Structural evaluation of sugar cane bagasse steam pretreated in the presence of CO₂ and SO₂

Roberta Cristina Novaes Reis Corrales¹, Fabiana Magalhães Teixeira Mendes¹, Clarissa Cruz Perrone¹, Celso Sant’Anna²,³, Wanderley de Souza²,³, Yuri Abud², Elba Pinto da Silva Bon⁴ and Viridiana Ferreira-Leitão¹,⁴*

Abstract

Background: Previous studies on the use of SO₂ and CO₂ as impregnating agent for sugar cane bagasse steam treatment showed comparative and promising results concerning the cellulose enzymatic hydrolysis and the low formation of the inhibitors furfural and hydroxymethylfurfural for the use of CO₂ at 205°C/15 min or SO₂ at 190°C/5 min. In the present study sugar cane bagasse materials pretreated as aforementioned were analyzed by scanning and transmission electron microscopy (SEM and TEM), X-Ray Diffraction (XRD) and Infrared (FTIR spectroscopy) aiming a better understanding of the structural and chemical changes undergone by the pretreated materials.

Results: SEM and TEM data showed that the structural modifications undergone by the pretreatment with CO₂ were less pronounced in comparison to that using SO₂, which can be directly related to the combined severity of each pretreatment. According to XRD data, untreated bagasse showed, as expected, a lower crystallinity index (CI = 48.0%) when compared to pretreated samples with SO₂ (CI = 65.5%) or CO₂ (CI = 56.4%), due to the hemicellulose removal of 68.3% and 40.5%, respectively. FTIR spectroscopy supported SEM, TEM and XRD results, revealing a more extensive action of SO₂.

Conclusions: The SEM, TEM, XRD and FTIR spectroscopy techniques used in this work contributed to structural and chemical analysis of the untreated and pretreated bagasse. The images from SEM and TEM can be related to the severity of SO₂ pretreatment, which is almost twice higher. The crystallinity index values obtained from XRD showed that pretreated materials have higher values when compared with untreated material, due to the partial removal of hemicellulose after pretreatment. FTIR spectroscopy supported SEM, TEM and XRD results. CO₂ can actually be used as impregnating agent for steam pretreatment, although the present study confirmed a more extensive action of SO₂.

Keywords: Sugar cane bagasse, CO₂ and SO₂ steam pretreatment, SEM and TEM microscopy, XRD and FTIR spectroscopy

Background

There is a growing urgency to develop novel bio-based products and other innovative technologies that can overcome the widespread dependence on fossil fuels [1]. Unlike gasoline, ethanol is a renewable energy source produced through fermentation of sugar. In Brazil, ethanol is produced largely from sugar cane juice, known as first generation (1G) ethanol. The residual lignocellulosic biomass from the 1G ethanol industry (sugar cane bagasse and leaves) is, presently, for a collection of reasons, the most promising resource for the production of lignocellulosic (2G) ethanol [2]. However, although the sugar-ethanol industry generates bagasse in large quantities during the process of extraction of the sugar cane juice it is mostly used for co-generation, accounting for approximately 3% of the electricity available in Brazil [3].

Lignocellulosic biomass is mainly composed of cellulose, hemicellulose and lignin. The predominant component of lignocellulosic biomass is cellulose, a linear β (1,4)-linked chain of glucose molecules. It is non-toxic, renewable, biodegradable, modifiable and has great potential as an...
excellent industrial material [4,5]. The elementary fibrils are composed of crystalline and amorphous regions. Hemicelluloses are made up of C5 and C6 sugar, such as xylose, arabinose, galactose, glucose and mannose. Lignin accounts for about one fourth of the lignocellulosic biomass and is the third most abundant biopolymer only after cellulose and hemicellulose.

According to Fengel and Wegener [6], four elementary fibrils of cellulose are held together by a monolayer of hemicellulose, which generate 25 nm wide thread-like structures that are enclosed in a matrix of hemicellulose and lignin (associated with each other through physical interactions and covalent bonds).

The main steps for ethanol production from lignocellulosic biomass are pretreatment, hydrolysis, fermentation and distillation/purification. The pretreatment should enhance the fiber accessibility and consequently facilitate the subsequent steps of enzymatic hydrolysis and fermentation [7].

The raw material pretreatment step could represent up to 20% of the total costs of cellulosic ethanol production [8]. According to Galbe and Zacchi [9], an effective pretreatment should (a) improve cellulose digestibility; (b) produce low concentrations of degradation products derived from sugars and lignin; and (c) have a low energy demand.

