Physical-chemical Characterization of Wheat Straw during a Continuous Pretreatment Process

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Abstract

The changes occurring during the depolymerization of lignocellulosic biomasses are not yet fully understood. Synchrotron micro-Fourier Transform InfraRed (µ-FTIR), Raman Spectroscopy (RS), X-Ray Diffraction (XRD), and X-Ray Fluorescence (XRF) were used for better characterization of Wheat Straw fibers during a continuous pretreatment process: Conditioning (C), Extrusion (E), Steam Explosion (SE), and Enzymatic Hydrolysis (EH). µ-FTIR revealed functional groups as phenylpropanoid polymers, ethers, and aliphatic alcohol. RS revealed acetoacetate, methyl and phenol groups after SE. The crystallinity index (CrI) was: 54.5%, 27.1%, 31.6%, and 26.7% for C, E, SE and EH respectively. The silica content was: 2.5%, 2.7%, 1.9% and 5.6% for C, E, SE and EH respectively. Keywords: Synchrotron µ-FTIR; Raman spectroscopy; Wheat straw; Steam explosion; Silica content

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1. Introduction

Pretreatment of Lignocellulosic Biomass (LB) comprises a key stage in biorefinery schemes for glucose production and its subsequent transformation into bioethanol or bio-based, high-value-added products [1-3]. LB is an abundant source of cellulose, hemicellulose, and lignin. Cellulose is a glucose-based polymer, while hemicellulose is a polymer that is composed of different monosaccharides, including xylose, arabinose, and mannose, among others. Lignin is a polymer containing mainly aromatic groups. LB has an annual worldwide production estimated at 10–50 billion tons of dry lignocellulose, accounting for about one half of the global biomass [4]. In this regard, wheat (*Triticum aestivum* L.) is the world’s most widely grown crop, cultivated in >115 nations under a wide range of environmental conditions. Worldwide production of Wheat Straw (WS) is calculated at >850 Tg per year [5]. WS can be decomposed into ethanol through biochemical processes that mainly consist of four stages: a) pretreatment; b) hydrolysis; c) fermentation, and d) downstream process [6]. In this context, the WS conversion process is particularly difficult due to its recalcitrance. This affects subsequent biorefining steps, such as enzymatic hydrolysis and fermentation, in parameters such as product yields and concentration, hydrolysis rate, enzyme loading, waste products, and fermentation toxicity, thus and production economics [6,7].

An Important factor that renders difficult the conversion of LB into biofuels is the high degree of cellulose crystallinity, which reduces the enzymatic hydrolysis [8]. The degree of cellulose crystallinity can be modified during the pretreatment by thermomechanical or physicochemical exposure [8]. Several pretreatment routes have been proposed depending on the type and composition of LB [9, 10]. The majority of the pretreatments studied included thermochemical treatments such as diluted acid treatment, steam explosion (SE), and hydrothermal process (HP) [11]. SE is one of the most cost-effective and widely used pretreatment methods for WS [12, 13].

Recently, several optical methods, including Attenuated Total Reflectance-Fourier Transform InfraRed (ATR-FTIR) spectroscopy and Raman Spectroscopy (RS), have been employed to evaluate the structural changes and composition of LB during pretreatment processes [11, 14]. RS can be utilized to analyze viscous reaction mixtures without the interference of the water present in aqueous samples. In addition, RS has been employed as an alternative for the structural analysis of biomass during and after the pretreatment process and its posterior enzymatic hydrolysis [14]. To date, few reports have quantified component concentrations with the identification of functional groups during biorefinery processes using RS [14]. However, with the recent advances in instruments with higher detector sensitivity and laser performance, the use is possible of RS with a high degree of accuracy, for instance, in the monitoring of enzymatic hydrolysis and yeast fermentation [14-17].

