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# Ozonolysis combined with ultrasound as a pretreatment of sugarcane bagasse: Effect on the enzymatic saccharification and the physical and chemical characteristics of the substrate

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## HIGHLIGHTS

- Combination of $O_3$-NaOH-Ultrasound as pretreatment of sugar cane was evaluated.
- $O_3$-NaOH-Ultrasound pretreatment promotes high delignification of biomass.
- $O_3$-NaOH-Ultrasound pretreatment occurs at ambient temperature and pressure.
- Glucose yield of 391 mg/g (95%) was obtained after pretreatment.
- Changes in fiber due to the pretreatment have been demonstrated.

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## ABSTRACT

Sugarcane bagasse (SCB) was treated in three stages using ozone oxidation (O), washing in an alkaline medium (B) and ultrasonic irradiation (U). The impact of each pretreatment stage on the physical structure of the SCB was evaluated by its chemical composition, using an infrared technique (FTIR-ATR), and using thermogravimetric analysis (TGA/DTG). The pretreatment sequence O, B, U provided a significant reduction of lignin and hemicellulose, which was confirmed by changes in the absorption bands corresponding to these compounds, when observed using infrared. Thermogravimetric analysis confirmed an increased thermal stability in the treated sample due to the removal of hemicellulose and extractives during the pretreatment. This pretreatment released 391 mg glucose/g from treated SCB after the enzymatic hydrolysis, corresponding to a yield of 94% of the cellulose available.



## 1. Introduction

Demand for ethanol is likely to increase over the coming decades worldwide due to the mandatory ethanol/gasoline blend that has been adopted in countries around the world. First generation bioethanol is produced by distillation from crops such as wheat, corn, sugar cane and sugar beet. Together Brazil (sugar cane) and the USA (corn) account for approximately 83% of world production of ethanol. Environmental organizations have proposed to limit biofuel produced from "food crops", due to concerns about food prices and the impact of land use. Ethanol can also be produced from other energy raw materials, which do not compete with food markets, e.g. cellulosic ethanol (a second generation biofuel), which can be produced from a wide range of feedstock, including agricultural residues.

In the 2014/2015 season, Brazil produced 632 million tons of sugarcane, which resulted in 35 million tons of sugar and a total of 28 billion liters of ethanol (anhydrous + hydrated), generating about 190 million tons of sugarcane bagasse (UNICA, 2016). A part of this material can be burned for electricity generation, but a considerable part (more than 16 million tons) still remains at the ethanol production plant (Moretti et al., 2014). Due to its high carbohydrate content, this raw material can be used for cellulosic ethanol production with a projection of producing up to 3 billion liters per year of cellulosic ethanol (Moretti et al., 2014). According to UNICA, (2016), two plants were built in Brazil with capacity to produce in total, up to 182 million liters of 2G ethanol per year.

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However, cellulosic ethanol requires a more complex production process (cellulose hydrolysis). The lignocellulosic material is highly recalcitrant due to the presence of lignin, crystalline cellulose and cross-links between hemicellulose and lignin hindering the access of the enzyme to the substrate and reducing the yield of the hydrolysis, making the process economically unviable (Pandey et al., 2000). Therefore, it is necessary to perform pretreatment of the pulp in order to change the structure of the lignocellulosic material. An ideal pretreatment must alter the fiber structure without loss of carbohydrates, should increase the efficiency of the enzymatic hydrolysis, must not form byproducts that inhibit fermentation and must also have low cost (Mosier et al., 2005).

Ozone treatment has been shown to be effective in the breakdown of the plant cell wall. Ozone is highly reactive with compounds having conjugated double bonds and functional groups having high electron density, such as lignin and has the advantage that it does not generate compounds which are inhibitors of enzymes or of the fermentation process (Travaini et al., 2013). Ultrasound works by means of a phenomenon called acoustic cavitation and has been used in various studies as a pretreatment of lignocellulosic materials (Liu et al., 2007; Sun et al., 2004; Velmurugan and Muthukumar, 2011). Alkaline pretreatment of lignocellulosic materials has also been reported as a pretreatment for the deconstruction of lignocellulosic materials (Fan et al., 1987; Bi et al., 2016).

