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Review

# A critical review of analytical methods in pretreatment of lignocelluloses: Composition, imaging, and crystallinity

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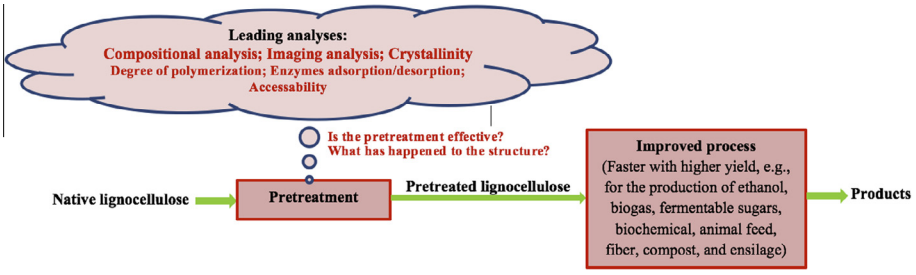
## HIGHLIGHTS

- Pretreatment of lignocelluloses are among the crucial steps in a number of bioprocesses.
- Compositional, imaging, and crystallinity measurements are widely used for analysis.
- Advantages, drawbacks, approaches, practical details, and points are presented.
- The methods need special care and preparations, discussed in this paper.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Lignocelluloses are widely investigated as renewable substrates to produce biofuels, e.g., ethanol, methane, hydrogen, and butanol, as well as chemicals such as citric acid, lactic acid, and xanthan gum. However, lignocelluloses have a recalcitrance structure to resist microbial and enzymatic attacks; therefore, many physical, thermal, chemical, and biological pretreatment methods have been developed to open up their structure. The efficiency of these pretreatments was studied using a variety of analytical methods that address their image, composition, crystallinity, degree of polymerization, enzyme adsorption/desorption, and accessibility. This paper presents a critical review of the first three categories of these methods as well as their constraints in various applications. The advantages, drawbacks, approaches, practical details, and some points that should be considered in the experimental methods to reach reliable and promising conclusions are also discussed.



## 1. Lignocelluloses and pretreatment

Pretreatment is a process in which the carbohydrates, particularly cellulose, get ready for an enzymatic or microbial attack (Fig. 1). This process is mainly used to improve the ethanol and biogas production; however, it can also be used to improve the production yield of all biochemicals from lignocelluloses as well as for the improvement of animal feed, fiber properties, and compositing (Ghasemi et al., 2013; Jeihanipour et al., 2010a; Karimi and Pandey, 2014; Salehian et al., 2013). This pretreatment can be a physical process such as milling; a chemical treatment by, for example, alkali, acids, or cellulose solvents; biological pretreatment, e.g., by white-rot fungi or lignin-degrading enzymes; or a combination of these processes (Karimi and Chisti, 2015; Kumar and Wyman, 2013). Several reviews and book chapters have been presented, describing different pretreatment processes (e.g., Kumar and Wyman, 2013; Shafiei et al., 2015; Taherzadeh and Karimi, 2008); however, none of them was focused on the analyses used to investigate the pretreatment effects on the lignocelluloses. This is the main purpose of this paper.

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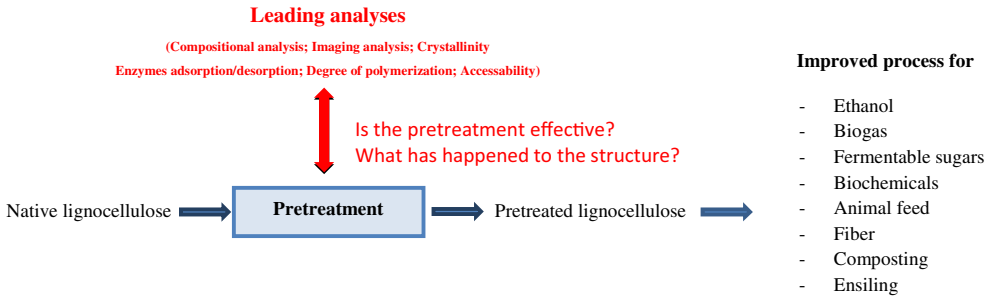


**Fig. 1.** Analysis of the pretreatment of the lignocelluloses for different purposes.

A number of analyses have been developed to investigate the effects of the pretreatment on the lignocelluloses and mainly to describe the improvements on the enzymatic or microbial hydrolysis. These analyses indicate alterations in composition, crystallinity (e.g., by FTIR and X-ray), pore size (e.g., by Simon staining), surface properties (e.g., by SEM, TEM, and AFM), enzymes adsorption/desorption, and degree of polymerization. This paper presents the details of half of these methods and makes a critical review on their applications and constraints.

In these analyses, different terms and expressions are used to describe the untreated lignocelluloses properties, including "compact structure", "inaccessible carbohydrates", "high crystalline cellulose", "cellulose crystallinity", "total crystallinity", "cellulose type I", and hemicellulose and lignin barriers. On the other hand, the terms such as "open up structure", "modified structure", "porous structure", "accessible structure", "sponge like structure", "cellulose type II", "amorphous celluloses", "amorphous carbohydrates", and "lower cellulose degree of polymerization" are used to indicate the pretreated lignocelluloses. However, not all of these terms have a scientific and well-defined meaning. Some of these terms that have scientific meanings are defined and described herein.

## 2. Compositional analysis of untreated and pretreated biomass

Compositional analysis of lignocellulose, before and after pretreatment, is used in almost all second-generation biofuel production studies, except in some investigations on biogas production that use volatile solids (VS) instead. Accurate and reliable analysis is important to evaluate the biofuel production. The product yield, recoveries, techno-economical evaluations, and feasibility studies are typically based on the carbohydrate content. It is also very crucial when considering running a plant on an industrial-scale (Sluiter et al., 2010; Templeton et al., 2010). Historically, the analytical methods are used for the analysis of the cellulose and lignin content, rather than the details of molecular entity. Therefore, it is difficult to compare the compositional results of one type of plant to another (Barton, 1988). Even for one type of biomass, besides complexity and variability of the lignocelluloses by themselves, a wide compositional variation reported in the literature may indicate the presence of errors in the analytical methods. It might be less problematic to evaluate the effectiveness of one type of pretreatment, as the compositional data can be compared even though the absolute values are not accurate enough. Some of the challenges in the compositional analysis are indicated in Fig. 2.

Different methods were developed for the analysis of lignocelluloses for specific applications and industries. The analysis of woody biomass using the Technical Association of Pulp and Paper Institute's (TAPPI) standards is optimized for the pulp and paper industries, in which cellulose is the most desired fraction. The Association of Official and Analytical Chemists International (AOAC) standards are suitable for herbaceous and foods analysis in the forage and feed industries, which are interested in measuring the digestibility of food and feed, in which digestible fiber is the most desired fraction (Agblevor and Pereira, 2013; Theander et al., 1995).