Synchrotron micro-Fourier Transform InfraRed (µ-FTIR) was used in order to evaluate structural changes and functional groups after the conversion process. A synchrotron is a high-energy electron storage ring that has 100–1,000 times higher brightness than a conventional thermal global source [18]. Synchrotron radiation infrared microspectroscopy allows the in situ analysis of biomass samples at the molecular level, combining spatial and chemical information from IR absorbance to produce a chemical map that can be linked to a particular morphology or functional group [19]. Kirtania [20] explained that the beamline capacity made it possible to focus on single particles to obtain low noise measurements without mixing with KBr; additionally, the IR beam is not focused on any distinct particle; thus, it measures the bulk surface functional groups, which comprise the spectral average in several particles. To our knowledge, scarce literature can be found that deals with synchrotron-based IR beam focus in biomass analysis [20, 21]. However, these studies did not apply a pretreatment process of WS for glucose production. Yu [21] presented the results of the mapping of the tissue of a wheat seed. Kirtania [20] reported the in situ study of the pyrolysis of three types of woody biomass to observe changes in the surface functional groups with temperature ramping by synchrotron-based IR beam.

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Moreover, the silica content present in WS is an emerging and important factor that can affect the operation of the pretreatment process, because it can form precipitates that are difficult to remove, causing instrumental defects. These defects have been previously reported in paper pulping industries and wastewater treatment processes [22].

This study discusses structural modifications in WS during a sequential, continuous extrusion-steam explosion pretreatment and its posterior enzymatic hydrolysis employing synchrotron µ-FTIR and other characterization techniques, such as Raman Spectroscopy (RS), Scanning Electronic microscopy (SEM), X-Ray Diffraction (XRD), and X-Ray Fluorescence (XRF) in order to identify functional groups, usable by-products, structural changes, and the silica content of WS in a continuous conversion process for glucose production. To our knowledge, this is the first time that a synchrotron source has been used to analyze a WS after a continuous conversion process. The conversion process consisted of four stages: Conditioning (C); Extrusion (E); Steam Explosion (SE), and Enzymatic Hydrolysis (EH). The complete conversion process was denominated the CESEE process (Fig. 1).

2. Experimental

2.1. Materials
Winter Wheat Straw (WS) (\textit{Triticum aestivum} \textit{L.}) was obtained from local farmers in La Barca Province, Jalisco, Mexico (20º 15' 30" to 20º 26' 45" North latitude and 102º 20' 40" to 102º 21' 20" West longitude). The WS was harvested using conventional hay-harvesting equipment. Straw composition was determined according to AOAC INTERNATIONAL methods [42] with 44.45 ± 2.35, 19.23 ± 4.20, 5.78 ± 0.57 and 10.34 ± 1.1 of cellulose, hemicellulose, lignin and ash respectively (% w/w dry weight basis). The WS sample employed in this research was composed of internodes of plant stalk.

2.2. Conversion process description
A block diagram of the complete conversion process is presented in Fig. 1. The pretreatment process consisted of four stages: i) Conditioning (C); ii) Extrusion (E); iii) the Steam Explosion process (SE), and iv) Enzymatic Hydrolysis (EH) to assess pretreatment effectively. The complete process was termed CESEE. Samples were taken at the end of each CESEE stage. Each stage of CESEE is described as follows: C stage: at conditioning stage, WS was firstly milled with a hammer mill (Azteca 301012), classified with a vibratory sieve (Alcon, México) using 5, 6, 8, 12, 16, 20, 30, and 40 US mesh trays, and stored at room temperature. WS retained on 6–30 US mesh was taken for further experimentation; E stage: the characteristics of the extruder are 0.40 m in length with 5 turns-extrusion screw exhibits a 3:1 reduction; SE stage: the steam explosion was carried out in a tubular continuous reactor 4” in diameter with a dual-valve system discharging into a 90 L blow-down tank, processing 3 kg of WS (dry basis)/h. Saturated steam was fed directly into the reactor at 10 bar pressure (185°C). Residence time was fixed at 30 min [43], and the EH stage: was performed in 250-ml flasks with 4% w/v solids load, and initial pH of 4.6, and 14 FPU/g cells as the initial enzyme dosage of the Accellerase \textsuperscript{TM} 1500 cocktail, kindly provided by Genencor. Flasks were incubated at 50°C for 48 h in an orbital shaker (ThermoScientific MaxQ7000 Benchtop; Thermo Scientific, Marietta, OH, USA) [43].