Various pretreatment processes, which can disrupt cellulose crystallinity and increase the porosity of the biomass, have been shown to be effective in enhancing the enzymatic hydrolysis of sugarcane bagasse. These processes include the use of: mechanical milling, steam explosion, liquid hot water, dilute acid, alkali, alkaline hydrogen peroxide, organic solvents, microwave-acid/alkali, biological process and ionic liquid. However, existing pretreatment methods are still encountering many problems such as cost-effectiveness, high power usage, environmental contamination, formation of enzyme inhibitors or ethanol fermentation and high consumption of chemicals (Mosier et al., 2005; Bi et al., 2016). Besides, some of them require corrosion-resistant and high quality reactors, due to acid and/or pressure. Consequently, there is still space for new processes that can contribute to overcoming the existing bottlenecks. The use of ozone in the pulp and paper industry and ozone and ultrasound for waste water treatment have been shown to be technically feasible by numerous reports in the literature in recent years (Travaini et al., 2016; Mahamuni and Adewuyi, 2010). But, to date, there are no reports which address the feasibility of the use of ultrasound combined with ozone for pretreatment of SCB. The advantage over other pretreatments is that they can operate under mild conditions (room temperature, atmospheric pressure), production of inhibitors is negligible and there is no need for corrosion-resistant and high quality reactors. In this economic sense, the strategy of this pretreatment is a novelty, compared to those already available.

The paper industry promotes the delignification of pulps by a process frequently composed of more than one discrete step called a bleaching sequence. So, it will be interesting to use the same strategy for pretreatment of biomass aiming at improving the efficiency of process. Most reports have used a pretreatment based on ozone (O) and ultrasound (U). The objective of the current work was to combine these two different operational parameters, with alkaline washing (B) and to evaluate the sequence O, B, U as a pretreatment to enhance the enzymatic hydrolysis of sugarcane bagasse. The yield of the enzymatic hydrolysis and the concentration of inhibitor compounds were evaluated using HPAEC-PAD. The impact of different treatments on the physical structure of the sugarcane bagasse was evaluated using the chemical composition, attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy, and thermogravimetric analysis (TGA).

## 2. Material and methods

### 2.1. Materials

Sugarcane bagasse (SCB) was donated by Usina Vale, from the village of Onda Verde, São Paulo State, Brazil. 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). The ultrasonic irradiation was performed using an ultrasonic probe from Fisher Scientific, Model 50 Sonic Dismembrator, operating at a frequency of 22 kHz and power of 50 W.

### 2.2. Treatment with ozone, alkali and ultrasound

The experiment followed the selected sequence of pretreatments such as ozonization, alkalinization and ultrasonication. 20 g of dry SCB were moistened with 10 ml of distilled water and packed in glass columns (2.7 cm diameter × 50 cm length) and subjected to treatment with ozone (O), using the method described by Travaini et al. (2013). The ozone flow was 32 mg $O_3$/minute for 60 min. Aliquots of 2 g of the treated SCB were transferred to flasks (250 ml) containing 40 ml of NaOH 0.1 mol $L^{-1}$ (B) and left to stand for 2 h. After that, the material was treated with ultrasound irradiation (U) for 5 min. After each pretreatment stage, the soluble phase was obtained by centrifugation or filtration and used for the quantification of total phenolic compounds (TPC), inhibitors furan, furfural (F) and hydroxymethylfurfural (HMF). Similarly, after each pretreatment stage the insoluble fraction (solid) was air-dried and used in chemical composition analysis, structural analysis by infrared (ATR-FTIR) and thermogravimetry (TGA and DTG), and additionally, enzymatic hydrolysis. Experiments of SCB without OBU treatment were performed as a control. All experiments were conducted at least in duplicate.

### 2.3. Chemical composition analysis

The structural carbohydrates, cellulose, hemicellulose and lignin composition and ash of the bagasse were determined by the NREL (National Renewable Energy Laboratory – USA), Laboratory analytical procedures NREL/TP 510-42618 (Sluiter et al., 2008).

The quantification TPC was performed by the Folin–Ciocalteu reagent. The determination of F and HMF was performed using HPLC with a UV–vis Dionex oven (HPLC 3000) at 35 °C, P680 pump and isocratic program of water and methanol (90:10) at 1.0 ml/min. A reverse phase C-18 column (EC 250/4.6 Nucleosil 100-5 CN) was used and 20 μL injected with detection at 276 nm with a Dionex UVD (340V) detector.

Quantification of glucose, xylose and arabinose was carried out using high-performance anion-exchange chromatography – with pulsed amperometric detection (HPAEC-PAD) (Thermo Scientific, Dionex, ICS-5000) with a CarboPac® PA-1 anion exchange column at the 25 °C and 30 °C compartments, diluents prepared with ultra-pure deionized water 18 MΩ and degassed with $N_2$, with a flow of 1 ml/min with solvent A (ultrapure water) and B (500 mM NaOH). Isocratic elution was used for column cleaning from 0 to 12 min with 95.2% A, from 12 min to 16 min 100% B was used and from 16 min to 30 min 95.2% of A was used to end the process.

### 2.4. Physical analysis of the material

A portion of the insoluble fraction of the treated and untreated SCB was used for structural analysis by infrared (ATR-FTIR).