Most of the biomass analytical methods are not suitable for the material balance and for quantifying the individual carbohydrates required for the biofuel evaluation and to investigate the pretreatments suitability (Burkhardt et al., 2013). A general procedure on the determination of carbohydrates, acetate, lignin, and ash in biomass, presented by Sluiter et al. (2008b), provided by the National Renewable Energy Laboratory (NREL), is extensively used and cited in the literature for application in the second generation biofuels

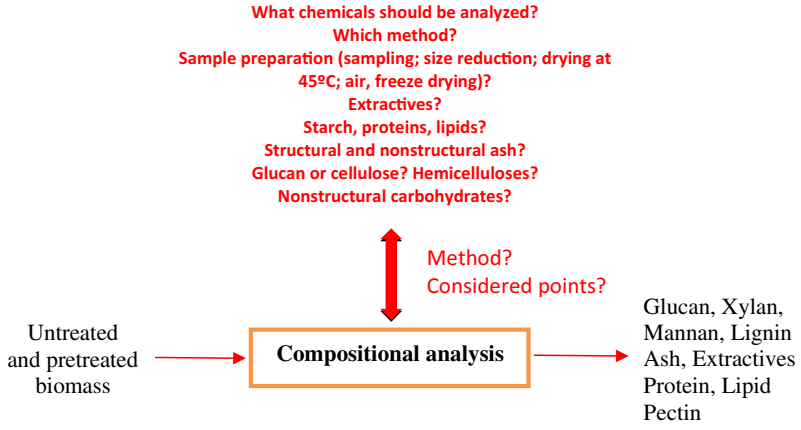


**Fig. 2.** Compositional analysis and challenges.

and chemicals, in which the polysaccharides are the most desirable fraction. This method is very similar to the American Society for Testing and Materials method (ASTM E1758-01), which is a general and standard method for the determination of carbohydrates. The method is based on a two-stage acid hydrolysis of the carbohydrate polymers to monomeric sugars. Biomass is first subjected to hydrolysis using 72% sulfuric acid solution, followed by dilution of the mixture to obtain 4% sulfuric acid hydrolysis at 120 °C (autoclaving) in a sealed vessel. After the hydrolyses, the mixture is neutralized by liming, and the released simple sugars are measured by HPLC. The acetyl groups released can also be detected by HPLC.

In this method, lignin is classified as acid-insoluble (AIL) and acid-soluble (ASL) fractions. Acid-soluble material is the fraction of lignin that has a low molecular weight and is solubilized in the acid hydrolysis. It is determined using the UV–Visible spectrophotometer. The acid-insoluble lignin, on the other hand, is the high molecular weight lignin that cannot be dissolved during the acid hydrolysis and is measured gravimetrically. This type of lignin is determined by the filtration of materials after the acid hydrolysis, drying at 105 °C, and ashing at 575 °C (Sluiter et al., 2008b). This method is simple; however, special care should be taken to get reliable results. Sampling, particle size, moisture content, presence of other impurities such as protein, soil, or other debris can highly affect the results (Sluiter et al., 2010; Templeton et al., 2010).

### 2.1. Sample preparation

Sample preparation is an important part of the method, and different biomass need different methods. A suitable sample preparation process is suggested by the ASTM E1757 (2008) and TAPPI T264 (2002–2003), and a modified procedure is presented by Hames et al. (2008). The samples should be dried before the analysis, as the moisture will dilute the added acid; thus, less than 10% moisture is recommended.

Air-drying is suggested for the preparation of large quantities (>20 g) of samples in ambient humidity that allows for drying to less than 10% moisture. The samples should be first cut into small pieces, spread out on a surface, and air-dried until the weight is stabilized to less than 1% in 24 h. The best suggested method for very wet samples is convection oven drying at 45 °C, and avoiding microbial contamination is important. An alternative to air- and oven drying for very wet biomass is lyophilization (freeze-drying). The method is particularly suitable when the risk for microbial growth during drying is high (Hames et al., 2008). It should be noted that a part of the volatile components, e.g., resin acids, could be lost as a result of drying. To avoid the loss of volatile constituents, therefore, Karl–Fischer (dissolving water in methanol) and Nuclear magnetic resonance (NMR) spectroscopy are suggested (Hames et al., 2008); however, they are rarely used for the analysis of pretreated lignocelluloses.

On the other hand, the Sluiter et al. (2008b) procedure was optimized for a specific particle size range; thus, the reduction of sample sizes is unavoidable. Some laboratory mills, e.g., ball milling, can damage the biomass by heating to high temperatures. Thus, knife milling to less than 2 mm is the recommended method. Sieving (between 20 and 80 meshes) is suggested for the analysis of homogeneous materials, e.g., woods, when fractionation has not occurred. However, sieving is not a good choice for nonhomogeneous substrates, e.g., herbaceous feedstocks, as the whole sample is different from the sieved sample. For instance, when the sample contains soil, the soil appears in the particles with less than mesh 20, and if the fraction between mesh 20 and 80 is analyzed, the results will not be accurate (Agblevor and Pereira, 2013; Hames et al., 2008).

### 2.2. Non-structural carbohydrates

Two types of carbohydrates may be available in biomass: structural and non-structural. The carbohydrates that are bound in the biomass are called structural, and those that can be removed by extraction (water or ethanol) or washing are called non-structural (Hoch et al., 2003; Yu et al., 2011). The Sluiter et al. (2008b) method is suitable for structural, but not for non-structural carbohydrates. For instance, when sorghum or sugarcane, with some residual sugar, is subjected to the analysis, the free sugars should first be analyzed and removed, and then the glucan and hemicellulosic parts can be analyzed (Sluiter et al., 2008b). This should be considered also for the substrates that contain appreciable amounts of pectin (e.g., citrus wastes), tannin (e.g., acorn), and extractives (e.g., pinewood).

### 2.3. Ash measurement

Ash can be determined by combustion of biomass in a furnace at 575 °C (ASTM E1755 or TAPPI T211 om-07). However, it should be noted that there are two types of ash in the lignocellulosic samples: non-extractable and extractable. Non-extractable ash is the inorganic materials that are bound to the structure of the lignocelluloses, whereas extractable ash is the inorganic chemicals that adhere to the biomass and can be removed by washing with water. The typical form of extractable ash is soil, contaminating the lignocelluloses (Sluiter et al., 2008a). Moreover, the method is not suitable for lignocelluloses with a high ash content (>10%), e.g., bark of various trees. It should be noted that the samples that contain minerals might neutralize part of the sulfuric acid and considerably affect the analytical results (Sluiter et al., 2010).