2.3. Characterization of WS fibers

2.3.1. Scanning Electronic Microscopy (SEM) analysis
Scanning Electron Microscopy (SEM) (JSM-7800F, JEOL) was utilized for morphological analysis of samples. The SEM was operated at 15 kV. WS samples were cut, using a microtome, into 10-µm-thick slices and were placed on black adhesive tape in order to analyze the WS transversal section. Images were acquired at 800X.
2.3.2. Raman analysis

DXR-Raman microscope spectrophotometer (Thermo Scientific) equipped with a detector laser of 780-nm wavelengths (Class I) was employed for sample analysis. WS samples obtained from each process were placed into a 20-μL microtube containing distilled water, and was then frozen at −5ºC during 1 day. The samples were cut in a microtome at −5ºC using a serrated steel knife, obtaining samples 5-μm thick. The samples were placed into a sample holder for their later analysis. Spectra were obtained by mapping a specific number of points (15 × 15) with a 5-μm step size. The samples were scanned with a collective exposure time of 20 sec. Preview exposure time was 10 sec, and sample exposures at spectral range utilized 800 cm⁻¹–3,300 cm⁻¹. Sampling with a 20X microscope objective (bright field/dark field) was used for sample focus, and laser power of the sample was 24 mW.

2.3.4. Synchrotron analysis

Samples after C, SE, and EH were analyzed by synchrotron µ-FTIR. Samples were frozen in water inside a 200-μl microtube (Eppendorf) and then were cut in a cryogenic microtome using a diamond knife obtaining samples of 5-μm in thickness. The samples were covered with a CaF₂ window, lyophilized, and, finally analyzed by µ-FTIR. The µFTIR end-station is equipped with a Thermo Nicollet Nexus infrared spectrometer, MCT 50 μm (700-4000 cm⁻¹) and InGaAs Detector (11700-3800 cm⁻¹).

2.3.5. X-ray analysis

X-Ray Diffraction (XRD) was used to calculate the degree of crystallinity of the samples during CESEE. The cellulose crystallinity of C, E, SE, and EH were analyzed in an X-ray diffractometer (D2 PHASER; Bruker, USA) with CuKα generated at 40 kV and at 30 mA, which provides a wavelength of 1.54 Å. The scan range ranged from 10–45º (2 theta), with step size and scan speed of 0.020º and 0.02º/sec, respectively. The crystallinity index was calculated according to [44]:

\[
CrI = \left( \frac{I_{002} - I_{AM}}{I_{002}} \right) \times 100 \quad (\text{Eq. 1})
\]

Where \( I_{002} \) and \( I_{AM} \) are the intensity of crystalline and amorphous phase localized at 22.7 and 18º in 2θ, respectively.

Crystallite size was calculated in a direction perpendicular to its Miller plane using the Scherrer equation as follows:

\[
t_{002} = \frac{\lambda}{\beta_{002} \cos \theta} \quad (\text{Eq. 2})
\]

Where \( t_{002} \) is crystallite size in Miller plane 002, \( \lambda \) is an x-ray wavelength (\( \lambda = 0.154 \) nm for CuKα), \( \theta \) is the bragg angle of the reflection in radians, \( \beta_{002} \) is the pure integral of the width of the reflection at one half maximum height in the 002 plane also in radians, and \( K \) is the Scherrer constant considered as 1.

X-Ray Fluorescence (XRF) in Ar atmosphere (Panalytical Epsilon 3-XL with Rh tube) was utilized to determine the samples SiO₂ content throughout the process. Before XRF measurement all samples were manually milled in agate mortar to homogenize the particle size. After milled, 10 g of each sample were pressed to obtain specimens with 38 mm of diameter and 10 mm thickness.

