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Integrated Ozonation-Enzymatic Hydrolysis Pretreatment of Sugarcane Bagasse: Enhancement of Sugars Released to Expended Ozone Ratio

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Abstract: The combined effects of three key ozonation process parameters on the integrated ozonation-enzymatic hydrolysis pretreatment of sugarcane bagasse (SCB) were investigated, with emphasis on the relationship between sugar release and ozone consumption. A lab-scale fixed bed reactor was employed for ozonation at varying ozone doses (50, 75 and 100 mg O₃/g SCB), particle sizes (420, 710 and 1000 μm) and moisture contents (30, 45 and 60% *w/w*) in multifactorial experiments, keeping a residence time of 30 min. The ozonated SCB showed a reduction in the content of acid-insoluble lignin from 26.6 down to 19.1% *w/w*, while those of cellulose and hemicellulose were retained above 45.5 and 13.6% *w/w*, with recoveries of 100–89.9 and 83.5–72.7%, respectively. Ozone-assisted enzymatic hydrolysis allowed attaining glucose and xylose yields as high as 45.0 and 37.8%, respectively. The sugars released/ozone expended ratio ranged between 2.3 and 5.7 g sugars/g O₃, being the higher value achieved with an applied ozone input of 50 mg O₃/g SCB and SCB with 420 μm particle size and 60% moisture. Such operating conditions led to efficient ozone utilization (<2% unreacted ozone) with a yield of 0.29 g sugars/g SCB. Overall, the amount of sugars released relative to the ozone consumed was improved, entailing an estimated cost of ozonation of USD 34.7/ton of SCB, which could enhance the profitability of the process.

Keywords: lignocellulosic biomass; moisture content; ozone dose; particle size; pretreatment; sugar yield

1. Introduction

Nowadays, lignocellulosic biomass constitutes one of the most abundant and renewable resources to fuel biotech processes devoted to producing a portfolio of numerous target high added-value bio-products, such as ethanol, biogas, hydrogen, polymers and carboxylic acids, to give a few examples [1]. Sugarcane bagasse (SCB), the major by-product derived from the sugarcane industry, has been studied extensively as it possesses high potential as a feedstock for biorefineries. The worldwide production of sugarcane was reported to be about 2.015 billion tons in 2018 [2]. Around 450 kg of dry SCB are generated from each ton of sugarcane, being currently burned in combined heat and power systems [3]. There exists, however, a surplus of sugarcane lignocellulosic biomass that can be utilized for biorefining purposes. To that end, the efficient release of fermentable sugars by enzymatic hydrolysis requires that SCB is pretreated to tackle its complex and recalcitrant nature

imposed by its structural composition (mainly lignin) of 30–50% cellulose, 25–32% hemicellulose and 25–30% lignin [4–6].

Ozonation is one advanced oxidation process approach that has been long proven as a pretreatment step to make lignocellulosic biomass more suitable for further biological processes, with biomass deconstruction and delignification being the main mechanisms by which ozone decreases the recalcitrance of lignocelluloses [7,8]. Indeed, it is well known that enzymatic hydrolyzability can be enhanced by previously subjecting the material to ozonation [9–11]. Several studies have shown that the pretreatment of SCB by ozonation is technically feasible [12–16]. Some advantages of ozonation include the fact that (i) it can be produced on-site on demand which reduces/avoids handling and transport costs and issues related to storage; (ii) oxidation reactions take place at room temperature and atmospheric pressure; (iii) ozone molecules attack lignin selectively due to its aromatic structure, preserving cellulose [6,9]. Furthermore, the concomitant generation of potential inhibitors for downstream biological processes, i.e., furfural and 5-hydroxymethylfurfural (HMF), is minimized or avoided in the best case when ozonation is properly applied [17,18]. This is particularly important to circumvent negative aspects related to the application of an additional detoxification step, such as sugar loss and the generation of solid wastes (gypsum and lime sludge) and inorganic rich wastewaters [19]. By contrast, ozonation pretreatment requires relatively high ozone dosages, which in turn demand high-energy input to be produced, i.e., 36 MJ/kg of ozone [1,20]. According to Bhattarai et al. [17], most studies applying ozonation to lignocellulosic biomass usually reported a relatively long ozone residence time of 60 min or greater to achieve substantial biodigestibility, although excessive pretreatment exposure times may result in inhibitory side effects on further biological downstream processes, e.g., enzymatic hydrolysis [9,20,21] and dark fermentation [22]. Therefore, optimization of ozonation in terms of reducing the amount of expended ozone required for effective pretreatment is of utmost importance to achieve technical and economic profitability on a wide-scale application.