### 2.4. Analysis of samples containing protein

Lignin measurement interferes with proteins; thus, for accurate measurements of lignin from lignocelluloses containing considerable amounts of protein, the quantification of the protein is essential (Agblevor and Pereira, 2013; Sluiter et al., 2010). The proteins can be simply determined by measuring the nitrogen content (e.g., by Kjeldahl method) and then converting by a multiplier (N factor). The Lowry, Biuret, and Bradford methods can also be used. However, in the case of wastes such as municipal solid wastes, it should be noted that proteins can partly dissolve in the water during the extraction, while more resistant proteins such as keratin cannot be dissolved. In the method presented by Sluiter et al. (2008b), unhydrolyzed proteins result in an overestimation of lignin.

### 2.5. Starchy lignocelluloses

While considerable amounts of starch are available in the lignocelluloses (e.g., municipal solid waste, potato wastes, and fruit wastes), the compositional analysis of lignocelluloses is a challenging task. Starch is hydrolyzed to glucose under conditions that are less severe than that for cellulose. Under the severe hydrolysis conditions, e.g., the hydrolysis method presented by Sluiter et al. (2008b) for carbohydrate analysis, the starch converts into glucose but a part of the glucose is also destroyed into other products, e.g., hydroxymethyl furfural. Thus, a separate hydrolysis is necessary for the starch analysis. This can be carried out by enzymatic hydrolysis using alpha-amylase (Moore et al., 2015), considering that the enzyme has access to the starch. Then, the starch content can be subtracted from the total glucan detected by the Sluiter et al. (2008b) method.

### 2.6. Using pure sugars as models

In acid hydrolysis, the polymeric carbohydrates, e.g., glucan, mannan, and xylan, are converted into their monomeric and oligomeric sugars as well as a variety of degradation products (Taherzadeh and Karimi, 2007). Thus, a loss factor is necessary to correct these losses. In the procedure presented by Sluiter et al. (2008b), the monomeric sugars are considered as models in accounting for the degradation. Thus, similar to the lignocellulose sample, pure sugar is hydrolyzed simultaneously, and the degradation is taken into account for the loss of the carbohydrates. Without conducting the analysis on the pure sugar, no accurate detection is possible (Sluiter et al., 2008b).

The water molecules available in the sugars, e.g., when using monohydrate glucose, should be considered in the calculations.

### 2.7. Analysis of samples containing chemicals

The procedure optimized with the hydrolysis uses one stage with 72% acid and a second stage using 4% acids. Any dilution and neutralization or increase in the acidity can affect the results. Thus, this procedure is not suitable for the analysis of the lignocelluloses pretreated with acid, base, or other catalyst in which a fraction of these chemicals remain in the biomass. On the other hand, as ASL is measured by UV spectroscopy, accurate extinction coefficient for Beer's law should be applied. Spectroscopy has interferences from a high number of non-lignin chemicals having absorbency in the UV region, including the sugar degrading products, e.g., HMF, furfural, and caramels.

### 2.8. Extractives and extraction

Extractives are a mixture of different chemicals, including resins, proteins, phytosterols, fats, waxes, salts, and a number of non-volatile hydrocarbons available in minor portions. When available, free sugars are also involved as extractives. As mentioned, extractives interfere with some component analysis, particularly lignin. Thus, lignocelluloses with high extractive contents should be extracted first; otherwise, unacceptable results may be obtained. As an example, bark of a pinewood, containing 19% extractives, was analyzed using the Sluiter et al. (2008b) method for the lignin content, with and without the separation of extractives (Burkhardt et al., 2013). The lignin content was 52.7% when the extractives were not separated, whereas the lignin was 34.5% when the extractives were extracted before the analysis. This means that there was more than 50% error in the lignin analysis when the extractives were not separated before the lignin analysis.

The TAPPI T204 method is available for wood that involves sequential extraction of the non-polar compounds with benzene/-toluene, followed by benzene/ethanol extraction, and finally with water. However, this method is time consuming, unsafe, and rarely used for the analysis of pretreatment and biofuels. A simple and standard method, suggested for the determination of extractives in ASTM E1690 and modified by Sluiter et al. (2008c), is widely used for the analysis of pretreated materials for biofuel. According to this method, the extractives are simply extracted in a Soxhlet extractor by water or a solvent, which is usually ethanol. Inorganic and nitrogenous materials as well as sugar acids and nonstructural sugars can be extracted by water, while waxy and oily materials can be extracted by ethanol. Unlike similar methods, e.g., the Dionex ASE method, the Soxhlet extractor was suggested for use with different types of lignocelluloses (Agblevor and Pereira, 2013). However, recently, detailed analysis by Burkhardt et al. (2013) showed that this extraction method is also challenging; thus, it is necessary to refine the method to provide reproducible quantification of the carbohydrates.

### 2.9. Analysis of monosaccharides

Refractometry, colorimetry, and methanolysis as well as gas chromatography (GC), gas chromatography/mass spectrometry (GCMS), HPLC, alditol acetate gas–liquid chromatography (GLC), anion exchange chromatography (AEC), high-pH anion-exchange chromatography with pulsed amperometric detection (HPAE/PAD), NMR, proton NMR spectroscopy, near-infrared (NIR) spectroscopy and partial least squares (PLS) multivariate, thermogravimetric analysis (TGA), and capillary electrophoresis (CE) were used for the analysis of monosaccharides and carbohydrates (Agblevor and Pereira, 2013; Barton, 1988; Carrier et al., 2011; Payne and Wolfrum, 2015). Refractometry, which has a high interference with other chemicals, even minerals, may be used as a rough approximation for the pure sugars. Colorimetric methods, e.g., dinitrosalicylic acid (DNS) method (Miller, 1959), cannot differentiate between the types of monosaccharides, although they are simple and fast.

For sugar analysis with GC and GCMS, the sugars should be derivatized first, e.g., by aldonitrilacetates, making them volatile (Kacik and Kacikova, 2009). This method is suggested for the analysis of trace sugars. Other analytical methods, e.g., NMR spectroscopy, are also suitable; however, they are very expensive, specialized, and not commonly used for the analysis of lignocelluloses and pretreated materials (Agblevor and Pereira, 2013). HPLC is suggested as a reliable method for the analysis of monosaccharides for biomass analysis. It is widely used in this area. HPLC, with a high resolution and suitable baseline, is necessary for the separation of monomeric sugars released from the lignocellulose. However, no single HPLC column is available for the detection of all the sugars. A strong acid-exchange material (e.g., Aminex HPX-87H, Bio-Rad) is suitable for the monomeric sugars, ethanol, butanol acetone, glycerol, furfural, acetic acid, and hydroxymethyl furfural, but not for the oligomeric carbohydrates.

