



## Short Communication

# Influence of ozonolysis time during sugarcane pretreatment: Effects on the fiber and enzymatic saccharification



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## HIGHLIGHTS

- Secondary cell wall is the structure most affected by ozonolysis treatment.
- High yield conversion of cellulose using OBU treatment.
- Fiber changes during increase in ozonolysis treatment time were observed.
- SEM and TEM techniques demonstrated changes in fiber cells after ozonolysis treatment.

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## ABSTRACT

Modifications in sugarcane bagasse (SCB) from ozonolysis (O) NaOH (B) and ultrasound (U) (OBU) treatment for cellulosic ethanol production by enzymatic hydrolysis, were evaluated when increasing the exposure time of SCB to ozone. The lignin, cellulose, and hemicellulose after treatment were quantified: lignin removal and a consequent increase in cellulose content were shown using an infrared spectroscopic technique (ATR-FTIR) and chemical characterization. X-ray diffraction analysis (XRD) proved that OBU treatment does not affect the crystalline cellulose portion and electron microscopy techniques established that the fiber region most affected by the OBU treatment was the secondary cell wall, where the greatest lignin content is located. For OBU-60 treatment the lignin content was reduced and consequently there was a significant increase in cellulose content. After enzymatic hydrolysis, this pretreated SCB released 418 mg glucose/g, corresponding to six times more than untreated SCB and a yield of 93% of the cellulose available.

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## 1. Introduction

The search for renewable energy has grown in recent decades, as a strategy to replace fossil fuels and for green energy policies. The lignocellulosic biomass has great potential for power production because it has low cost, is renewable, it is readily available and has high fermentable sugars content for ethanol production and is the main material used in a biorefinery (Travaini et al., 2016a). In the 2015/16 season, for example, Brazilian ethanol production reached 30.2 billion liters (UNICA, 2016) and, in 2015, the sugar plants injected, into the interconnected system, 5% more

electrical energy than in 2014 (EPE, 2016). Biomass from sugarcane has an advantage over other lignocellulosic materials because it does not compete directly with food crops or require more field space. This is because, even after the use of sugarcane bagasse as an energy source in the co-generation of electricity, there is a considerable excess of residual biomass in the mills, as well as an abundance of straw deposited in the fields. This can be converted into ethanol by enzymatic hydrolysis and the subsequent fermentation of its polymerized sugars.

The fiber of sugarcane bagasse structure contains approximately 50% cellulose, 25% hemicellulose and 25% lignin (Travaini et al., 2014). However, the lignocellulosic material is highly recalcitrant, thus hindering the access of hydrolytic enzymes (Pandey et al., 2000). The cellulose present in vegetable fiber is divided into: (i) a crystalline portion formed from cellulose chains connected by a hydrogen bond in an organized way, and (ii) an amorphous

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portion of cellulose, less organized, more soluble and more easily degradable. To overcome lignocellulosic recalcitrance, different pretreatments of biomass are being investigated, such as steam explosion (STEX) (Scholl et al., 2015), liquid hot water (LHW) (Zhuang et al., 2016) and microwaves (Binod et al., 2012). This work has a particular focus on unpressurized processes, and among the possibilities in this area, ozonolysis is a promising alternative.

The ozone is a powerful electrophile due to an electron deficiency in one of the terminal oxygens during resonance. Thus, an ozone reaction with the lignocellulosic substrate mainly affects compounds with high electron density, such as double C–C bonds and aromatic rings, making the ozone attack the lignin structure in preference to the carbohydrates (Travaini et al., 2016a). The degradation of lignin and the consequent breakdown of the lignocellulosic structure increases the efficiency of the enzymatic hydrolysis procedure, making cellulosic ethanol production possible.

The alkali treatment was reported to be effective in the removal of hemicellulose and lignin fragments (Chaudhary et al., 2012) while treatment with ultrasound in an aqueous medium produces a phenomenon known as acoustic cavitation that can disrupt cell walls and facilitate the penetration of solvents in the cellulosic materials improving the alkaline effect in the treatment (Velmurugan and Muthukumar, 2012). The breakdown of lignin caused by ozonolysis, added to the effects of washing by alkaline assisted by ultrasound results in an efficient treatment.