Recently, Rosen et al. [20] observed that a short ozone pretreatment of 15 min led to a low delignification efficiency (~20%) using shredded mixed municipal trimmings of trees, bushes and grass as the lignocellulosic waste, while it accommodated a four-fold improvement of saccharification efficiency compared to the untreated material. The authors, therefore, concluded that full delignification and long pretreatment time could be redundant since short ozonation with partial delignification could sustain efficient sugar release. Peretz et al. [23] also stated that total lignin mineralization is not necessary for efficient enzymatic activity of cellulase from the observation that partial decomposition of a highly-concentrated tannic acid solution (a lignin model compound with a proven inhibitory effect on cellulase activity) through very short ozonation was enough to alleviate its inhibitory action, thereby reducing the total ozone needed from 54.31 to 1.16 kg. The selective fractionation of lignin may also contribute to enhance enzymatic susceptibility [24]. Perrone et al. [21] found that the highest loss of lignin during the ozonation of SCB occurred after 20 min of ozone exposure, which corresponded to 11.1% of the total time of pretreatment. Hence, one may surmise that the amount of ozone required to improve the sugar yield of ozonated SCB could be further optimized, provided that a controlled ozonation treatment is properly applied, being rather focused on the concomitant reduction of lignin and hemicellulose [21,25].

According to the literature, besides the type of lignocellulosic material, reactor configuration, moisture content, particle size, ozone flow rate and inlet concentration, and exposure time are key design-process parameters affecting ozone consumption, and in turn, ozonation pretreatment efficiency [4,24,26,27]. Ozone consumption, particle size and moisture content are considered the most important ozonation parameters affecting mass transfer rate and reaction extent. However, knowledge on the combined effect of such key process parameters on the process is still limited, deserving further attention in order to make ozonation a more efficient and cost-effective pretreatment process. This study aimed to investigate the combined effects of particle size, moisture content and ozone dose on the ozonation and subsequent enzymatic hydrolysis of SCB, with an emphasis on determining possible structural and morphological changes and their relationship with the amounts of sugars released and

ozone consumed. It is expected that the results discussed herein could be helpful for establishing operating conditions, leading to an enhancement in the sugars released/ozone consumption ratio during the integrated ozonation-enzymatic hydrolysis pretreatment of SCB.

2. Materials and Methods

2.1. Substrate

SCB was kindly provided by a sugar mill located in Veracruz, Mexico. First, the collected material was subjected to solar drying for 48 h, achieving a 4–6% *w/w* moisture content. Then, it was ground using a conventional hammer mill and sieved through a series of sieves to obtain average particle size distributions of 420, 710 and 1000 μm . Finally, the SCB was stored in plastic bags at room temperature until used. This conditioning step was applied only once at the beginning of the experimental investigation, and all the experiments were conducted using the same pre-conditioned SCB.

2.2. Ozonation: Experimental Set-Up and Operating Conditions

A lab-scale fixed-bed reactor was employed for ozonation (Figure 1). The reactor consisted of a glass tube (diameter 6.5 cm, height 30 cm) containing a cylindrical porous stone diffuser (diameter 3.5 cm, height 22.5 cm) fixed in the center to avoid channeling and uneven ozone exposure while enhancing mass transfer conditions via microbubble mediated ozonation [28]. Ozone was produced from oxygen by a G11 generator (Pacific Ozone Technology, Benicia, CA, USA) and fed at the bottom of the reactor. Ozone consumption was estimated as the difference of ozone concentration in the gas phase at the inlet and outlet of the reactor, which was determined by the 465H ozone monitor (Teledyne instruments, City of Industry, CA, USA). The combined effects of the process parameters on the ozonation-enzymatic saccharification of SCB were investigated through a multifactorial statistical experimental design 2^3 with two factorial levels (−1 and 1) and two central points (0) for each factor (Table 1). The coded levels, −1, 0 and 1, were 420, 710 and 1000 μm for particle size, 30, 45 and 60% *w/w* for moisture content, and 50, 75 and 100 $\text{mg O}_3/\text{g SCB}$ for ozone dose, respectively. Particular attention was paid to the determination of ozone consumption and possible changes in hemicellulose, cellulose and lignin fractions after ozonation and to the assessment of glucose and xylose yields after the enzymatic hydrolysis. Both the consumed ozone amount ($\text{mg O}_3/\text{g SCB}$) and the ozone consumption efficiency as the quotient of grams of consumed ozone by SCB to the total grams of fed ozone, expressed in percentage (%), were used as process indicators. Experiments were performed at room temperature and atmospheric pressure.