For monomeric and oligomeric sugars, the amine-bonded material (e.g., Nucleosil 5-$NH_2$), Ca-loaded ion-exchange material (such as Aminex HPX-42A, Bio-Rad), Pb-loaded ion-exchange columns (e.g., Supelcogel Pb and Aminex HPX-87P) provide a high resolution and selectivity. However, the ion-exchange columns have problems with sulfate and low pH. In our laboratory, the treatment and neutralization of the samples with barium hydroxide or $PbNO_3$ and then filtration was used to improve the detection and quantification of the sugars. The refractive index (RI) is the most common detector for the sugar analysis by HPLC, which is the least expensive detector. High-pH anion-exchange chromatography with pulsed amperometric detection (HPAE/PAD), which is much more expensive, can provide a better resolution. However, the PAD detector is very sensitive and needs 50–100-fold dilutions of the hydrolyzate samples, which are typically accompanied by a high error.

Partition chromatography with gradient elution using Prevail carbohydrate column and then detection using evaporative light scattering detector (ELSD) is another option, showing an improved baseline resolution for the analysis of the biomass hydrolyzates (Agblevor et al., 2004). A highly reliable standard is also required. For instance, uronic acids can be measured by the NREL procedure, but it cannot be accurately measured by the HPLC because of the lack of commercial standards for uronic acids (Agblevor and Pereira, 2013).

### 2.10. Lignocellulose type consideration

The procedure presented by Sluiter et al. (2008b) is optimized for the analysis of the typical lignocelluloses. However, it is not optimized for the characterization of all feedstocks, e.g., municipal solid waste and algae (Agblevor and Pereira, 2013), while it is reported in the literature that it is carelessly used for the analysis of a number of non-recommended substrates. In some cases,

complicated reactions have taken place, affecting the analysis results. In the case of rice straw, for example, containing a high amount of silica, the lignin condenses on the silica particles resulting in a higher apparent lignin content. The measurements may be improved by ashing the lignin (Theander et al., 1995). Special care should also be taken for the analysis of more complex and mixed chemicals. For instance, Salix glauca leaves (containing 4% fat, 11% protein, and 6% ash (Scotter, 1972)), sweet sorghum biomass (containing 5.7% ash, 3.3% protein, 9.8% free sucrose, 7.3% starch (Stefaniak et al., 2012)), manure (containing high amounts of protein and urine, and extractives), and municipal solid wastes (containing high amounts of protein, lipid, and extractives) (Ghanavati et al., 2015) cannot be analyzed accurately using the routine analytical methods.

## 3. Imaging analysis

Lignocelluloses are three-dimensional nanocomposites and a dynamic mixture of multifunctional components. Compositional analysis is not enough to investigate the effects of a pretreatment on a lignocellulose. For instance, it is not enough to know how much lignin has a biomass; it is also important to know where the lignin is located and how it interacts with the other components, e.g., celluloses and hemicelluloses. On the other hand, lignin re-localization and cell wall delamination by pretreatments are likely to be as important as lignin removal in the improvement of lignocelluloses hydrolysis.

Microscopic and nanoscopic analyses of the lignocelluloses and comparisons with the pretreated samples can be used to qualitatively predict and understand the susceptibility of the lignocellulosic materials to subsequent hydrolysis. However, getting an effective characterization using the surface imaging is challenging.

As indicated in Table 1, lignocelluloses have a multi-scale complexity; thus, small and large scales give different overviews. Furthermore, different parameters and treatment can be performed prior to the imaging to improve the quality of the analysis. Some staining methods are suggested for highlighting the cell wall structures and their components. Different microscopy approaches can also be used to understand the changes on the cell wall structure by pretreatment, enhancing the cellulose accessibility (Fig. 3).

Currently, SEM, TEM, and AFM are widely used to investigate the lignocellulosic material structures at nanoscale. Although they are mainly qualitative, they can provide us with an insight into the structure that cannot be inferred from the other analysis. They can show images of the microfibrils and cell walls. Other imaging, e.g., Confocal Laser Scanning Microscopy (CLSM) and Electron Microscopy (EM), are also powerful tools used for imaging the structure of the lignocelluloses, but less frequently used for the analysis of pretreatments.

**Table 1**
Dimension of analysis needed for the observations of the different parts of the lignocelluloses.

| Lignocellulosic parts | Size order of magnitude |
|---|---|
| Differing cell types | $10^{-4}$ m |
| Vascular bundles and pith | $10^{-5}$ m |
| Primary and secondary cell walls | $10^{-6}$ m (μm) |
| Microfibers comprise the walls | $10^{-9}$ m (nm) |

### 3.1. Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) is among the powerful tools widely used to investigate the lignocelluloses surfaces (Amiri and Karimi, 2015). Surface characterization, morphology, and analysis of microstructure can be performed by SEM. When equipped with an energy-dispersive spectroscopy (EDS), it can also indicate the elemental composition with 1–3% accuracy. Using SEM, it is possible to see the surface erosions, deconstruction, and re-localization of the cell wall components and to get an idea of the accessibility and the enzymatic hydrolysis improvements (Donohoe et al., 2011). However, the samples for SEM are required to be conductive; furthermore, the electron beam may damage the samples. These two drawbacks limit its application. In order to make the samples conductive, they can be coated with a vaporized metal (e.g., gold) or carbon (Yarbrough et al., 2009).

To achieve reasonable conclusions, oven drying, which results in significant deformation and collapse of the surfaces, should be avoided. Among the drying methods, freeze drying, which is a vacuum sublimation of the frozen sample, is among the best option to avoid the surface tensions. Before subjecting to the freeze dryer, rapid freezing of the biomass sample in liquid nitrogen is suggested (Donohoe et al., 2012). Staining may also be used for highlighting the different parts. For instance, $KMnO_4$, which is a preferential lignin stain, can be used to highlight the location of the lignin within the cell wall (Chundawat et al., 2011; Donohoe et al., 2008; Karp et al., 2015). Two rat monoclonal antibodies, designated as LM10 and LM11, may also be used for staining the SEM samples (McCartney et al., 2005).

Besides qualitative surface analysis, a surface roughness factor, called "SEM roughness index," can be analyzed and calculated from the SEM micrographs (Banerjee et al., 2009). Corresponding to the increase in the hydrolysis, an increase in the "SEM roughness index" was detected after the pretreatment of the corn stover (Ciesielski et al., 2014).

Investigating the SEM images can give different valuable information from the biomass; furthermore, comparing the untreated and pretreated samples may lead to different insight into the biomass. SEM imaging is used in a large number of studies in the pretreatment of lignocelluloses, but many of them contain speculations. However, there are some studies that support the observations and conclusions by using other analysis, e.g., the studies led by the Biomass Surface Characterization Laboratory (BSCL), NREL. A summary of some of the observations is presented in Table 2.