Previous studies on steam pretreatment of bagasse employed CO2 as impregnating agent to replace the traditionally used SO2 [10]. The use of CO2 was previously investigated in order to explore some advantages of this gas over SO2, such as high availability in the first-generation ethanol plants, low toxicity, low corrosivity and low occupational risk [10,11]. Although the use of CO2 provided equivalent results in comparison to those obtained when SO2 was used as impregnating agent, higher temperatures or longer times were necessary. Comparative results concerning glucose release and inhibitors formation (furfural and hydroxymethylfurfural – HMF) from steam pretreated bagasse were obtained under the conditions: 205°C/15 min using CO2 or 190°C/5 min using SO2. As previously reported by authors [10], the use of SO2 resulted in 79.7% of glucose after enzymatic hydrolysis and provided the formation of 0.80 g/100g of furfural and 0.18 g/100g of HMF (dry bagasse). When CO2 was employed, the yield of glucose reached 86.6% and the values for furfural (0.9 g/100g) and HMF (0.2 g/100g) were very similar to those reported for SO2.

FTIR spectroscopy and electron microscopy have been used for the analysis of structural and morphological modifications in the biomass after pretreatment [12-14]. The present work evaluated structural and chemical changes of SO2 and CO2 steam pretreated sugar cane bagasse in comparison to the untreated material using electron microscopy, X-ray diffraction (XRD) and infrared spectroscopy (FTIR).

Results and discussion

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM)
The use of scanning electron microscopy as an analytical technique proved to be of great importance and versatility for studying the biomass structure. Figure 1 shows the morphological characteristics of the steam pretreated bagasse in the presence of CO2 or SO2 as well as of the untreated material, obtained by scanning electron microscopy (SEM).

Untreated bagasse sample (Figure 1A, B, C) presents a rigid and compact morphology, while the ones submitted to pretreatment with SO2 (Figure 1D, E, F) or CO2 (Figure 1G, H, I) exhibited a more disorganized morphology, with greater exposure of the fibers.

After pretreatment, the most exposed cell wall structure allows for a greater accessibility to hydrolytic enzymes, which facilitates the hydrolysis of lignocellulosic biomass. Transmission electron microscopy (TEM) has been used as a suitable method to determine the effect of pretreatment within the plant cell wall [15]. TEM images of untreated sugar cane bagasse clearly showed that the primary cell wall (PCW), secondary cell wall (SCW) and middle lamella (ML) were well preserved (Figure 2A, B). These structures were bonded strongly together giving rise to a typical highly compact architecture of cell walls. As is a thicker and more rigid structure in the bagasse, the SCW, where cellulose microfibrils are arranged in parallel position, is responsible for cell wall integrity (Figure 2B). The pretreated CO2 samples show, in the cell wall, large pores with different size and shape (Figure 2C, D). Remarkably, most of the pores were formed in the outer region of the cell wall. When SO2 was used as impregnating agent, the secondary cell wall, especially the outer region, was also severely disrupted leading to the appearance of large irregular shaped pores (Figure 2E, F) as a result of partial solubilization of ultrastructural cell wall components. Similar results were recently reported by Chundawat and co-workers [15] in corn stover after ammonia fiber expansion (AFEX) treatment.

The AFEX pretreatment strategy revealed that cellulose hydrolysis increased roughly five-fold when compared to untreated samples. In addition, when both CO2 and SO2 were employed, coalescent particles with round or elongated shapes were found in the cell wall (Figure 2D, F). They seem to be formed by the process of coalescence of cell wall matrix components (hemicelluloses and lignin). The change in cell wall morphology observed when bagasse was pretreated with either CO2 or SO2 result in the increase of the cell wall porosity. At nanoscale, the limited cell wall matrix porosity is considered an important factor that impairs cellulase penetration and accessibility to cellulose fibrils, therefore, contributing to biomass recalcitrance [16,17].
Data from SEM and TEM showed that both pretreatments were effective with respect to structural changes, increasing the surface exposure of the bagasse samples. However, the morphology of CO$_2$ pretreated material is more preserved than that of the SO$_2$ material. This result can be directly related to the higher combined severity for the pretreatment using SO$_2$ (1.7), when compared to combined severity of the pretreatment with CO$_2$ (0.9) as impregnating agent. It is important to emphasize that the combined severity factor [18] takes into account besides time and temperature of the steam pretreatment, the acidity generated in the reaction media by the formation of sulfuric and carbonic acid and the release of organic acids, such as acetic acid from the raw material, as indicated by the pH drop after pretreatment (pH 1.7 (SO$_2$); pH 3.8 (CO$_2$)) [10].