Some studies using ozone as a biomass pretreatment obtained 80% (Li et al., 2015) and 84% (Travaini et al., 2016b), cellulose conversion to glucose after the enzymatic hydrolysis of the pretreated material. The combination of ozonolysis (O) and subsequent washing with NaOH (B) in ultrasound (U), produced excellent results with glucose conversion yields of 94% of the cellulose available, in previous work (Perrone et al., 2016). Ozonolysis in biomass treatment has been widely reported and the use of this oxidant has produced good results due to low formation of inhibitor compounds and also high glucose yields obtained during the enzymatic hydrolysis. However, there are no studies that demonstrate the effect of increased content of ozone during the treatment on the fiber. In this study, the modifications caused in the lignocellulosic material fiber due to an increasing ozonolysis time during sugarcane pretreatment were evaluated. The impact of different treatments on the physical structure of the sugarcane bagasse was evaluated by chemical compositional analysis. The X-ray diffraction technique (XRD) was used to verify changes in crystallinity. Infrared spectroscopy (ATR-FTIR) was used for the analysis of chemical changes in the functional groups from lignin, cellulose and hemicellulose, and microstructural analysis to understand the physical variations in the material morphology after treatments was done using scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The yield of the enzymatic hydrolysis was also evaluated using high-performance anion-exchange chromatography (HPAEC-PAD).

## 2. Material and methods

### 2.1. Sugarcane bagasse and pretreatment

Sugarcane bagasse (SCB) was collected from Usina Vale, in Onda Verde, São Paulo State, Brazil (2013/2014 harvest). The SCB was washed three times with hot distilled water at 40 °C to remove any soluble sugar. The SCB was ground and sieved to obtain fibers less than 3 mm long. These fibers were dried in a fan oven at 40 °C for 24 h. Ozone gas was obtained using the Radast 10C generator, (Ozoxi-ozone, São Paulo State, Brazil) and the ozone flow was 32 mg O<sub>3</sub> min<sup>-1</sup>, the same way as in the previous study (Perrone

et al., 2016). In each test, 20 g of bagasse was pretreated in ozonolysis with exposure times of 5, 10, 20, 40, 60, 90, 120 and 180 min. After the process of ozonolysis, 2 g of this material were transferred to flasks (250 mL) containing 40 mL of NaOH 0.1 mol L<sup>-1</sup> (B) and left to stand for 2 h. After that, the material was treated with ultrasound irradiation (U) for 5 min. using an ultrasonic probe (Fisher Scientific, Model 50 Sonic Dismembrator), operating at a frequency of 22 kHz and power of 50 W.

### 2.2. Physical analysis

ATR-FTIR spectra were collected using a Perkin-Elmer FTIR Spectrum Two. For ATR-FTIR analysis, approximately 0.1 g of the dried SCB samples were compressed to form discs (13 mm diameter and 1 mm thickness). The disc samples were pressed against the ATR interface with the same pressure for each sample. The air was used as background and all spectra were recorded in an average of 4 scans at a resolution of 4.0 cm<sup>-1</sup>. Due to the heterogeneity of the SCB, the disc position was changed so that the infrared light could be pointed at four different places. XRD patterns of SCB were obtained on a Rigaku® diffractometer model MiniFlex 300 using CuK $\alpha$  radiation ( $\lambda = 1.54 \text{ \AA}$ ) in an angular range of 5–40° at 2 $\theta$  min<sup>-1</sup> at 25 °C. The crystallinity index (I<sub>cr</sub>) was calculated following the method proposed by Seagal (Terinte et al., 2011). Structural carbohydrates, cellulose, hemicellulose and lignin fractions and ash of the bagasse were determined using the National Renewable Energy Laboratory–USA (NREL) analytical procedures NREL/TP 510-42618 (Sluiter et al., 2008).

### 2.3. Scanning electron microscopy (SEM)

The bagasse was fixed in 2.5% glutaraldehyde in a 0.1 mol L<sup>-1</sup> phosphate buffer (pH 7.3) for 48 h at room temperature. Next, the material was washed with distilled water and post-fixed in 1% osmium tetroxide diluted in distilled water for 30 min at room temperature. Following fixation, the bagasse was dehydrated in a series of ethanol washes, critical point-dried with CO<sub>2</sub>, and sputter coated with gold (Bal-Tec SCD 050). The samples were examined using an FEI Quanta 200 scanning electron microscope (FEI Company, Eindhoven, Netherlands) with an accelerating voltage of 12.5 kV.