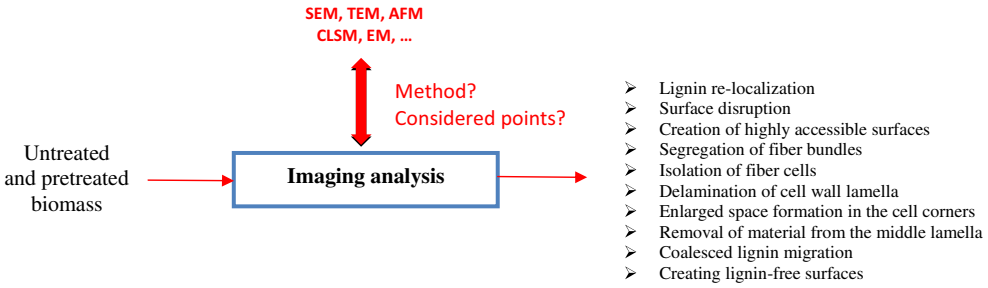


**Fig. 3.** Imaging for understanding the changes due to pretreatments.

**Table 2**
Some SEM imaging observations for the pretreated lignocelluloses.

| Lignocellulose and pretreatment | Observation after the pretreatment by SEM | Refs. |
|---|---|---|
| Switchgrass pretreated with ammonia fiber expansion, soaking in aqueous ammonia, lime, dilute sulfuric, and liquid hot water | - The smooth cell wall surfaces become extremely irregular from the apparent deposition of the re-localized cell wall matrix<br>- Lignin re-localization into lignin-rich globules, apparent at high magnifications (20,000×)<br>- The surface is deeply etched and removal of the cell wall<br>- No penetration of extensive erosion far into the cell wall<br>- Creation of highly accessible surfaces for the cellulase<br>- A homogeneous surface texture in the treated sample, creating an even relocation and removal of lignin<br>- The pretreatment etched away the cell wall matrix while the microfibrils are exposed on the cell wall structures | Donohoe et al. (2011) |
| Switchgrass pretreated with NaOH | - Segregation of fiber bundles and isolation of the fiber cells<br>- Weakening of the particle mechanical integrity<br>- Increased fragmentation creating smaller clusters of cells and individual fibers<br>- Loosened and segregated particles<br>- Low density structure is observed as lighter areas within the cell wall<br>- Severe deconstruction by intracell wall nanofibrillation and delamination<br>- Reduced contrast of the cell walls, indicating lower lignin content<br>- Switchgrass contains more compact cellulose microfibrils than corn stover | Karp et al. (2015) |
| Corn stover pretreated with ammonia fiber expansion | - No observable modification in the epidermal cells, parenchyma cells, vascular bundles, or lignified sclerenchyma cells<br>- A fibrous network of cellulosic macrofibrils was observed in the untreated walls<br>- Untreated biomass contains occasional cytoplasmic remnants as well as crevices and cracks that formed the cell wall surface<br>- Never-dried untreated biomass contains 42 nm with cellulose macrofibril, while the air dried sample width was 23 nm* | Chundawat et al. (2011) |
| Corn stover pretreated with NaOH | - Color changes from light brown to white, indicating lignin removal<br>- Particle fragmentation and cell separation by pretreatment may be attributed to the lignin removal and depolymerization from the shared compound in the middle lamella<br>- Structural deformation of the fiber cells by pretreatment<br>- More frequent bends, longitudinal twisting, and kinks in the fibers were observed after the pretreatment, as a result of the weakening of the cell walls and partial collapse of the lumens | Karp et al. (2014) |

* Zeiss LSM Image browser was used for the calculation of the cell wall lumen perimeter and the enclosed area.

### 3.2. Transmission electron microscopy (TEM)

TEM is also among the leading imaging techniques for nanoscale investigation and visualization of the internal structure of the lignocelluloses (Table 3). It is a powerful tool for detecting the cell wall deconstruction by pretreatments. The staining of the samples for the traditional TEM, e.g., by $KMnO_4$ labeling, could help to provide insight into the changes that occur in the lignocelluloses by pretreatments (Donohoe et al., 2008). Furthermore, the cell wall components, e.g., xylan and lignin, can be visualized by immune-TEM (Donohoe et al., 2009). Moreover, electron tomography, an extension of TEM, is used to capture the three-dimensional structure of the lignocelluloses with nanometer resolution (Ciesielski et al., 2013). Donohoe et al. (2012), researchers at NREL, presented suitable methods for the sectioning, preparation, and staining of the samples for obtaining better results in order to understand the lignocelluloses structure by TEM.

The sample preparation, involving the stabilization of the biomass specimens in a resin and obtaining suitable thickness by sectioning, should be done carefully; otherwise, the results obtained may be useless (Yarbrough et al., 2009).

### 3.3. Atomic force microscopy (AFM)

FM, also known as scanning force microscopy (SFM), is used to investigate the topographic, physical, and chemical properties of the lignocelluloses at nanometer resolution that can be used for better understanding of the characteristic features. In a typical AFM, the interaction forces a 1–10 nm probe tip, allowing the sample to be measured. Using the topography imaging, it is possible to follow the structural changes of the biomass fibers (e.g., fiber surface features and fiber cross sectional area) throughout the hydrolysis or pretreatment process (Clarke et al., 2013).

Cell wall boundaries, middle lamellas, and arrangement of the microfibrils can be observed as close as possible to the native state (Yarbrough et al., 2009). It can also be used to investigate the surface properties, e.g., measuring the roughness of a biomass surface. Furthermore, AFM can be used to directly evaluate the interaction forces between the different components (cellulose, lignin, and hemicellulose) as well as the cellulase and the components. Hydrophilic and hydrophobic regions of the lignocelluloses can also be mapped by AFM.

SEM and TEM can provide two-dimensional images, while high resolution three dimensional images can be obtained by AFM without the necessity of sample preparation, staining, dehydration or metal coating (Sant'Anna and de Souza, 2012). For instance, freeze drying, which is shown to be the best way for the sample preparation for SEM, can still be accompanied with some changes in the structure of the biomass (Yarbrough et al., 2009); however, this is not necessary for the AFM analysis.

It is also possible to combine AFM with a variety of optical microscopy techniques, e.g., fluorescent microscopy, for further applicability. One of the limitations of AFM is very low scanning speed. On the other hand, the tip artifacts and data misinterpretation may be observed as a result of an unwanted effect, which is a problem with the auxiliary gap-width-sensing mechanism (Yarbrough et al., 2009). Moreover, the analysis of the AFM images is more difficult than that of the SEM and TEM images.