**X-ray diffraction (XRD)**

Figure 3 shows diffractograms of untreated bagasse (A) and sugar cane bagasse pretreated with SO$_2$ (B) or CO$_2$ (C). As can be observed, all samples exhibit typical cellulose diffraction peaks, where the highest peak corresponds to the 002 crystallographic planes. The crystallinity index was calculated according to Equation 1 (Methods session). The untreated sugar cane bagasse showed a lower crystallinity (CI = 48.0%) when compared to samples pretreated with SO$_2$ (CI = 65.5%) and CO$_2$ (CI = 56.4%). Many studies indicate that there is an increase in the value of this index when the biomass is subjected to pretreatment by steam explosion [19]. The phenomenon is due mainly to the removal of a certain amount of lignin and hemicellulose (amorphous substances) and not necessarily due to changes in the crystalline structure of the biomass.

As expected the crystallinity index for the bagasse pretreated with SO$_2$ (65.5%) was higher than that of CO$_2$ pretreated bagasse (56.4%). Indeed, it was observed a more effective removal of hemicellulose to the liquid fraction (63.8%: 7.0% xylose as oligomers and 56.8% of monomeric xylose) using the SO$_2$ steam pretreatment than that using CO$_2$ that showed 40.5% hemicellulose removal: (21.5% of xylose in oligomeric form and 19.0% in monomeric form) [10]. In this work, the increase of the crystallinity index in the pretreated samples is explained by the partial removal of hemicellulose fraction. The amount of glucose released...
from cellulose (amorphous region) was not relevant in the pretreatment step.

The high-pressure steam modifies the plant cell wall structure, yielding a dark brown material from which partially hydrolyzed hemicelluloses are easily recovered by water-washing, leaving a water-insoluble fraction composed of cellulose, residual hemicelluloses and a chemically modified lignin [20].

**FTIR spectra**

In order to understand the changes in the chemical structure after pretreatment, infrared spectra (Figure 4) of the untreated sample (A) and pretreated samples (B and C) were obtained. The assignments given to the absorption bands were referred to the collection of literature Table 1 [21-26].

The band at 1514 cm⁻¹ has been chosen as an internal standard, since this band is present in all spectra and it is well defined.

The main features of these spectra are attributed to the presence of lignin, hemicellulose and cellulose; the natural components of lignocellulose fibers. Infrared spectra of pretreated samples are similar to the untreated ones, which show that the pretreatment conditions did not promote drastic changes in the chemical structure. The values in Tables 1, 2, 3 and 4 represent the relative absorbance of main functional groups stretching (O-H, C-Ph, C=C, OCH₃, C=O).

The absorption at 2920 cm⁻¹ could be attributed to C-H aliphatic axial deformation in CH₂ and CH₃ groups from cellulose, lignin and hemicellulose.

Usually the absorptions of O-H stretching occur in 3100–3600 cm⁻¹ range. The band observed at 3386 cm⁻¹
seems to be characteristic of OH groups present in lignin and carbohydrates. From Table 3, it could be observed that this band has higher values of relative absorbance in the case of pretreated samples when compared to untreated one. This result could be attributed to the chemical changes observed when sugar cane bagasse is pretreated with SO2 or CO2.

The relative absorbance of the bands of primary and secondary OH groups at 1051 cm\(^{-1}\) and 1165 cm\(^{-1}\) of untreated sugar cane bagasse is lower than the pretreated ones (Tables 1 and 3). It is worth noting that the relative absorbance of pretreated bagasse with SO2 is even higher than the one pretreated with CO2. This could be explained by the fact that SO2 provides a lower pH and consequently a higher combined severity, which resulted in a more exposed structure.

According to Nada and co-workers [13], the band at 2852 cm\(^{-1}\) is assignable to vibration of OCH3 groups, which is commonly present in lignin (Table 2). This OCH3 group could also be attributed to acetyl from...
hemicellulose. It could be observed that only a slight increase of 6.5% and 5.5% of the relative absorbance of OCH3 groups after pretreatment using SO2 or CO2, respectively, as expected after an efficient pretreatment process.

When comparing the relative absorbance of the OCH3 group and OH group (Tables 2 and 3), it is possible to note that there was a significant increase in the relative absorbance after pretreatment of OH groups (31.9% and 28.8%, using SO2 and CO2 in the pretreatment, respectively), while this is not observed for the OCH3 groups (6.5% and 5.5%, using SO2 and CO2, respectively). This data indicate the conversion of lignin methoxyl groups into phenolic groups during pretreatment [25].