### 2.4. Transmission electron microscopy (TEM)

The bagasse was fixed in a 2.5% glutaraldehyde and 4% paraformaldehyde solution in a 0.1 mol L<sup>-1</sup> phosphate buffer (pH 7.3) for 24 h at room temperature and post-fixed in 1% osmium tetroxide in the same buffer for 2 h at room temperature. After washing with distilled water, the material was block stained in 0.5% uranyl acetate for 2 h at room temperature. Next, the samples were dehydrated in a graded acetone series and embedded in Araldite® resin. Ultrathin sections were cut with a Leica ultramicrotome (EM UC7; Leica Microsystems, Wetzlar, Germany) and stained with uranyl acetate and lead citrate. The analyses were performed using a Tecnai Spirit transmission electron microscope (FEI Company, Eindhoven, The Netherlands). SEM and TEM analysis were performed at the Electron Microscopy Center of the Biosciences Institute (UNESP, Botucatu-SP, Brazil).

### 2.5. Enzymatic hydrolysis

Enzymatic hydrolysis of 0.25 g of both dried untreated and pretreated bagasse was carried out in glass flasks with rubber stoppers with 10 mL of acetate buffer 0.1 mol L<sup>-1</sup>, pH 5.0, containing cellulase (Prozyn®, São Paulo, Brazil) at a dosage of 32 FPU per gram of bagasse (dry basis). An enzyme reaction was performed at 60 °C,

150 rpm for 24 h. After hydrolysis, samples were filtered through a 0.22 µm filter and the liquid used for qualitative and quantitative analysis. Cellulose (or hemicellulose) conversion as calculated by using the equation: % glucose (or xylose) conversion =  $[(c * v * 0.9)/m] \times 100\%$ , where *c* is the concentration (g L<sup>-1</sup>) of sugars in the enzymatic liquor hydrolyzed, *v* (L) is the volume of liquor, and *m* is the glucan (cellulose or hemicellulose) content (g). Glucose and xylose were quantified using an ICS 5000 Dionex HPAEC-PAD ionic chromatograph with anionic column CarboPac PA-1 (Perrone et al., 2016).

### 3. Results and discussion

#### 3.1. Ozonolysis influence in the sugarcane pretreatment

Due to the recalcitrance of the SCB cell wall, its deconstruction by pretreatment is necessary for subsequent efficient enzymatic hydrolysis. The treatment sequence ozonolysis (O), NaOH (B) and ultrasound (U) was chosen for additional ozonolysis tests because this pretreatment sequence of sugarcane bagasse gave the best yields in the previous study (Perrone et al., 2016). The structural carbohydrate and lignin compositions of each ozonolysis time treatment are shown in Table 1.

A reduction in the lignin and hemicellulose content was achieved for all treatments compared to the untreated SCB. The lignin content in the SCB is 24.5%, and after 180 min of ozonolysis, it was reduced to about 12%. The highest lignin loss occurred between 10 and 20 min of ozone exposure, and from that treatment (OBU-20) on the enzymatic yield increases. Hemicellulose is slightly affected by the treatment, mainly due to the alkaline hydrolysis of intermolecular ester bonds between lignin and hemicelluloses after the NaOH washing process (Chaudhary et al., 2012). The treatment with NaOH and ultrasound (OBU-0) does not significantly modify the material, and a small decrease in the hemicellulose content can be seen. But, by increasing the ozonolysis treatment time, the hemicellulose content decreases from 18.9% (SCB-0) to 14.7% (OBU-180).

On the other hand, there is an increase in cellulose content from 44.8% to 60.6% after 90 min in ozonolysis and decreasing to 56.8% at 180 min. The reduction in the cellulose content observed between the OBU-90 and OBU-180 sample can be related to the degradation of carbohydrates by direct ozone reaction or due to the action of OH<sup>•</sup> on random glucosidic linkages, resulting in the formation of carboxylic groups from carbohydrates (Travaini et al., 2014). This observation indicates that ozonolysis treatment degrades lignin but, after a given time, excess ozone can affect the carbohydrate portion, leading to a reduction of cellulose content as observed in the chemical characterization (Table 1).