Table 1. Experimental design for the ozonation of sugarcane bagasse.

Run	Coded Levels			Values		
	Particle Size	Ozone Dose	Moisture Content	Particle Size (μm)	Ozone Dose ($\text{mg O}_3/\text{g SCB}$)	Moisture Content (% <i>w/w</i>)
T1	0	0	0	710	75	45
T2	−1	1	−1	420	100	30
T3	−1	−1	1	420	50	60
T4	1	−1	−1	1000	50	30
T5	0	0	0	710	75	45
T6	1	1	1	1000	100	60
T7	−1	−1	−1	420	50	30
T8	1	1	−1	1000	100	30
T9	−1	1	1	420	100	60
T10	1	−1	1	1000	50	60

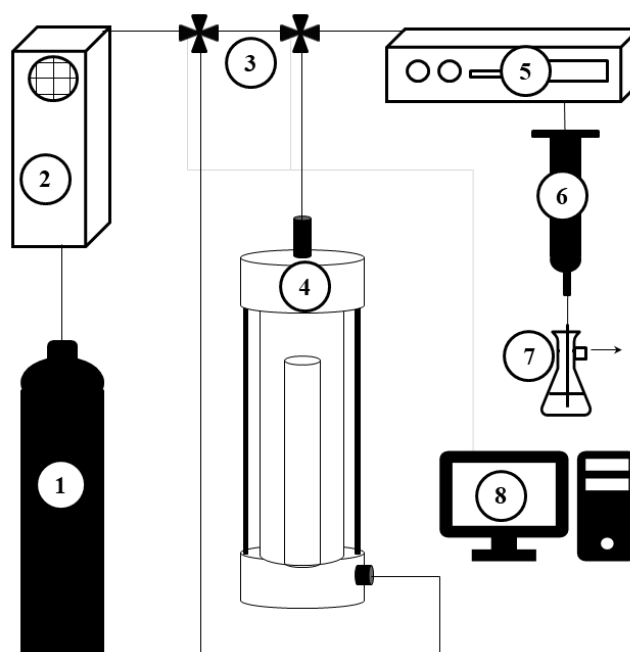


Figure 1. Scheme of the experimental set-up used for ozonation. (1) Oxygen tank, (2) ozone generator, (3) electronic shut-off valves, (4) fixed-bed reactor, (5) on-line ozone monitoring device, (6) ozone destructor, (7) potassium iodide (KI) solution and (8) control and measurement system.

Ozone dose ($\text{mg O}_3/\text{g SCB}$) was defined as a function of ozone concentration in gas phase ($C_{\text{O}_3,g}$) in mg/L , gas flow (Q_g) in L/min , reaction time (t_r) in min and bagasse mass (BM) in g , as expressed in Equation (1). In all the experiments, BM was fixed at 28 g of material, while Q_g and t_r were kept constant at 1.5 L/min and 30 min , respectively. Ozone dose was, therefore, only a function of ozone concentration, which varied accordingly, i.e., $31, 46$ and $62 \text{ mg O}_3/\text{L}$, depending on conditions.

$$\text{Ozone dose} = \frac{C_{\text{O}_3,g} * Q_g * t_r}{BM} \quad (1)$$

Before SCB was fed to the reactor, its moisture content was adjusted to the desired value by adding distilled water and stored hermetically for 18 h to avoid water evaporation. After ozonation, bagasse was dried in an oven at $30 \text{ }^\circ\text{C}$ for 48 h and stored for further structural characterization and enzymatic hydrolysis.