## 4. Cellulose crystallinity measurement

Unlike starch and hemicellulose, cellulose has a crystalline structure. Its crystallinity is believed to play a major role in its

**Table 3**
Some TEM imaging observations for the pretreated lignocelluloses.

| Lignocellulose | Observation after the pretreatment by TEM | Refs. |
|---|---|---|
| Switchgrass pretreated with ammonia fiber expansion, soaking in aqueous ammonia, lime, dilute sulfuric, and liquid hot water | - Uniform staining pattern across the cell wall layers in the untreated sample indicated uniform distribution of lignin without gaps in the lamella<br>- Cells attached to the lumen surfaces in the untreated samples, but not in the pretreated samples<br>- Lignin re-localization by pretreatment<br>- Globules of coalesced lignin-rich material on the cell wall surfaces and newly formed delamination/pore zones<br>- Delamination of the cell wall lamella<br>- Lignin coalescence in the middle lamella, cell corners, and delamination gaps<br>- Increase in porosity across the width of cell walls by pretreatment<br>- Striking and extensive delamination in the cell walls<br>- Decrease in staining, correlated to the lignin loss<br>- Irregular surface scalloped<br>- Enlarged space formation in the cell corners<br>- Removal of material from the middle lamella<br>- Swollen cell walls and a uniform lignin across the layers of the cell wall<br>- No extensive delamination | Donohoe et al. (2011) |
| Switchgrass pretreated with NaOH | - Delamination of cell walls and generation of extremely low density regions<br>- Pretreated samples containing lower lignin are lighter than the native cell walls<br>- Delamination and intracell wall nanofibrillation resulting in destruction<br>- The reduced contrast of the cell walls by pretreatment is a result of lignin removal<br>- Cellulose microfibrils delamination and disruption | Karp et al. (2015) |
| Corn stover pretreated with ammonia fiber expansion | - Inner cell wall ultrastructure was investigated by TEM<br>- Compound middle lamella and secondary cell walls (S1–S3) were clearly observed<br>- Lignin enriched regions were black/dark gray, while hemicelluloses and cellulose enriched regions were lighter gray<br>- Compound middle lamella collapsed and shifted into the cell corners and outer surfaces by pretreatment<br>- Cellulose microfibrils from the secondary cell walls were shifted outwards<br>- Elementary microfibrils were darker, coated with a thin layer of lignin and/or hemicellulose<br>- Coalescence of inter-lamellar lignin packed between adjacent cellulosic fibrils<br>- Major porosity by the pretreatment was observed in the compound middle lamella and the S1 secondary walls<br>- Extensive interconnectedness of the pore network and increased porosities were observed by pretreatment | Chundawat et al. (2011) |
| Corn stover pretreated with NaOH | - Decreasing staining density (generated by $KMnO_4$) indicated a reduced lignin content in the cell wall by pretreatment<br>- Pretreatment modified the cell wall surfaces by creating a lignin-free surface layer<br>- At high severity pretreatment, lignin was largely coalesced into discrete structures with surrounding area without lignin | Karp et al. (2014) |

biological conversion. An evidence of this claim is very low enzymatic and microbial digestibility of the pure natural cotton fibers, in which no lignin and hemicellulose is present. Neither compositional nor imaging can be used for investigating the crystallinity of the lignocelluloses. Crystallinity is among the parameters that is widely measured and related to the bioconversion of the lignocelluloses (Fig. 4) (Karimi et al., 2013; Shafiei et al., 2015).

Cellulose, a linear β-D-glucan, is the dominant carbohydrates of the plant cell walls. A part of the β-D-glucan in the plants is also

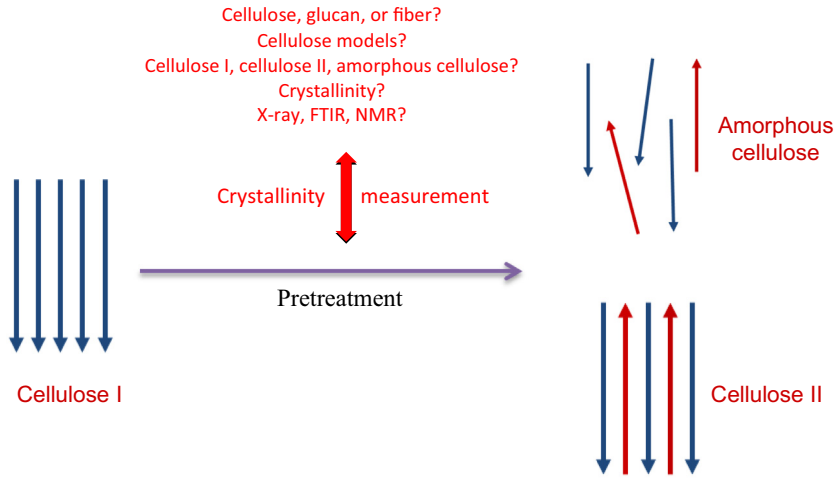


**Fig. 4.** Pretreatment and cellulose models and crystallinity.

available in the hemicellulose. Unlike the glucan in the cellulose, which is mainly crystalline, the hemicellulosic glucan is an amorphous polymer. Therefore, lignocelluloses contain different glucans, categorized as crystalline and amorphous. By mistake, in some literature, cellulose and glucan are equally used, whereas a dominant part of the glucans is in the form of cellulose and a minor part in hemicellulose. For instance, Sluiter et al. (2008b) provide the glucan content, but not the cellulose; however, the measured glucan is widely reported as cellulose.

Cellulose, by itself, consists of regions with a low molecular order (called amorphous regions or cellulose), regions with a very high crystalline order (called crystalline cellulose), and a small amount of matter with an intermediate order (Ciolacu et al., 2011; Klemm et al., 2005). The amorphous regions are able to adsorb the water; moreover, their chemical, enzymatic, and microbial hydrolysis are easier and faster than the crystalline regions (Karimi et al., 2013; Kumar and Wyman, 2013). The amorphous cellulose can be obtained from the crystalline cellulose by a number of physical processes, e.g., ball milling, chemical treatments, e.g., alkaline or acid, and dissolution in some cellulosic solvents, e.g., concentrated phosphoric acid. However, in the presence of water, the constructed amorphous cellulose is thermodynamically unstable and partly changed to crystalline cellulose (Isogai and Atalla, 1991).

### 4.1. Different models of cellulose

Different models are suggested for the crystalline cellulose, i.e., cellulose I, II, III, and VI (Klemm et al., 2005). Cellulose I, also known as Meyer–Misch model, describes the crystalline form of the native cellulose (Mark and Meyer, 1929). This model, developed in 1923, assumes a monoclinic unit cell. It was then further completed by Atalla and Vanderhart in 1984 (Atalla and Vanderhart, 1984), suggesting that the native cellulose is a composite of two different crystal units, cellulose $I_\alpha$ (with one-chain triclinic structure) and cellulose $I_\beta$ (a two-chain monoclinic structure). Cellulose $I_\alpha$ is dominant in cotton, while cellulose $I_\beta$ is dominant in the lignocelluloses (Karimi et al., 2013).