Approximately 100 mg of residue were pressed to formulate a 12 mm diameter and 1 mm thick disk. The spectra were collected on a Perkin Elmer spectrophotometer (FT-IR Spectrum Two – UART Two, with ATR). The sample disk was pressed on the crystal surface at the same pressure for all the samples using 4 scans in the range 2000 $cm^{-1}$ to 600 $cm^{-1}$ and a resolution of 4.0 $cm^{-1}$. Due to the heterogeneity of the bagasse, the light beam was directed at least 8 positions and an average spectrum for each sample was constructed.

Powder X-ray diffraction (XRD) patterns of sugarcane bagasse were obtained on a Model 300 MiniFlexRigaku® diffractometer using Cu Kα radiation ($\lambda = 1.54\ \theta$) in an angular range of 5–40° at 2θ/min and 25 °C, the crystallinity index (CrI) was calculated by the Segal method (Terinte et al., 2011).

Scanning electron microscopy (SEM) was performed using a FEI Quanta 200 scanning electron microscope (FEI Company, Eindhoven, Netherlands) with an accelerating voltage of 12.5 kV. The bagasse was fixed in 2.5% glutaraldehyde in 0.1 M 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 an ethanol series, critical point-dried with $CO_2$, and sputter coated with gold (Bal-Tec SCD 050).

Thermal stability of sugarcane bagasse was measured using thermogravimetric analysis (TGA) and differential thermogravimetry (DTG) in a Perkin–Elmer TGA-4000 thermogravimetric balance. About 10 mg of dried sugarcane bagasse was placed in a ceramic sample holder and heated at 50 °C/min in the temperature range of 30–700 °C, under nitrogen flow (20 mL/min).

### 2.5. Enzymatic hydrolysis

Enzymatic hydrolysis of 0.25 g of both dried untreated and pretreated bagasses were carried out in glass flasks with rubber stoppers with 10 mL of acetate buffer 0.1 M, pH 5.0, containing cellulase (Prozyn®, São Paulo, Brazil), a dosage of 32 FPU per gram of bagasse (dry basis). An enzyme reaction was performed at 60 °C, 150 rpm. To adjust the time required for hydrolysis of SCB, an initial experiment was conducted using 32 FPU/g of the untreated pulp, for 0, 6, 12, 24 and 36 h. After that, 24 h hydrolysis was established as optimal hydrolysis time. After hydrolysis, samples were filtered through a 0.22 μm filter and the liquid used for qualitative and quantitative analysis. Cellulose (or hemicellulose) conversion was calculated by using the equation: % glucose (or xylose) conversion = $[(c * v * 0.9)/m] \times 100\%$, where $c$ is the concentration (g/L) of sugars in the enzymatic liquor hydrolyzed, $v$ (L) is the volume of liquor, and $m$ is the glucan (cellulose or hemicellulose) content (g).

## 3. Results and discussion

### 3.1. Composition of bagasse and sequence of pretreatment

The structural carbohydrate and lignin composition of the untreated SCB was 41.3 ± 2.7 cellulose, 19.3 ± 0.6 hemicellulose and 30.7 ± 2.7 lignin. Due to the recalcitrance of the SCB cell wall, its deconstruction by chemical or biological treatments is necessary for efficient enzymatic hydrolysis. Based on previous results (data not shown) with ozone followed by washing in alkali or acid, the alkali was chosen due to the higher amount of sugar released in the enzyme hydrolysis. So, the combination of O, B and U, was chosen as the sequence of pretreatment of sugar cane bagasse.

Table 1 shows the effects of treatment on the soluble and insoluble fractions of the biomass. It is possible to observe an apparent increase in cellulose content due to the removal of hemicellulose, lignin and extractives after treatment of the samples. The phenolic compounds are formed from the breakdown of lignin, so the TPC in the pretreatment can be related to the degree of lignin degradation. After the pre-treatments O and B the soluble fraction presents TPC concentration 11 times greater than the treatment with only ozone (O), which means less phenolic compounds in the insoluble fraction. The presence of phenolic compounds can inhibit the action of cellulases (Ximenes et al., 2010) and their exclusion can contribute to an increase in the hydrolysis rate of the insoluble fraction. The furan compounds (furfural and hydroxyl methyl furfural) were not detected in any of the treatments used in this study, which is in agreement with Travaini et al. (2013).

It can be seen that the increase in cellulose is significantly higher in samples treated with ozone, alkali and ultrasound. However, hemicellulose and lignin content are dramatically reduced after each step of the treatment. Each treatment acts differently and their combination in a sequence resulted in an optimal deconstruction of lignocellulosic material. Ozonolysis is a chemical oxidative treatment of lignocellulosic materials which has been recently used as a pretreatment of biomass for second generation ethanol (García-Cubero et al., 2010; Travaini et al., 2013). The ozone treatment has been shown to be effective in the breakdown of the plant cell wall mainly due to its high oxidation potential ($E° = +2.07$ V) and its ability to degrade rapidly leading to the formation of hydroxyl radicals, which, in turn, have higher oxidation potential than ozone ($E° = +3.06$ V). It reacts preferably with lignin rather than carbohydrates, due to the aromatic structure and the large number of carbon double bonds in lignin. Lignin removal without inhibitory compound formation, increases the accessibility of the enzymes to polysaccharides wherein fermentable sugars are released (Travaini et al., 2013). The data show that ozone delignification preserves the majority of the cellulose while solubilizing lignin which has been reported in the literature (Travaini et al., 2013).