The bands at 1604 cm⁻¹ and 1633 cm⁻¹ are attributed to C-Ph and C=C, respectively. These bands are generally found in the lignin aromatic structure. The relative absorbance of these vibrations are higher (around 9.3%) after pretreatment (Table 4), which confirms the conversion of lignin methoxyl groups into phenolic groups. The band at 1735 cm⁻¹ is referred to the acetyl groups present in the hemicellulose. The pretreated bagasse with CO2 or SO2 exhibits a higher relative absorbance when compared to untreated sample 16.9% and 15.5%, respectively [10].

The signal at 897 cm⁻¹ is attributed to β-glycosidic linkages between monosaccharide units and it is also higher for the pretreated samples (8.8% and 4.4% with SO2 and CO2, respectively), as expect after bagasse fibers exposure.

**Conclusions**
The analysis by SEM, TEM, XRD and FTIR spectroscopy of steam pretreated bagasse in the presence of CO2 at 205°C/15 min or SO2 at 190°C/5 min showed significant differences amongst the untreated and the pretreated materials. It was observed in the outer region of the cell wall (SCW), upon pretreatment, the formation of large pores with different sizes and shapes which were more

| OCH₃ group bands (cm⁻¹) | Untreated bagasse | Pretreated bagasse with SO₂ | Pretreated bagasse with CO₂ |
|------------------------|------------------|-----------------------------|-----------------------------|
| 2852                   | 0.85             | 0.87                        | 0.89                        |
| 1624                   | 0.88             | 0.95                        | 0.93                        |
| 1427                   | 0.85             | 0.94                        | 0.91                        |
| Mean value             | 0.86             | 0.92                        | 0.91                        |

**Table 3 Relative absorbance of O-H group (cm⁻¹) according to the FTIR spectrum of the sugar cane bagasse**

| OH group bands (cm⁻¹) | Untreated bagasse | Pretreated bagasse with SO₂ | Pretreated bagasse with CO₂ |
|-----------------------|-------------------|-----------------------------|-----------------------------|
| 3386                  | 0.3              | 0.51                        | 0.50                        |
| 1375                  | 0.79             | 0.93                        | 0.90                        |
| 1165                  | 0.51             | 0.71                        | 0.65                        |
| 1051                  | 0.29             | 0.61                        | 0.57                        |
| Mean value            | 0.47             | 0.69                        | 0.66                        |

**Table 4 Relative absorbance of aromatic ring (cm⁻¹) according to the FTIR spectrum of the sugar cane bagasse**

| Aromatic ring bands (cm⁻¹) | Untreated Bagasse | Pretreated bagasse with SO₂ | Pretreated bagasse with CO₂ |
|---------------------------|-------------------|-----------------------------|-----------------------------|
| 1604                      | 0.88              | 0.97                        | 0.94                        |
| 1633                      | 0.89              | 0.99                        | 0.98                        |
| Mean value                | 0.88              | 0.98                        | 0.96                        |
prominent when SO$_2$ was the impregnating agent. It was also observed in both cases the formation, in the cell wall, of coalescent particles with round or elongated shapes likely formed by lignin and/or hemicellulose.

The morphology of CO$_2$-pretreated bagasse was more preserved than that of the SO$_2$-pretreatment, likely due to its lower combined severity factor of 0.9 in comparison to that of SO$_2$, pretreatment of 1.7. It was also observed that both impregnating agents, SO$_2$ or CO$_2$, behaved in a quite similar way.

The crystallinity index values obtained from XRD patterns showed that pretreated materials have higher values (CI (SO$_2$) = 65.5%, CI (CO$_2$) = 56.4%) when compared with untreated material (CI = 42.5%), due to the partial removal from the bagasse of its hemicelluloses content. The results of FTIR spectra also showed changes in the chemical structure of materials pretreated with CO$_2$ and SO$_2$, mainly in OCH$_3$, OH and C=O groups; which supported the data from the XRD, SEM and TEM analysis.

**Methods**

The use of SO$_2$ and CO$_2$ as an impregnating agent for sugar cane bagasse treatment was previously studied [10]. In the present study the most promising pretreated materials were selected for further studies on structural modifications. Bagasse samples steam pretreated in the presence of CO$_2$ (205°C/15 min) and SO$_2$ (190°C/5 min) and also untreated bagasse were submitted to the following techniques: electron microscopy, X-ray diffraction and infrared spectroscopy. All samples were sieved (< 1.8 mm) before analyses.