3. Results and Discussion

3.1. SEM analysis

Figures 1C₁, 1E₁, 1SE₁, and 1EH₁ depict the transversal SEM micrograph of WS fibers after CESEE stage. Fig. 1C₁ presents the micrograph of WS after C stage. Fig. 1C₁ illustrates a defined structure of a longitudinal-cross section of epidermal cells, parenchymal cells, and vascular bundles (phloem and xylem). Fig. 1E₁ reveals the micrograph of WS after E stage, where the fibers underwent stretched of

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the structural stretch, but no disruption was observed; however, in this stage, it is not possible to describe the components of the skeletal structure of WS fibers. Fig. 1SE, exhibits the micrograph of WS after SE where the structure is completely disordered, presenting a clear difference compared with raw material. Finally, Fig. 1EH, demonstrates completely amorphous WS fibers after EH, indicating that structure was degraded. It is not possible describe the morphology of sample.

Kristensen et al. [11], conducted a similar study of WS fibers after SE (198 ºC for 6 min), observing large pieces of nearly intact straw, but also a large fraction of individual fibers that had been compacted together. Also, the same study demonstrated that WS fibers do not undergo disruption after pretreatment, attributing the increase of WS digestibility to the re-localization of lignin.

**3.2. Raman analysis**

Figures 1C, 1E, 1SE, and 1EH present the mapping zones for Raman analysis employing an objective of 20X, where it was possible to obtain a homogeneous analysis. In our experience, a horizontal surface permitted to obtain spectra with low distortion compared with analysis carried out on a non-homogeneous surface.

Fig. 2 displays Raman spectra after each CESEE stage, while Tab. 1 enlists the Raman frequencies (cm\(^{-1}\)) associated with the macromolecules observed on the spectra. Raman band assignments were based on literature related with the analysis of biomolecules of cellulose, xylan, and lignin. The bands associated with cellulose were 899, 1095, and 1461 cm\(^{-1}\) [23-25]. Cellulose is a linear chain of \(\beta\)(1→4)-D-glucose units that bond with hydrogen side-by-side in parallel fashion to form microfibrils of varying thicknesses [26]. The Raman band at 899 cm\(^{-1}\) was associated with an antisymmetric ring stretch of amorphous cellulose; this band is very sensitive to the amount of crystalline vs. the amorphous structure of cellulose [15]. The Raman band at 1095 cm\(^{-1}\) was attributed to asymmetric C-O-C stretching with heavy atom (CC and CO) stretching modes. The band at 1461 cm\(^{-1}\) was associated with CH\(_2\) and H-O-C bending vibration. The Raman band associated with amorphous cellulose (899 cm\(^{-1}\)) reduced its intensity during the process and it was slightly perceptible after SE and EH indicating that amorphous cellulose was removed from raw material. Raman bands associated with crystalline cellulose (1095 and 1461 cm\(^{-1}\)) are perceptible in all stages of process indicating that crystalline cellulose is present in all stages of process. The reduction of the signal of amorphous cellulose after EH suggests that the enzyme used in this research was able to hydrolyze a higher percent of amorphous cellulose.

The Raman band associated with xylan was located at 1120 cm\(^{-1}\). Xylan represents one of the major non-cellulosic components in the cell wall of higher plants. Mazeau and Charlier [27] explained that xylan is a generic substance that describes a series of hemicelluloses, which have in common a linear backbone consisting of polyxylosyl residues, but that are more or less decorated by a variety of substituents, the most common being acetyl groups, L-arabinosyl, and D-glucosyluronic acid residues. The band at 1120 cm\(^{-1}\) was attributed to O-acetyl-4-O-methylglucurono-d-xylan chain units of xylan [28, 29]. This Raman band was reduced after SE, presenting a weak signal.
The Raman bands observed in the spectra attributed to lignin were at 1600 and 1630 cm\(^{-1}\). A report [30], described lignin as an amorphous, non-polar macromolecule comprising phenyl-propane units. Also, the structure of lignin depends on the source. Moreover, the extraction method modifies the structure of the lignin prior to analysis. Lignin typically comprises three basic units: trans-coniferyl alcohol; trans-synaptic alcohol, and trans-p-cumaryl alcohol [30]. The Raman band at 1230 cm\(^{-1}\) is typical of C-aryl-O bands of lignin. The band at 1330 cm\(^{-1}\) was associated with C=O stretching vibrations from coniferil alcohol lignin. The Raman band at 1600 cm\(^{-1}\) was associated with the vibrations of the modes of phenylpropane and acetoacetate units of lignin. The Raman band at 1660 cm\(^{-1}\) was associated with the solubilization of lignin in reaction broth during this stage as was theorized previously by [11]. This result demonstrates that lignin is removed easily by simple mechanical forces through mixing during EH and after SE. Kristensen et al. [11], attributed this phenomenon to less strongly bind of lignin to carbohydrate polymers compared with its native linkages.