Leaving the SCB after the ozone treatment in an alkaline medium resulted in a continued solubilizing of xylan and lignin while preserving cellulose. Alkaline pretreatment of lignocellulosic materials causes the hydrolysis of intermolecular ester bonds crosslinking xylan and lignin (Chaudhary et al., 2012), which can contribute to the extraction of these compounds. Also, an increase in internal surface area, a decrease in the degree of polymerization and a decreased crystallinity has been reported from the treatment of lignocellulosic materials by dilute NaOH (Fan et al., 1987). However, only alkali does not modify the biomass enough to ensure good conversion of cellulose. So, a combination with another treatment is necessary to increase the cellulose conversion.

The treatment with U further improved both the removal of hemicellulose and lignin from SCB (Table 1), which is in agreement with various reports from studies with lignocellulosic materials (Sun et al., 2004; Liu et al., 2007; Velmurugan and Muthukumar, 2011). Ultrasound is sound with a frequency greater than 16–20 kHz and which produces a rapid cycling of pressure called acoustic cavitation, resulting in the formation of intense vibrations in the liquid system. Collapse of cavitational bubbles causes a rapid rise in local temperature and pressure, which may lead to the generation of free radicals that promote certain reactions. Thus, both these physical and chemical effects on the biomass being sonicated have the ability to increase the surface area of the pretreated lignocellulosic biomass and this results in an intensification of the enzymatic hydrolysis (Subhedar and Gogate, 2013). The treated bagasse generated a higher amount of ash than untreated bagasse, probably due to higher inorganic content in this sample because of the alkali treatment.

### 3.2. Enzymatic hydrolysis

The amount of fermentable sugars (glucose and xylose) released after enzymatic saccharification of sugarcane bagasse is the most

**Table 1**
Composition of soluble and insoluble fractions after pretreatment.

| Pretreatments | | | | Composition after pretreatment | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | Soluble fraction (mg/g) | Insoluble fraction (%)[a] | | | |
| Sequence | Media[b] | $O_3$[c] | U[d] | TPC | Cellulose | Hemicel. | Lignin | Ash |
| O | $H_2O$ | Yes | No | 0.8 ± 0.2 | 43.6 ± 3.1 | 15.2 ± 1.2 | 27.3 ± 1.4 | 1.9 ± 0.1 |
| OB | NaOH | Yes | No | 6.7 ± 0.1 | 48.9 ± 1.0 | 14.1 ± 1.1 | 24.9 ± 0.4 | 2.7 ± 0.6 |
| OBU | NaOH | Yes | Yes | 6.5 ± 0.1 | 51.8 ± 2.2 | 11.2 ± 0.6 | 22.3 ± 0.8 | 3.1 ± 0.3 |
| Untreated Sugarcane bagasse | | | | ND | 41.3 ± 2.7 | 19.3 ± 0.6 | 30.7 ± 2.7 | 2.7 ± 0.1 |

ND = not detected;
[a] w/w.
[b] 0.1 mol $L^{-1}$;
[c] 32 mg $min^{-1}$;
[d] 5 min. of ultrassound.

important factor in evaluating the pretreatment efficiency. An experiment was carried out at a fixed dosage of enzyme (FPU 32) with different hydrolysis times (6, 12, 18, 24 and 36 h) using the untreated bagasse as reference. A conversion of about 16% of untreated pulp, measured as glucose, was achieved in 24 h and stabilized at that level (Fig. S1). Consequently, 24 h of hydrolysis was established as the optimal hydrolysis time for all substrates.

Table 2 shows that the release of glucose increased with each step of the pretreatment sequence, so that the best yield for the enzymatic hydrolysis was observed for the OBU sequence. This 3-stage pretreatment resulted in the release of 391 mg glucose/g bagasse, corresponding to a 94% yield, which represents one of the highest percentages of hydrolysis of cellulose reported to our knowledge. With the OB sequence only, 354 mg glucose/g bagasse (85% yield) was released, which can be considered a satisfactory amount of sugar released and this may be an advantageous treatment if U is not available. Like glucose, the release of xylose was observed in all treatments.

The increase in the pretreated bagasse cellulose conversion may be due to the ability of OBU pretreatment to increase the surface area of bagasse samples, which leads to increased contact between the cellulases and the substrate. This produces an increase in the glucose released (Plácido and Capareda, 2014).