Cellulose II, called regenerated cellulose, is another model, describing the cellulose crystalline structure prepared by precipitating the dissolved cellulose into an aqueous medium. This is the form that is typically detected in the pretreated lignocelluloses. Historically, cellulose II is prepared using the mercerization process, treating native cellulose in NaOH (17–20%). Microbial and algal cellulose typically followed this model. The cellulose in this model has a nonparallel arrangement, and it is more stable thermodynamically than the cellulose I (Chundawat et al., 2011).

Cellulose III can be obtained by treating the native cellulose with cold ammonia or an organic amine, followed by subsequent removal of these reagents. Cellulose III can be reverted into the native form by hot water treatment. Cellulose IV is obtained by treating the cellulose in a suitable liquid at high temperatures (e.g., glycerol at 260 °C or high temperature water) and under tension (Hori and Wada, 2006; Klemm et al., 2005).

Despite the different studies on cellulose and the presentation of different models since 1858, cellulose seems to have a too complex structure to be fully understood and modeled (Park et al., 2010). It should be noted that these are just "models," and generally one should not expect a model to cover the whole real structure. Furthermore, evaluation by following the crystalline cellulose with models is not perfect or easily possible.

### 4.2. Changes in the crystallinity by pretreatment

It is repeatedly reported that the cellulose's "crystallinity reduction" or "lower crystallinity" resulted in a higher rate of bioconversions for the lignocelluloses (Goshadrou et al., 2011; Hall et al., 2010; Jeihanipour et al., 2010b; Ostovareh et al., 2015; Poornejad et al., 2013; Salehian and Karimi, 2013; Teghammar et al., 2012). However, this is not always the case, as several investigations have showed that a higher digestibility is obtainable with more crystallinity. Moreover, in several cases, no relationship between degradability and crystallinity was found. In those cases where a higher crystallinity resulted in a higher digestibility, other factors, e.g., accessible surface area, porosity, lignin and hemicellulose content, and particle size, were the most influencing factors. As an example, in lime pretreatment of corn stover, the amorphous parts are removed, resulting in a highly crystalline residue, which is amenable to enzymatic hydrolysis as it has a highly porous structure (Kim and Holtzapple, 2006). Therefore, the crystallinity is an important characteristic of lignocelluloses for digestibility but not the sole effective factor in all cases (Karimi et al., 2013; Shafiei et al., 2015).

The crystallinity indexes are often defined as the ratio of "crystalline region of cellulose" to "amorphous region of cellulose". This may be suitable for pure cellulose, but not for lignocelluloses. In lignocelluloses, however, "amorphous regions" are available not only for the cellulose, but also for the other parts, including lignin, proteins, pectin, and hemicellulose, or let us say all parts except crystalline glucans. Thus, the term "crystallinity index" definition based on the "amorphous regions" is not clear, although it is widely used in the literature.

The numerical values reported for crystallinity in the literature, often called crystallinity index, are highly dependent on the method of crystallinity measurement, data evaluation procedure, and perfectness of the sample (Terinte et al., 2011).

### 4.3. Methods for measurement of crystallinity

X-ray diffraction (XRD), infrared (IR) spectroscopy, Fourier transform (FT)-IR spectroscopy (FTIR), Raman spectroscopy, terahertz-time domain spectroscopy (THz-TDS), and magnetic resonance (NMR) are the most widely used methods (Park et al., 2010; Vieira and Pasquini, 2014). Among these methods, the most employed method is currently FTIR, XRD, and NMR (Ju et al., 2015).

Crystallinity index (CrI) is a traditional parameter used for the quantitative representation of the crystallinity, indicating the relative amount of the crystalline (ordered) and amorphous (less ordered) regions of a cellulosic structure. The concept of "apparent crystallinity" might also be preferable, as amorphous parts in the lignocelluloses are not only from the cellulose. The CrI values highly depend on the measurement method as well as the data analysis (Park et al., 2010).

#### 4.3.1. X-ray diffraction (XRD)

XRD provides information directly related to the crystal and amorphous parts of the cellulose. From the crystalline cellulosic parts, XRD shows strong signals, related to the crystalline unit cell distances, while the non-crystalline part of the cellulose represents broader and weak signals in the diffraction pattern (Terinte et al., 2011). Obtaining data from XRD is easier; however, the analysis of the data is rather challenging.

Three methods are typically applied to calculate the CrI, based on the XRD data. In 1959, Segal et al. (1959) developed a method for the analysis of the XRD data based on using the focusing and transmission techniques. In this method, known as Segel or peak height method, CrI is simply calculated by dividing the height of (200) peak (the maximum interference; $I_{200}$) and the height of the minimum among the (200) and (110) peaks (the intensity at 2θ = 18°; referred to $I_{AM}$):

$$\mathrm{CrI} = (I_{200} - I_{AM})/I_{200} * 100 \quad (1)$$

This equation was first developed by Ingersoll to study the orientation of the cellulose chains and lateral ordering around the long chain axes by photographical data (Segal et al., 1959). Nowadays, the Segal method is the most widely used method for the calculation of CrI in the lignocellulose for the biofuels and the pretreatments. However, the method has been shown to be not accurate enough, as the exact amount of the crystalline fraction may be more related to the peak area rather than its height (Ju et al., 2015). Furthermore, it has recently been shown that the analytical results are highly dependent on the crystallite size and cellulose polymorph, resulting in significant errors when the crystallite sizes are reduced, e.g., by pretreatment (French and Cintron, 2013).

The second method is based on deconvolution (curve fitting) of the peak for the crystalline and amorphous parts. The CrI, in this method, is calculated based on the intensity of the peaks at (1 1 0), (1 0 2), (2 0 0), or (0 0 4) for the cellulose $I_\beta$ and a single broad peak for the amorphous parts. Gaussian, Lorentzian, and Voigt are among the functions used for the deconvolution of the data. In this method, it is assumed that the peak broadening is mainly due to the increased amorphous contribution, but not the crystallite size and the non-uniform parts. Difficulty in selecting the proper peaks is the main challenge of this method (Ju et al., 2015).

The third common method, known as Ruland–Vonk or amorphous contribution subtraction method, is based on the ratio of the area above an amorphous profile, as a standard, to the total area:

$$\text{CrI}\ (\%) = (\text{Sc}/\text{St}) * 100 \quad (2)$$

where Sc is the area of the crystalline domain and St is the area of the total domain (Ciolacu et al., 2011). The amorphous profile can be obtained from a pattern measured from an amorphous material, e.g., a regenerated cellulose, milled pure cellulose, and lignin powder, or a polynomial function (Ju et al., 2015). The standard amorphous material should be similar to the amorphous components in the samples. This method is very sensitive to instrumental inaccuracies and difficult for comparative studies of samples of different origin; however, it has been widely used to determine the crystallinity of plant fibers (Thygesen et al., 2005). Generally, the method is difficult when analyzing samples that are mostly amorphous, as a result of having a broad diffraction profile (Park et al., 2009). There are several other methods, e.g., Hermans–Weidinger X-ray diffraction, Rietveld refinement, and Debye calculation method, which have been rarely used for the CrI analysis of the cellulose (Thygesen et al., 2005).