2.3. Enzymatic Hydrolysis

Enzymatic hydrolysis of un- and pretreated SCB was carried out using two enzymatic complexes simultaneously as follows: cellulases from *Trichoderma reesei* (Celluclast 1.5 L, Sigma, St. Louis, MO, USA) at a concentration equivalent to $38.5 \text{ filter paper units (FPU)/mL}$ and a cellulolytic enzyme mixture composed of arabinase, cellulase, hemicellulase, b-glucanase and xylanase (Viscozyme, Sigma) at an amount corresponding to 29.1 FPU/mL . Enzymatic hydrolysis was conducted using $5\% w/w$ in a 50 mM citrate buffer solution at $\text{pH } 4.8$, $50 \text{ }^\circ\text{C}$ and 150 rpm for 24 h . At the end of the enzymatic reaction, the resulting hydrolysate was centrifuged at 4000 rpm for 5 min , filtered through a $0.45 \text{ }\mu\text{m}$ filter and stored at $4 \text{ }^\circ\text{C}$ for further analysis. Glucose and xylose yields were calculated by Equation (2), where C_i is the concentration (in g/L) of sugar i ($i = \text{glucose, xylose}$) in the enzymatic hydrolysate, V is the volume (in L) of the hydrolysate, h_i is the correction factor of sugar i (0.90 for glucose and 0.88 for xylose) and m_i is the absolute content (in g) of cellulose (as glucose) or hemicellulose (as xylose) of the unpretreated SCB [21,29]. In order to relate the total sugars released after ozone pretreatment and

enzymatic hydrolysis to the total amount of ozone fed to the reactor, a normalized quotient, q_1 , was defined (Equation (3)).

$$\text{Sugar yield (\%)} = \frac{C_i * V * h_i}{m_i} * 100\% \quad (2)$$

$$q_1 = \frac{\text{total sugars released (g)}}{\text{fed ozone (g)}} \quad (3)$$

2.4. Analytical Methods

The content of acid-soluble lignin (ASL), acid-insoluble lignin (AIL), cellulose (as glucose) and hemicellulose (as xylose) was determined in the un- and pretreated SCB following the analytical procedure of the National Renewable Energy Laboratory (NREL) for the determination of structural carbohydrates and lignin in biomass [30]. The concentration of glucose, xylose, organic acids and furan derivatives was determined, in the liquid fraction obtained during enzymatic hydrolysis, by high performance liquid chromatography (HPLC) using Prostar Varian HPLC equipment equipped with a BioRad Aminex HPX-87H HPLC column and a refractive index (RI) detector. The column temperature was 65 °C and 5 mM sulfuric acid at a flow rate of 0.6 mL/min was used as the mobile phase. Confocal laser scanning microscopy (CLSM) was employed to observe physical changes in the surface of the bagasse. Bagasse fibers were cut in transverse and longitudinal sections of 10 µm thickness using a CM1850 cryostat (Leica, Nussloch, Germany) and observed in a TCS SPE confocal system equipped with a DM5500B-CS microscope (Leica, Heidelberg, Germany). A 405-nm laser was used for excitation and the emission spectra were acquired over 420–600 nm. The structure of SCB components was also analyzed by Fourier Transformed Infrared (FTIR) with a Cary 630 system (Agilent Technologies, Santa Clara, CA, USA) in the mid-infrared region at 4000–400 cm⁻¹ [31]. Water activity (A_w) in untreated SCB samples was determined by a 4TEV water activity meter (Aqua Lab, Riverside, CA, USA).

Mass loss is usually estimated as the difference of lignocellulosic material obtained before and after ozonation [4,32,33]. Since mass loss could also occur when compounds are solubilized or volatilized due to ozone reactions, the separation of soluble compounds (extractives) from the surface of bagasse was implemented to determine mass recovery efficiency. Representative samples of un- and pretreated SCB were dried in a stove at 30 °C for 48 h, achieving a moisture content of 4–6% *w/w* (dry basis). Then, a sample of 0.5 g of bagasse (W_0) was placed in a flask containing 40 mL distilled water and stirred at 240 rpm for 30 min. Then, all solids contained in the sample were recovered via filtration using a 0.2 µm porcelain filter crucible and dried in an oven at 105 °C until they reached a constant weight. The mass recovery was calculated by Equation (4), where R_i is the mass recovery (*w/w*) of bagasse i (i = untreated SCB, pretreated SCB), W (in g) is the dry weight of bagasse i after water-washing and oven-drying, W_0 (in g) is the weight of bagasse i added to the flasks and TS is the fraction of total solids in the sample i , previously known. The relative loss of mass of bagasse was calculated in triplicate using Equation (5), where R_{SCB} and R_{PSCB} stand for the mass recovery (*w/w*) of un- and pretreated SCB, respectively.