Ozone has been used in pretreatments with varying results depending on the procedure adopted and the enzymes used. Souza-Corrêa et al. (2013, 2014) obtained conversions between 65% and 78.8%, Travaini et al. (2013) obtained a 41.79% conversion and Pereira et al. (2016) obtained a 39% yield. However they used different ozonolysis conditions and different enzymes and activities. The enzyme activity FPU used in this work was higher than that reported in those studies.

Several processes of pretreatment of biomass by ultrasound have been reported with varying rates of cellulose conversion (Subhedar and Gogate, 2013). The combination of alkaline treatment with ultrasound has shown an increase in the cellulose conversion by cellulase. Velmurugan and Muthukumar, (2011, 2012), reported lignin removal between 75 and 90%, with a recovery of cellulose and hemicellulose ranging from 79 to 99%.

**Table 2**
Contents of glucose and xylose after enzymatic hydrolysis of pretreated bagasse.

| Pretreatments | | | | Enzymatic hydrolysis (mg/g) | | | |
|---|---|---|---|---|---|---|---|
| Sequence | Washing[a] | $O_3$[b] | U[c] | Glucose | Yield (%) | Xylose | Yield (%) |
| O | $H_2O$ | Yes | No | 66 ± 4 | 16 | 23 ± 1 | 12 |
| OB | NaOH | Yes | No | 354 ± 12 | 85 | 75 ± 10 | 38 |
| OBU | NaOH | Yes | Yes | 391 ± 7 | 94 | 79 ± 9 | 55 |
| Untreated Sugarcane bagasse | | | | 15 ± 2 | 3 | 4.5 ± 0.6 | 2 |

[a] 0.1 mol $L^{-1}$;
[b] 32 mg $min^{-1}$;
[c] 5 min. of ultrassound.

It is difficult to compare the results from different studies even though the techniques are identical. Conditions of work in the lab, variations in experimental parameters such as composition and activity of individual cellulases in the enzyme solution, the unit of activity for cellulase etc. are parameters that can contribute to different results. Furthermore, the synergistic effects between cellulases are influenced by the nature of the substrate, such as chemical composition, degree of crystallinity and solubility (Yang et al., 2007). However, the results obtained in this work show that a combination of ozone and ultrasound treatments in an alkali medium improved the saccharification when compared to the untreated SCB, and the glucose yield is one of the highest that has been reported.

The economic implications is a question that always comes up when addressing the pre-treatment of biomass, and are usually only marginally treated in scientific articles. Two things are needed for any technology to be suitable for use in the industry, (1) Technical feasibility and (2) Economical feasibility. From the academic viewpoint, it is always difficult for the researcher to dominate this second issue. As a scientist, one has to evaluate the first point, that is assessing the technical feasibility of the process on a small scale (on the bench). In a second stage, with commercial interest, one has to verify its economic viability in operation on an industrial scale. At the moment, our research is still at the first stage, which includes up to three different steps in the pretreatment.

The ozonolysis is already an established, well-known process, and it is already applied on a large scale in the bleaching of pulp in the paper industry, which is a very cheap final product. (Today there is estimated to be at least 28 ozone bleach plants in operation worldwide). It is also applied in the industrial water treatment for removing mainly phenolic compounds and compounds having conjugated double bonds and has been reported as one of the advanced oxidation processes (AOPs) with the best cost-benefit (Pera-Titus et al., 2004). However, practical application of ozonolysis as a pretreatment of biomass depends largely on cost reduction in ozone production. As reviewed by Travaini et al. (2016), an estimated cost of generation of 100 g ozone requires about 2.38 MJ of electrical energy, and the combustion of 100 g ethanol releases 2.67 MJ. From these figures, the production of ethanol alone does not appear to be economical using ozonolysis pretreatment with current process efficiency. Though, as ozone is common in the pulp and paper industry, a good way of getting around this apparent unviability comes, as suggested by Phillips et al. (2013), from the concept of integrating pulp and paper technology with bioethanol production. Both Kraft pulp and mechanical pulping mills for newsprint can be repurposed to bioethanol production and be economically attractive. Technical and equipment risk are minimal for pretreatment of different biomasses, even at high concentrations.