### 3.3. FTIR synchrotron analysis

This research presents a first synchrotron based IR results during a continuous conditioning-pretreatment and hydrolysis process of wheat straw samples. The capability of the beamline made it possible to focus on single particles to obtain low noise measurements without mixing with KBr. Fig. 3 exhibits the micro Fourier Transformed Infrared (µ-FTIR) spectra of samples obtained after the CESEE process. Tab. 2 displays the frequencies and the association of each component of WS fibers.

The µ-FTIR bands associated with cellulose were 1434, 1376, and 1338 cm\(^{-1}\) that are associated with CH\(_2\) in-plane bending vibrations, C-O stretching, and with C-H ring in-plane bending vibrations, respectively [21, 31-33]. These bands are presents in all stages of the process, showing that cellulose is present along the process. The FTIR band at 1066 cm\(^{-1}\) is also associated with cellulose and can be associated to β(1-3)-polysaccharide, which is a strong signal characterizing a high cellulose composition, these bands are perceptible along the process but the intensity increase after SE and EH [34, 35].

The FTIR band at 1743 cm\(^{-1}\) corresponds to the C=O group and comprises a typical band associated with hemicellulose [35]. This band is present with a strong intensity in non-treated WS; however, after SE and EH, the weakness signal indicates that hemicellulose was solubilized during SE.

FTIR bands associated with lignin were 1603 cm\(^{-1}\) and 1510 cm\(^{-1}\), which are associated with quadrant ring stretching (aromatic lignin) and semicircle ring stretching (aromatic lignin), respectively. These bands are present with a weak signal in the conditioning of WS (a); therefore, after SE and EH, these signals increased their intensities. These phenomena were attributed to an increase of aromatic skeletal vibrations and the semi-circles stretch of para-substitute benzene rings for 1603 and 1514 cm\(^{-1}\), respectively [34, 35]. The signals observed were attributed to aromatic ring lignin that is present in the sample surface after SE. This results permitted concluded that lignin is not strongly bonded to carbohydrate polymers after this stage. In addition, the result permitted concludes that lignin is relocated.

The FTIR band at 1167 cm\(^{-1}\) was associated with glycosidic linkage of the cellulose [34, 36]. The FTIR bands at 2950 and 2906 cm\(^{-1}\) were attributed to the stretching of C-H [20]. Kristensen [11] attributed the bands at 2950 and 2906 cm\(^{-1}\) to waxes that are reduced after SE.

Also, the hydrophilic tendency of raw WS fibers, steam-exploded fibers, and hydrolyzed fibers was reflected in broad absorption bands at 3413, 3343, and 3314 cm\(^{-1}\), which was related with the –OH groups presented in their main components that may include absorbed water, the aliphatic primary

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and secondary alcohols found in cellulose, hemicellulose, lignin, extractives, and carboxylic acids in extractives [11, 37, 38]. Also, the FTIR band at 1664 cm\(^{-1}\) was attributed to water absorbed in the cellulose [11, 35].

“Figure 3 here”

“Table 2 here”

### 3.4. X-ray analysis

X-Ray Diffraction (XRD) was utilized to calculate the crystallinity index (CrI) of samples during the process (see Tab. 3). Fig. 4 presents two well defined peaks at 16.1 and 22.7º in 2θ which represent the lattice planes 101 and 002 respectively and are characteristic of the typical cellulose I structure [39, 40]. Tab. 3 displays crystallite size in the 002 plane and the crystallinity index for each sample. All samples exhibit a crystallite size from 3.8–5.5 nm, which corresponds to previous reports for this type of materials [41]. The increase in the crystallite size after the extrusion process could indicate the reorganization of small crystals and amorphous regions during the mechanical strain to produce bigger crystals promoted by the compression of the atomic arrangements. Then, during SE process previous crystals remained after extrusion, working as nuclei for a new crystal growth front increasing the crystallite size as shown in Tab. 3. The increase in the crystallite size after SE can indicate that new atomic arrangements are produced after the cleavage of cellulosic bonds.