Enzymatic hydrolysis of the treated materials is also an important test for evaluating treatment efficiency. After performing hydrolysis, an increase in cellulose conversion into glucose can be seen for all the treated materials. The low yields of the enzyme hydrolysis in times less than or equal to 10 min can be related to the low lignin removal and or partial breakdown of lignin, generating compounds that potentially inhibit the enzymatic action. Conversion yield for the enzymatic hydrolysis increases from 31% in OBU-20 treatment to 80% in OBU-40 treatment and stabilizes at 93% in OBU-60 treatment; these results indicate that significant changes occur in the fiber after 20 min of ozonolysis treatment, leading to increasing in enzyme yield. However, the enzymatic yield decreases in OBU-120 and OBU-180 treatments (79% and 65%, respectively). According to Travaini et al. (2016b), reactions between ozone and carbohydrates are 10<sup>6</sup> times slower than between ozone and lignin. Moreover, the high lignin removal can directly affect the enzymatic hydrolysis because small amounts of lignin in the substrate can favor the enzymatic process (Zhuang et al., 2016). In these treatments (OBU-120 and OBU-180), possibly all the lignin is unstructured and, from this stage, the ozone begins to act in carbohydrate degradation. In addition, the absence of lignin in the substrate contributed to lower enzymatic efficiency.

#### 3.2. XRD analysis

Treatments under alkaline conditions and elevated temperatures, the cellulose chains expand due to base diffusion within the crystalline cellulose. After treatment, the chains will undergo rearrangements and this effect will modify the crystal structure and consequently cause decreases in Icr (Maryana et al., 2014). On the other hand, for treatments without changes in crystalline cellulose fraction, the Icr does not change. In the literature, many studies reported increases in Icr after treatment for both alkaline treatments (Velmurugan and Muthukumar, 2012; Velmurugan and Muthukumar, 2011; Binod et al., 2012) and acid treatments (Sindhu et al., 2010; Sindhu et al., 2013; Pereira et al., 2016). Using the OBU treatment sequence at ambient temperature and pressure a decrease in Icr is not expected. In fact, after each treatment, there was an increase in Icr for all samples when compared to the SCB-0.

The biggest increase occurs from 65% to 68% after 20 min of ozonolysis, and stays at approximately 68% until 180 min (OBU-180) (Fig. S1). This happens because the ozonolysis treatment causes the lignin structure to breakdown and the subsequent washing with NaOH removes hemicellulose, both being lignocellulosic amorphous components, while the most crystalline fraction, the cellulose remains (Moretti et al., 2016). This hypothesis is supported by the data from the compositional analysis, since, there was a decrease in the lignin and hemicellulose content and an

**Table 1**

The composition of insoluble fractions and content of glucose released from enzymatic hydrolysis after pretreatment process.

Pretreatment		Composition after pretreatment (%)			Enzymatic hydrolysis (mg g <sup>-1</sup> )	
	Ozonolysis <sup>a</sup>	Cellulose	Hemicellulose	Lignin	Glucose	Yield (%)
SCB-0	Untreated	44.8 ± 0.1	18.9 ± 0.1	24.5 ± 0.1	59 ± 7.8	13
OBU-0	0	45.6 ± 1.7	16.7 ± 1.0	23.1 ± 2.5	67 ± 11	15
OBU-5	5	50.7 ± 1.6	17.3 ± 0.4	22.9 ± 1.6	80 ± 9.3	18
OBU-10	10	52.8 ± 0.9	15.9 ± 0.2	22.2 ± 0.9	85 ± 6.4	19
OBU-20	20	54.7 ± 1.5	14.8 ± 1.6	15.5 ± 1.5	137 ± 12	31
OBU-40	40	58.1 ± 2.6	14.8 ± 0.8	15.7 ± 2.6	360 ± 22	80
OBU-60	60	58.4 ± 0.5	14.2 ± 0.3	14.3 ± 0.5	418 ± 9.2	93
OBU-90	90	60.6 ± 0.6	14.7 ± 0.9	12.8 ± 0.6	416 ± 14	93
OBU-120	120	58.1 ± 1.9	14.6 ± 0.7	11.9 ± 1.9	352 ± 8.7	79
OBU-180	180	56.8 ± 1.3	14.7 ± 0.3	12.1 ± 1.3	293 ± 19	65

<sup>a</sup> In minutes (32 mg min<sup>-1</sup>).

increase in the proportion of cellulose. In this case, the treated material resulted in a higher glucose yield after enzymatic hydrolysis indicating that an increase in cellulose availability, and lignin and hemicellulose removal are a more important factor for enzymatic hydrolysis than just decreasing the crystallinity of cellulose.