**Table 3**
The main characteristic absorption bands of lignocellulosic material.

| Vibration type and functional groups | Wavenumber ($cm^{-1}$) | References |
|---|---|---|
| Region of OH and C–H stretching vibrations | 3800–2700 | Poletto et al. (2012) |
| Cellulose and hemicelluloses core structures (–OH stretching) | 3400 | Sun et al. (2004) |
| Cellulose and hemicelluloses core structures (C–H stretching) | 2920 | |
| Region of "fingerprint" (different groups) | 1800–800 | Poletto et al. (2012) |
| C–O stretching (unconjugated ketone, ester or carboxylic groups) | 1727 | Hoareau et al. (2004) and Sun et al. (2004) |
| Aromatic skeletal vibrations, (lignin) | 1600 | Xu et al. (2013), Liu et al. (2007) and Sun et al. (2004) |
| Aromatic skeletal vibrations, (lignin) | 1510 | |
| Aromatic skeletal vibrations, (lignin) | 1440 | |
| Stretching of C–O–C from the β(1 → 4), glucosidic linkages | 1158, 1175 | Xu et al. (2013) and Sun et al. (2004) |
| Stretching of C–O in cellulose, hemicelluloses and lignin, or stretching C–O–C in cellulose and hemicelluloses (xylan) | 1035, 1049 | Liu et al. (2007) |
| Stretching of C–O–C from the β(1 → 4)-glucosidic linkages | 897 | Liu et al. (2007), Xu et al. (2013) and Sun et al. (2004) |
| Vibration of C–H out of plane in p-hydroxyphenyl units (Lignin) | 833 | Hoareau et al. (2004) |

The alkaline treatment does not involve large costs and has also been widely applied on an industrial scale for the bleaching of Kraft pulp in the paper industry. It has also been reported as a viable and efficient process, when combined with other processes in the pre-treatment of sugarcane bagasse to improve the efficiency of the enzymatic hydrolysis of biomass (Bi et al., 2016).

Ultrasound has been reported for the treatment of biomass and water treatment, but its implementation on an industrial scale has not been carried out yet due to low efficiency and high cost (Mahamuni and Adewuyi, 2010). Therefore, U can be an expensive step in our process, however, as mentioned before, the combination of ozone and alkaline alone, resulted in over 85% yield, making the process interesting even without US.

Although estimation of the treatment cost is an important aspect, the cost analysis of the process is very difficult, because the overall cost of the treatment process is represented by the sum of the capital, operating and maintenance cost (mainly power). For a full-scale system, these costs strongly depend on the nature and the concentration of the raw material, size and configuration of the reactor, location etc. Without data on these cost parameters, estimation is impossible. However, in a cost simulation exercise, electric power consumption was assessed. Under similar conditions to this study, 1 kg of treated bagasse would consume 125 kWh and 0.47 kWh, using US and $O_3$, respectively (see Table S1, Supplementary Material). Accordingly, considering the current cost of energy in Brazil (this changes a lot from country to country), there would be an estimated cost of R$ 60 (US$ 17) and R$ 0.22 (US$ 0.06) for the treatment of 1 kg of pulp using US and $O_3$, respectively.

### 3.3. Infrared analysis (ATR-FTIR)

FTIR spectroscopy is a simple, rapid and non-destructive technique which has been used for obtaining information on the chemical and structural composition of wood materials (Xu et al., 2013). Thereby, the samples were analyzed using FTIR-ATR in order to understand the effect of OBU delignification pretreatment on the chemical features of sugar cane bagasse which resulted in improvement of the enzymatic cellulose conversion. Increase in transmittance corresponds to a lower concentration of specific chemical function that absorbs the infrared radiation for that specific wave number ($cm^{-1}$). The main characteristic absorption bands of lignocellulosic material are listed in Table 3. From the infrared spectrum of the untreated bagasse, it was possible to compare the structural changes caused by the treatments.

According to Poletto et al. (2012), due to their complexity, the spectra can be separated into two regions: the 3800–2700 $cm^{-1}$ region corresponding to the OH and CH stretching vibrations and the 1800–800 $cm^{-1}$ or "fingerprint" region, corresponding to the stretching vibrations of different groups of wood components.

It can be observed in Fig. S2 that there is a strong broad band at around 3440 $cm^{-1}$ which is assigned to different OH stretching modes and another band at around 2920 $cm^{-1}$ related to methyl and methylene stretching groups present in the spectra of both sugar cane bagasses. However, these two bands are more attenuated in the OBU spectra. Since the absorption band at 3440 $cm^{-1}$ is caused by the stretching of the hydroxyl groups present in cellulose, hemicellulose and lignin, but cellulose has increased in the treated bagasse, the attenuated band might be attributed to the lignin and hemicellulose removed from the treated bagasse. On the other hand, the attenuation of band 2900 $cm^{-1}$ might be attributed to the removal of extractives from treated bagasse, since some compounds in organic extractives, like fatty acid methyl esters and phenolic acid methyl esters, contain methyl and methylene groups (Poletto et al., 2012).