On the other hand the crystallinity index (CrI), is a factor that can significantly affect EH because it is correlated proportionally with recalcitrant effect. The WS sample obtained from the conditioning stage presented a CrI of 54.5%, which that consistent with the CrI present by other authors [41]. The CrI of WS obtained after the extrusion process was reduced to 27.1% as expected, which can be attributed to the deformation of crystalline regions into amorphous regions as a consequence of mechanical effects. The sample obtained after SE shows a CrI of 31.6% that represents a slight increase compared with previous sample (sample E). The increase in the crystallinity is attributed to the removal of amorphous cellulose and hemicellulose during SE that is according with Raman analyses. Finally, the sample after the EH process presented a CrI of 26.7%. The slight decrease in the crystallinity can be attributed to the effect of the enzymatic complex on amorphous and crystalline cellulose indicating that crystalline cellulose is also hydrolyzed during the EH process as reported previously [41].

X-Ray Fluorescence (XRF) was applied to determine the content of SiO\(_2\) after each stage of the CESEE process (see Tab. 3). SiO\(_2\) is associated with the natural defense of plants against abiotic (e.g., heavy metal toxicity and salinity) and biotic (e.g., fungi and insects) stresses [22]. Silica content must to be taken into account due to the percentage present in WS (2–7%), is higher that percentage presented in Wood. Wood usually presents a very low content, one within the range of 0.0001–0.01%. The importance of SiO\(_2\) during biorefinery processes is due to that operative problems have been reported in paper pulp plants or in wastewater processes associated with SiO\(_2\) precipitates that affect control systems [22].

The WS sample obtained from the conditioning stage revealed a content of 2.53% of SiO\(_2\). The content of SiO\(_2\) in non-treated WS was reported previously as falling within the range of 2.00–7.30% [22]. After extrusion, the SiO\(_2\) content was 2.69%, demonstrating that this treatment did not significantly affect the SiO\(_2\) content. The sample taken after steam explosion showed that the SiO\(_2\) content was reduced to 1.96%; this reduction can be attributed to SiO\(_2\) solubility at high temperatures. It has been reported that SiO\(_2\) solubility can be increased by the effect of temperature. Finally, the SiO\(_2\) content found after EH was 5.63%; the high concentration of SiO\(_2\) after EH was attributed to the operative conditions (50ºC and low pH), which cannot successfully solubilize SiO\(_2\). In addition, the increase of SiO\(_2\) content during EH can be attributed by the hydrolysis of amorphous cellulose.

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“Table 3 here”

4. Conclusions

The structural changes and functional groups of WS that was subjected to sequential pretreatment process followed by enzymatic hydrolysis (CESEE) were documented. SEM analysis permitted observed that the structure of biomass was modified after E and SE but not disruption was observed. A clear disruption is observed after EH where it is not possible describe the final structure of biomass. Raman analysis permitted study the characteristic functional groups of biomass showing that amorphous cellulose was removed after SE and EH. Also, Raman analysis demonstrated that lignin can be easily removed by mechanical forces after SE. µ-FTIR revealed functional groups as phenylpropanoid polymers, ethers, and aliphatic alcohol. This research presents a first synchrotron based IR results during a continuous conditioning-pretreatment and hydrolysis process of wheat straw samples. Synchrotron studies showed that lignin was decomposed into aromatic compounds after SE and not only relocated. X-ray analysis permitted observes the modification of crystalline structure during process with a drastic reduction after E stage. The XRF analysis showed that SiO₂ is maintained inside the fibers during all the stages of process with the exception of SE where SiO₂ was solubilized due to high temperature. In addition, it can be theorized that part of SiO₂ is carried out to EH and maintained inside the reactor during this stage of CESEE process. It implies that silica can be inlays in EH reactor. The SE stage must be tested at higher temperatures in order to evaluate the possibility of obtain other chemical products. In addition, more studies of reaction broth are recommended in order to obtain more information about functional groups and silica content.