### 3.3. ATR-FTIR spectroscopy

The bands related to the infrared absorption by the aromatic rings from lignin structure are mainly found in the spectra in the region of 1600, 1510, 1426 and 833  $\text{cm}^{-1}$ . The first three bands correspond to aromatic skeletal vibrations (Liu et al., 2007) and the last one is due to the C–H bond in *p*-hydroxyphenyl moieties stretching out of plane. In addition to these bands associated with the lignin presence, various infrared absorption bands are present in the spectra, all attributed to cellulose/hemicellulose and some functional groups in the lignin. Bands associated with glycosidic linkages  $\beta(1\rightarrow4)$  are found at 897  $\text{cm}^{-1}$  (Adebajo and Frost, 2004). Moreover, the band at 1248  $\text{cm}^{-1}$  is attributed to absorption by C–O stretching in acetyl moiety in the hemicellulose. The strong band at 1035  $\text{cm}^{-1}$  is due to C–O stretching in cellulose, hemicellulose and lignin or to C–O–C stretching in cellulose and hemicellulose (Liu et al., 2007). Fig. S2 shows the ATR-FTIR spectra from SCB-0 and SCB treated for different times by ozonolysis. When comparing SCB-0 with the ozonolysed materials, decreases in the absorption band at 1248  $\text{cm}^{-1}$  can be seen, indicating the removal of hemicelluloses after treatment, probably due to NaOH washing. This observation is in agreement with the data from the compositional analysis (Table 1). Significant changes occur in the lignin structure and characteristic bands of this component at 833, 1426, 1510 and 1600  $\text{cm}^{-1}$  decrease and disappear almost entirely after 20 min of ozonolysis confirming the high degree of removal of this polymer after treatment. As noted by the structural characterization of the material and also by the Icr, a minimum of 20 min under ozonolysis treatment (in the conditions used in this work) are necessary for an effective change in the fiber material. The reduction of the absorption bands associated with lignin and slight changes in bands associated with hemicellulose and cellulose proves that the ozonolysis treatment is a selective treatment and especially degrades the lignin.

### 3.4. SEM and TEM analysis

Fig. S3 shows the SEM images from SCB-0, and OBU-5, OBU-60 and OBU-180 treatments. The images from SCB-0 show a smooth, preserved and intact surface. The increase in the ozonolysis treatment time intensified the damage to the fiber and some plates are fragmented and detached from the material surface, these changes being most intense in the OBU-180 treatment. SEM images confirm that increasing ozonolysis treatment time increased the disintegration of the material. Other authors, even using other treatment methods, also observed similar changes on the surface of the materials (Chandel et al., 2013; Moretti et al., 2016). These changes can increase the superficial area and lead to the appearance of a significant number of reactive sites in the material, which increases the accessibility of the enzyme and thus the efficiency of the hydrolysis (Zhou et al., 2016).

Fig. S4 shows TEM images from SCB-0 and OBU-5, OBU-60 and OBU-180 treatments. The electron density of each photograph can be used to indicate the abundance of cellular material in the primary cell wall (PW), secondary cell wall (SW), and middle lamella (ML) structures. SCB-0 images show no difference in the electron density from these structures, they are preserved and show no changes. Thus, when observing the OBU-5 the ML and PW structures are preserved and undamaged. In the image for OBU-60 treatment, ML disruption is not seen, but there is some disruption

in the SW. However the OBU-180 treatment shows both ML and SW disruption. Both OBU-60 and OBU-180 showing a significant electron density decrease in the SW structure, more intense from (a) to (b), as shown in Fig. S4. This is indicative of the component cell reduction. The greatest amount of lignin is contained in the SWs and the decrease in electron density means that ozonolysis treatment mainly affected the lignin in this structure. This data is confirmed by the reduction in lignin after ozonolysis treatment shown in Table 1.

## 4. Conclusion

The increase in ozonolysis time demonstrates the need to use a controlled amount of ozone during treatment to occur the desired disruption of the lignin in the fiber and an increase in the enzymatic hydrolysis yield. Excess of ozonolysis causes carbohydrate degradation. Larger amounts of cellulose available and also the best enzymatic hydrolysis yields were achieved with 40–90 min of ozonolysis, corresponding to production of about 64–144 mg ozone  $\text{g}^{-1}$  of sugarcane bagasse. Microscopy showed that the SW material is the most affected by the action of ozone.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.biortech.2016.11.043>.

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