In the "fingerprint" region, corresponding to the stretching vibrations of different groups of bagasse components, the spectra of both samples are quite different. The treated bagasse presents a notable decrease in transmittance for peaks attributed to lignin, such as 1727, attributed to ferulate and p-coumarate esters (Pan et al., 1998); 1600, 1510and 1440, attributed to the vibration of the aromatic ring (Saw et al., 2011); 1374, attributed to the syringyl group (Sahoo et al., 2011); 833, attributed to the C-H out of plane bending vibrations in the p-hydroxyphenyl propane units (Hoareau et al., 2004). All these functional groups are highly rich in electrons, which make them targets for the ozone attack. Besides that, the saponification of these esters is expected considering the alkaline pH used during delignification. Therefore, all these alterations in the FTIR-ATR spectra are consistent with the lignin removal detected by the chemical analysis (Table 1).

The deep decrease in transmittance around 1220–900 for both samples (more pronounced for the untreated sample) illustrated the typical signal pattern for the hemicellulosic (xylan) component (Sun et al., 2004), in which the strong band at 1035 is attributed to C–O stretching in cellulose, hemicelluloses and lignin or to C–O–C stretching in cellulose and hemicelluloses (Liu et al., 2007). Two low-intensity peaks at 1175 and 980 $cm^{-1}$ are attributed to arabinose substitution in the xylose residues, a characteristic typical of arabinosyl side chains (Sun et al., 2004). It is known that hydroxyl ions cause swelling of the cellulose; hydrolysis of ester bonds; breakage of intermolecular hydrogen bonds between cellulose and hemicellulose; and even stronger linkages, such as ferulic acid bridges between hemicelluloses and lignin; thus extracting the hemicellulose fraction of the material (Sun et al., 2004; Cyran et al., 2004; Xu et al., 2013). Therefore, all the spectra shown are in concordance with the chemical analysis and are indicative of

**Table 4**
TGA and DTG values of sugarcane bagasse components and OBU treated and untreated bagasses.

| Samples | $T_{onset}$ (°C) | $T_{endset}$ (°C) | ΔT(°C) | $T_{máx}$ (°C) |
|---|---|---|---|---|
| Lignin* | 237 | 510 | 273 | 324 |
| Cellulose* | 374 | 425 | 51 | 405 |
| Xylan* | 288 | 382 | 94 | 346 |
| OBU | 332 | 428 | 96 | 393 |
| *In nature* | 312 | 440 | 128 | 434 |

* Fractions extracted from bagasse.

hemicellulose, lignin and extractives having been released during the OBU pretreatment.

### 3.4. Thermogravimetric analysis (TGA and DTG)

Thermogravimetric analysis (TGA) gives the weight loss as a function of temperature, while DTG presents the derivative thermogravimetric curves. They provide important information on the thermal stability of material. TGA is a method of thermal analysis in which changes in physical and chemical properties of materials are measured as a function of increasing temperature (with constant heating rate). Changes in temperature affect the sample. So, data from the thermogravimetric analysis provide information regarding the nature and extent of degradation and the thermal stability of the materials (Brown, 1988). It also helps the understanding of intermolecular and intramolecular forces. Thus, the TG curve can help to elucidate the decomposition and differences of related materials.

Table 4 shows the values obtained by TGA and DTG for both bagasses before and after treatments and their individual components: lignin, cellulose and xylan. Each of these components has specific characteristics of thermal decomposition, which are based on types of intermolecular and intramolecular forces and their polymeric structures (Brown, 1988). Lignin and hemicellulose (composed principally of xylan) start degrading at lower temperatures than cellulose. Lignin has a wide range of degradation, starting at about 237 °C and ending at about 510 °C (Table 4). Xylan degrades in the range of 288 °C to 382 °C, which can be observed as a shoulder on the DTG of untreated bagasse (Fig. 1). Cellulose has a narrower range of degradation, between 374 °C and 425 °C. Thus, it can be inferred that the sample with the highest Tonset is more thermally stable and has a greater amount of cellulose. The OBU sample (Table 4 and Fig. 1) has a greater Tonset than that of the untreated sample. It also shows Tonset, Tendset and Tmax that are closer to the cellulose confirming the high level of cellulose in this sample. On the other hand, the untreated sample exhibits low Tonset and high Tendset, two thermal characteristics of lignin, indicating high content of this component in that sample. Both these results are in agreement with the results seen in the chemical characterization (Table 1) and the infrared spectroscopy (Fig. S2).

Fig. 1 shows the TGA curves for treated and untreated sugarcane bagasse. The TGA curves of the samples showed three mass loss steps. The first stage of mass loss, approximately 7%, occurred at around 100 °C for both bagasses and is attributed to the moisture of the material. The second stage of mass loss started first for untreated bagasses at around 312 °C and a bit later for treated bagasse at around 332 °C (Fig. 1 and Table 4). According to Mothé and De Miranda (2009), this stage is due to the elimination of organic extractives, such as fats, waxes, alkaloids, glycosides and terpenoids. This agrees with results of chemical data (Table 1) and corresponds to extractives that have been removed from the treated bagasse.