A considerable point, limiting the comparison of the data, is when quite different results are obtained by just different methods of data analysis, even for the same XRD data (Vieira and Pasquini, 2014). For instance, for a sample of Avicel cellulose, CrI was varied between 39% and 67%, depending on the method used to analyze the XRD data (Thygesen et al., 2005). The order of the CrI for one sample is: peak height method (Segel) > amorphous subtraction (Ruland–Vonk) > peak deconvolution method (Park et al., 2009).

#### 4.3.2. Fourier transform (FT)-IR spectroscopy

CrI can also be measured using FTIR by measuring the relative peak heights or areas (Park et al., 2010; Noori and Karimi, 2016; Amiri and Karimi, 2015). The crystallinity values of the pure cellulose have been determined and compared in several studies using the XRD, FTIR, NMR, and DSC methods, and very good agreement is observed (Ciolacu et al., 2011). Even in some references, FTIR is suggested to be a more advanced tool for the study of the cellulose structure (Fan et al., 2012).

Among the methods, FTIR is the simplest method with minimal sample preparation without the necessity of specific operation experience; however, it gives only relative values not the absolute values, as contributions from both crystalline and amorphous regions are present in the FTIR spectrum (Fan et al., 2012). Hydroxyl groups in the cellulose are mainly responsible for the intra- and inter-molecular hydrogen bonds and making high-order (crystalline) and low-order (amorphous) regions in the cellulose. All hydrogen bonds of cellulose, responsible for its chemical and mechanical properties, can be analyzed using FTIR. Fourier transform near-IR (FT-NIR) may also be used for the measurement of the same properties (Krongtaew et al., 2010). The relationship between the determination of cotton fiber crystallinity by FTIR or ATR-FTIR and XRD showed a high correlation of $R^2 > 0.90$. For pure cotton, an error of 5–10% or more should be considered (Liu et al., 2012).

In the FTIR spectra, the absorption band between 1420 and 1430 $cm^{-1}$ ($A_{1430}$) is assigned to a symmetric $CH_2$ bending vibration, known as the "crystallinity band", and the band appearing between 893 and 898 $cm^{-1}$ ($A_{898}$) is assigned to C–O–C stretching at β-(1→4)-glycosidic linkages, known as the "amorphous band" (Nelson and O'Connor, 1964).

O'Connor introduced the Lateral Order Index (LOI), as a ratio of $A_{1430}$ on $A_{898}$, to calculate the CrI. LOI, also defined as the empirical CrI, is sensitive to the amount of crystalline versus amorphous regions in the cellulose, and its lower value reflects a more disordered structure (O'Connor et al., 1958). Subsequently, Nelson and O'Connor analyzed cellulose I, II, III, and amorphous cellulose by FTIR (Nelson and O'Connor, 1964) and defined the Total Crystallinity Index (TCI), based on the ratio of the absorption band at 1372–2900. The results were matched well to the results from the XRD in many cases. However, no coherent results were shown for the TCI in the literature (Fan et al., 2012).

Besides crystallinity, cellulose $I_\alpha$ (3231 $cm^{-1}$) and $I_\beta$ (3429 $cm^{-1}$), Cellulose II, III, hydrogen bonding, some information at the supramolecular level regarding lignin, acetyl groups, even the chemical composition of the lignocelluloses can be obtained by FTIR. Fan et al. (2012) stated that "FTIR has been mostly successful in accurate analysis of both major (cellulose, hemicellulose and lignin) and minor (mineral, pectin, waxes) constituents of natural fibers." Change in chemical compositions, interface and hence properties of natural fibers and composites could also be effectively identified by using FTIR. However, we respectfully disagree with this statement for obtaining information for lignocellulosic materials, as dislocation of bands has occurred, the data are not always reproducible, and the absolute values are significantly different, making this method questionable for the indicated applications, particularly compositional analysis. However, to our knowledge, the qualitative analysis for the crystallinity changes by FTIR in the pretreatment always works well, but not by attenuated total reflection (ATR) FTIR.

#### 4.3.3. Nuclear magnetic resonance (NMR)

Solid-state $^{13}C$ nuclear magnetic resonance (NMR) was shown to provide a more accurate measure of the cellulose crystallinity. Besides crystallinity, a detailed structural elucidation of the lignocelluloses components, i.e., cellulose, hemicellulose, and lignin, are also possible by NMR analysis (Pu et al., 2013). Moreover, NMR provides useful information on the ultrastructure of the cellulose, particularly the ratio of the interior-to-surface of cellulose crystallites (Park et al., 2010). This method can also distinguish the cellulose chains located on the surface of the cellulose crystallites. Furthermore, it is possible to analyze the accessible and inaccessible fibril surfaces by NMR, which cannot be detected by XRD. Accessible fibril surfaces are the surfaces of fibrils that are in contact with the solvent or water, and the inaccessible fibril surfaces are the surfaces where the fibrils contacted each other or became distorted in their interiors. Para-crystalline regions of cellulose, which are less ordered than cellulose I and more ordered than

amorphous cellulose, can also be detected and analyzed by NMR (Pu et al., 2013). However, similar to XRD, CrI by NMR is highly dependent on the instrument and data analysis method used (Park et al., 2009).

For analysis of the data obtained by NMR, different methods are presented. Amorphous subtraction and peak deconvolution are more accurate methods for determination of cellulose crystallinity. Deconvolution can be performed by non-linear spectral fitting, using a combination of Lorentzian and Gaussian functions (Hallac et al., 2009). Recently, Park et al. (2009) suggested a procedure for amorphous subtraction, which is simpler and more straightforward. In this method, negative signal is avoided using a scale factor, and the CrI is determined as the ratio of the crystalline area to the total area. Compared with this method, the deconvolution method is less accurate, as it depends on the number of peaks and assumptions on the peak shape.

A serious challenge that can be inferred in the literature is using different methods and different data analysis, making the comparison of the pretreatment questionable.

## 5. Conclusion

Compositional analysis, imaging, and crystallinity are three methods to find the independent properties of lignocelluloses highly affect their bioconversions. The compositional analysis needs special care and consideration, including sample drying, size reduction, presence of non-structural carbohydrates, extractable ash, proteins, starch, and high extractive contents. Imaging can reveal some important aspects, e.g., lignin re-localization and cell wall delamination. SEM, TEM, and AFM are among the suitable and widely used methods. Crystallinity, another important biomass property, can be analyzed by XRD, FTIR, and NMR. FTIR is a suitable method for comparison purposes, while the crystallinity index values are more accurate by XRD and NMR analyses.

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