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Abbreviations

Ar       Argon
C        Conditioning
CESEE    Conditioning-Extrusion-Steam explosion-Enzymatic Hydrolysis
CrI      Crystallinity index
E        Extrusion
EH       Enzymatic hydrolysis
FPU      Filter paper units
IR       InfraRed
LB       Lignocellulosic biomass
RS       Raman Spectroscopy
SE       Steam explosion
SEM      Scanning Electronic microscopy
WS       Wheat straw
XRD      X-Ray Diffraction
XRF      X-Ray Fluorescence
µ-FTIR   Micro-Fourier Transform InfraRed
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[42] AOAC Official Methods 4.6.03 and 4.6.04
Figures and legends

![Diagram of continuous conditioning-extrusion-steam explosion pretreatment processes and subsequent enzymatic hydrolysis. SE (@ 10 bar and 185°C for 30 min) and EH (@ 50°C for 48 h).](image)

Figures C1, E1, SE1, and EH1 show the SEM micrographs of the transversal section of conditioning, extrusion, steam explosion, and enzymatic hydrolysis, respectively.

Figures C2, E2, SE2, and EH2 presents the SEM micrographs of a general view of conditioning, extrusion, steam explosion, and enzymatic hydrolysis, respectively.

Figures C3, E3, SE3, and EH3 depict the photograph of optical microscopy (20X) of conditioning, extrusion, steam explosion, and enzymatic hydrolysis, respectively.
Figure 2  Raman spectra of WS after each stage of the process: (C) Conditioning; (E) Extrusion; (SE) Steam Explosion (@ 185°C, 30 min) and (EH) Enzymatic Hydrolysis (@ 50°C, 48 h).

Figure 3  µ-FTIR spectra of WS after each stage of the process: (C) Conditioning; (E) Extrusion; (SE) Steam Explosion (@ 185°C, 30 min) and (EH) Enzymatic Hydrolysis (@ 50°C, 48 h).

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Figure 4 X-ray spectra of WS after each stage of the process: (C) Conditioning; (E) Extrusion; (SE) Steam Explosion (@ 185°C, 30 min), and (EH) Enzymatic Hydrolysis (@ 50°C, 48 h).
### Tables and captions

**Table 1** Summary of Raman frequencies (cm\(^{-1}\)) of macromolecules

<table>
<thead>
<tr>
<th>Frequency (cm(^{-1}))</th>
<th>Contributor</th>
<th>Band assignment</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1630</td>
<td>Lignin</td>
<td>Ring conjugated C=C stretch of coniferaldehyde</td>
<td>[24]</td>
</tr>
<tr>
<td>1601</td>
<td>Lignin</td>
<td>Aryl ring stretching, symmetric</td>
<td></td>
</tr>
<tr>
<td>1461</td>
<td>Cellulose</td>
<td>CH(_2) bending vibration, HOC bending vibration.</td>
<td>[24, 25]</td>
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<td>1377</td>
<td>Cellulose</td>
<td>CH(_2) bending vibration, HCC, HCO and HOC bending</td>
<td></td>
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<td>1267</td>
<td>Lignin</td>
<td>Guaiacyl ring breathing Caryl-O stretching</td>
<td></td>
</tr>
<tr>
<td>1230</td>
<td>Lignin</td>
<td>C-aryl-O stretching</td>
<td>[24]</td>
</tr>
<tr>
<td>1153</td>
<td>Xylan</td>
<td>The breathing vibration of the glucopyranose rings</td>
<td></td>
</tr>
<tr>
<td>1095</td>
<td>Cellulose</td>
<td>Asymmetric COC stretching, heavy atom (CC and CO) stretching</td>
<td></td>
</tr>
<tr>
<td>1045</td>
<td>Xylan</td>
<td>C-C and C-O stretching</td>
<td></td>
</tr>
<tr>
<td>1149, 1120, 1050</td>
<td>β-D-Glucose</td>
<td>C-O stretching</td>
<td>[25]</td>
</tr>
<tr>
<td>1374, 1017, 1468</td>
<td>Xylose</td>
<td>C-C and C-O stretching</td>
<td></td>
</tr>
</tbody>
</table>
### Table 2 Summary of μ-FTIR frequencies of macromolecules