As the thermal degradation of the polysaccharides lignin, cellulose and hemicellulose occurs at temperatures very close to each other, is not possible to detect separate peaks (Mothé and De Miranda, 2009). The treated material shows a greater thermal stability and has a higher degradation temperature than the untreated material, which may be related to its increased cellulose content (Table 1, Fig. 1) and also to the increased crystallinity of the cellulose (Fig. S3). The DTG graph (Fig. 1) shows that the untreated pulp presents a shoulder at about 380 °C, characteristic of the degradation of hemicellulose and lignin. The absence of this shoulder in the treated sample indicates the removal of these components during the treatment, which is in accordance with the chemical data (Table 1). According to Poletto et al., (2012) the degradation of one component with lower molecular weight, such as the extractives, may accelerate the degradation process of the other wood components, such as the cellulose. So, this peak is prominent for untreated bagasse, as it has more extractives together with hemicellulose and lignin.

### 3.5. Scanning electron microscopy (SEM)

The effect of pretreatment on the ultrastructural changes and the deconstruction of the substrate were characterized by comparing SEM of OBU pretreated and untreated sugar cane bagasse. Fig. S3a, shows the anatomy of untreated SCB which is compact, thick-walled fiber cells with highly ordered fiber morphologies, indicating a preserved structure of the cell wall. On the other hand, Fig. S3b, shows a significant morphological change of the treated

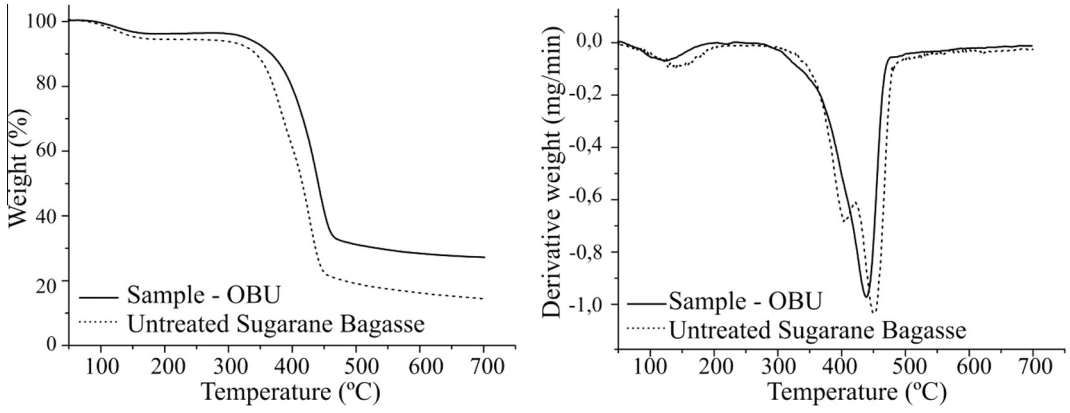


**Fig. 1.** TGA (left) and DTG thermograms of untreated sugarcane bagasse and sample OBU.

bagasse, the surface of fibers has become more open, rough, porous and fluffy, which is associated with the destructive effect of pretreatment on the cell wall. This effect can be attributed to the hemicellulose and lignin fractions solubilized after pretreatment. Similar alterations were observed by Travaini et al. (2013) with an $O_3$ pretreatment and Bi et al. (2016) with an $O_2$-NaOH pretreatment. The changes observed in the cell wall are consistent with the delignification detected by chemical and physical analysis and could explain the facilitation of the enzymatic hydrolysis of the treated bagasse.

### 3.6. XRD test

The ratio between the crystalline and amorphous regions determines the crystallinity index of the cellulose, which affects the enzymatic saccharification of fiber. Fig. S4 shows the diffraction patterns of untreated, only ozone, OB and OBU pretreated sugarcane bagasse. All samples show the XRD patterns for cellulose with three typical peaks, at 2θ of around 16, 22 and 35°. The crystallinity index of pretreated SCB increased with pretreatment, from 66.2 for the untreated SCB to 70.5 for the OBU pretreated bagasse. The results indicate that the pretreatment removed the contents other than cellulose efficiently. Many studies indicate that this phenomenon is mainly due 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. In this work, the increase of the crystallinity index in the pretreated samples was consistent with the delignification detected by the chemical and physical analysis and this could contribute to a better understanding of cellulases action on the treated bagasse.

## 4. Conclusion

This work has shown that the combinations of ozone, NaOH and ultrasound (OBU) as pretreatment of sugarcane bagasse resulted in good removal of lignin maintaining the cellulose and improving the enzyme hydrolysis of the biomass, resulting in a very high glucose yield (95% cellulose conversion). The FTIR and thermogravimetric analysis (TGA and DTG) methods used to characterize the bagasse samples were shown to be appropriate for evaluating differences in the structures as a result of the pretreatment and strongly corroborated the chemical composition results.

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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.2016.06.072.

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