf{C}\mathbf{S}^{\mathbf{T}} + \mathbf{E} \tag{1}$$

where **D** $(r \times c)$ is the data matrix (reshaped image), **C** $(r \times n)$ is the matrix of the relative amounts or concentrations, $\mathbf{S}^{\mathbf{T}}(n \times c)$ is the pure spectra matrix and **E** is the error matrix, *i.e.*, the residual variation of the data set that is not related to any chemical contribution [34]. The implementation of MCR-ALS is based on the iterative calculation of **S** and **C** as follows:

$$\hat{\mathbf{S}}\mathbf{T} = (\hat{\mathbf{C}}\mathrm{T}\hat{\mathbf{C}})^{-1}\hat{\mathbf{C}}\mathbf{T}\mathbf{D} \tag{2}$$

$$\hat{\mathbf{C}} = \mathbf{D}\hat{\mathbf{S}}(\hat{\mathbf{S}}\mathbf{T}\hat{\mathbf{S}})^{-1} \tag{3}$$

where ^ indicates an estimated value [36]. An initial estimate for either the relative concentration or the spectral profile matrix is required to initiate these algorithms, and iterations are carried out until convergence is achieved. One initial estimate useful for