<table>
<thead>
<tr>
<th>Frequency (cm⁻¹)</th>
<th>Contributor</th>
<th>Band assignment</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>3413, 3343, 3314</td>
<td>OH groups</td>
<td></td>
<td>[39]</td>
</tr>
<tr>
<td>2950, 2906</td>
<td>C-H stretching</td>
<td></td>
<td>[20]</td>
</tr>
<tr>
<td>1732</td>
<td>Xylan</td>
<td>C = C stretch vibration in hemicellulose</td>
<td>[34, 41]</td>
</tr>
<tr>
<td>1606</td>
<td>Xylan</td>
<td>Aromatic skeletal vibration</td>
<td></td>
</tr>
<tr>
<td>1559; 1512</td>
<td>Lignin</td>
<td>Very strong aromatic ring stretch, aromatic C-O stretch</td>
<td></td>
</tr>
<tr>
<td>1511</td>
<td>Lignin</td>
<td>Semicircle ring stretching (Aromatic lignin)</td>
<td></td>
</tr>
<tr>
<td>1461</td>
<td>Xylan</td>
<td>C-H-deformation Methyl and Methylene</td>
<td></td>
</tr>
<tr>
<td>1467</td>
<td>Lignin</td>
<td>C-H-deformation Methyl and Methylene</td>
<td></td>
</tr>
<tr>
<td>1431</td>
<td>Cellulose</td>
<td>In plane bending vibrations</td>
<td>[34]</td>
</tr>
<tr>
<td>1429</td>
<td>Lignin</td>
<td>Aromatic C = C stretch</td>
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</tr>
<tr>
<td>1373</td>
<td>Cellulose</td>
<td>Weak C-O stretching</td>
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<tr>
<td>1338</td>
<td>Cellulose</td>
<td>C-H rings</td>
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<tr>
<td>1251</td>
<td>Xylan</td>
<td>Acetylated Hemicellulose</td>
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<tr>
<td>1246</td>
<td>Xylan</td>
<td>Acetylated Hemicellulose</td>
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<td>1160</td>
<td>Xylan</td>
<td>Glycosidic linkage</td>
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<tr>
<td>1157</td>
<td>Lignin</td>
<td>C-O-C ring vibrational stretching</td>
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<tr>
<td>1050</td>
<td>Xylan</td>
<td>C=O stretching</td>
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</tr>
</tbody>
</table>

### Table 3 Crystallinity parameters and Si content of wheat straw after each stage of process obtained by XRD and XRF respectively.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Crystallinity Index [%]</th>
<th>Crystallite size [nm]</th>
<th>Si [%] *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conditioning</td>
<td>54.5</td>
<td>3.8</td>
<td>2.5</td>
</tr>
<tr>
<td>Extruded</td>
<td>27.1</td>
<td>4.1</td>
<td>2.7</td>
</tr>
<tr>
<td>Steam Explosion</td>
<td>31.6</td>
<td>4.4</td>
<td>1.9</td>
</tr>
<tr>
<td>Enzymatic Hydrolysis</td>
<td>26.7</td>
<td>5.5</td>
<td>5.6</td>
</tr>
</tbody>
</table>

*Obtained by XRF
Table of contents
In a biorefinery scheme, the changes occurring during the pretreatment of lignocellulosic biomasses are not yet fully understood. Synchrotron micro-Fourier Transform InfraRed, Raman Spectroscopy, X-Ray Diffraction, and X-Ray Fluorescence were used for better characterization of wheat straw fibers during a continuous pretreatment process: Conditioning, Extrusion, Steam Explosion, Enzymatic Hydrolysis.