**Scanning electron microscopy (SEM)**

Scanning electron microscopy (SEM - FEI / Inspect S50 model) was used to observe modifications on bagasse fibers. Samples were adhered to carbon tape and sputter coated with gold (sputter Emitech / K550 model) and observed in the SEM through the use of an acceleration voltage of 20 kV. SEM images were obtained on different areas of the samples to guarantee the reproducibility of the results.

**Transmission electron microscopy (TEM)**

Transmission electron microscopy (FEI Tecnai G2 12 Spirit) was used to observe the ultrastructural changes within the cell wall. Each condition (untreated material, pretreated with SO$_2$ and CO$_2$ material) was analyzed in triplicate. Each individual sample was studied by an unbiased random selection of fibers that represent the total population. Samples were dehydrated in an increasing acetone series and embedded in Spurr resin. Ultrathin sections, 70nm, were obtained in the LEICA ultramicrotome and deposited onto copper grids. The sections were stained with 5% uranyl acetate and lead citrate and observed in the TEM with an acceleration voltage of 120 kV.

**X-ray diffraction (XRD)**

Crystallinity of the cellulose fibers was evaluated by X-ray diffraction by means of a Diffractometer MiniFlex – Rigaku and filtered copper K$_\alpha$ radiation ($\lambda = 0.1542$ nm) by a monochromator at 30 KV voltage and 15 mA electric current, with a speed of about 2 degrees per minute and scanning at an angle (2θ) in the range of 2-60. The crystallinity of lignocellulose biomass accounts for the relative amount of total crystalline cellulose in the solid component. The crystallinity is strongly influenced by the composition of biomass; the relative amount of lignin, hemicellulose and cellulose varies according to the nature of the biomass. The crystallinity index (CI) was obtained from the ratio between the intensity of the 002 peak (I$_{002}$, 2θ = 22.5) and the minimum dip (I$_{am}$, 2θ = 18.5) between the 002 and the 101 peaks according to Equation 1 [14,27].

\[
\%CI = \left( \frac{I_{002} - I_{am}}{I_{002}} \right) \times 100
\]

Where $I_{002}$ is the intensity of plane 002 and $I_{am}$ is related to the amorphous structure.

**Infrared spectroscopy (FTIR)**

The infrared spectra (wave numbers in cm$^{-1}$) were obtained on a Magma - IR 560 E.S.P – Nicolet spectrophotometer, by means of a KBr disk containing 3% finely ground samples. Thirty-two scans were taken of each sample recorded from 4000 to 400 cm$^{-1}$ at a resolution of 4 cm$^{-1}$. The relative absorbance values were obtained with four decimal units; however, only two decimal units were plotted in the data showed in Tables 1, 2, 3 and 4.

**Abbreviations**

FTIR: Fourier transform infrared spectroscopy; SEM: Scanning electron microscopy; TEM: Transmission electron microscopy; XRD: X-Ray diffraction spectroscopy; CI: Crystallinity index.

**Competing interests**

The authors declare that they have no competing interests.

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**Author details**

1National Institute of Technology, Ministry of Science and Technology, Av. Venezuela, 82, sala 302, CEP 20081-312 Rio de Janeiro - RJ, Brazil.
2National Institute of Metrology, Standardization and Industrial Quality, Av. Nossa Senhora das Graças, 50 – Xerém, CEP 25250-020 Duque de Caxias - RJ, Brazil.
3National Institute of Science and Technology in Structural Biology and Bioimagers, Federal University of Rio de Janeiro, Av. Pedro Calmon, 550,
Prédio da Reitoria - sala 801, Ilha do Fundão - CEP 21941-001, Rio de Janeiro – RJ, Brazil. *Department of Biochemistry, Institute of Chemistry, Federal University of Rio de Janeiro. Av. Athos da Silveira Ramos, 149, bloco A, Ilha do Fundão, CEP: 21941-909 Rio de Janeiro - RJ, Brazil.

Authors’ contributions
ML and CP designed and carried out the experiments of pretreatment, the characterization of biomass, the discussion of results and revision of the manuscript. RC carried out SEM and XRD experiments and prepared the manuscript. FM carried out FTIR spectroscopy experiments and analyzed the results. CS and YA performed TEM analysis and discussed the results. EB and WS discussed the results and reviewed the manuscript. All authors read and approved the final version of the manuscript.

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