$$R_i = \frac{W_i}{W_0 TS} \quad (4)$$

$$\text{Relative mass loss (\%)} = \frac{R_{SCB} - R_{PSCB}}{R_{SCB}} * 100\% \quad (5)$$

Crystalline and amorphous fractions in bagasse were determined by a Panalytical X-ray diffractometer (Empyrean) employing Cu K α radiation ($\lambda = 1.542 \text{ \AA}$). The crystallinity index (I_{cr}) was calculated according to Equation (6), where I_{002} is the maximum intensity of 002 peak at $2\theta = 22.5^\circ$ and I_{am} is the intensity at $2\theta = 18^\circ$ of the amorphous fraction [34,35].

$$I_{cr} = \left(\frac{I_{002} - I_{am}}{I_{002}} \right) * 100 \quad (6)$$

2.5. Data Analysis

Individual and interaction effects on the response variables as well as the curvature effect between the two factorial levels were evaluated through an analysis of variance (ANOVA) with a 95% confidence interval. Statgraphics Centurion XV software was used for the analysis of the multifactorial statistical experimental design.

3. Results and Discussion

3.1. Effects of Ozonation on the Composition of Sugarcane Bagasse

As shown in Table 2, SCB was composed, on dry weight basis (% *w/w*), of cellulose $50.59 \pm 0.68\%$, hemicellulose $18.71 \pm 0.99\%$, ASL $3.07 \pm 0.22\%$ and AIL $26.60 \pm 1.01\%$. This structural composition, corresponding to the average and standard deviation values of the three different particle sizes tested, is similar to those reported by previous studies characterizing the chemical composition of SCB, with values of 35.2–49.5% for cellulose, 15.84–31.85% for hemicellulose, 3.13–4.46% for ASL and 19.54–22.53% *w/w* for AIL [4,15]. The differences in the structural fractions of the used SCB are attributed to the different geography, harvest season, variety and the heterogeneous nature of the biomass.

After ozonation, structural effects on the SCB were confirmed by changes in AIL, ASL, hemicellulose and cellulose fractions (Table 2), as well as by the loss of mass. All treatment conditions experienced a decrease in AIL. More specifically, the AIL content decreased from 26.6 down to 19.15% *w/w*, corresponding to a maximum degradation of 28%, which was sustained by the run T2 (420 μm particle size, 30% *w/w* moisture content, 100 mg O_3/g SCB ozone dose). The degradation of AIL could be explained by the high selectivity of ozone to react with the aromatic functional groups present in lignin [13,36]. By contrast, the ASL content increased from 3.07 up to 6.18% *w/w* (in run T5; 710 μm particle size, 45% *w/w* moisture content, 75 mg O_3/g SCB ozone dose), equivalent to an increment of 3.11%. This increment in ASL is higher than the 1.29% obtained by Barrera-Martinez et al. [6] using an ozone dose of 54 mg O_3/g SCB, an exposure time of 120 min and a moisture content of 50% *w/w*. In another study, Travaini et al. [14] pretreated SCB using a moisture content of 40%, an ozone dose of 210 mg O_3/g SCB and a reaction time of 120 min, obtaining an increment in the ASL content from 3.13 to 7.21%. On the other hand, the hemicellulose fraction changed from 18.71% to a minimal content of 13.6% *w/w* (run T5), corresponding to a maximum degradation of 27%. The degradation of hemicellulose could be explained by the reaction of ozone with phenolic compounds present in lignin; in other words, since lignin and hemicellulose are linked by ester and ether bonds, when these bonds are broken, hemicellulose is also reached by the ozone reaction [37]. Cellulose had minor changes regardless of the pretreatment conditions, with obtained recoveries values between 90 and 100%. Similar results were found by previous studies [13,38]. Taking into account the results obtained under the different experimental conditions assessed, higher AIL degradation occurred at higher ozone doses (75 and 100 mg O_3/g SCB) and smaller particle sizes (710 and 420 μm), which is in accordance with Travaini et al. [14] who decreased AIL in SCB from 8.75 to 6.49%, increasing ozone concentration from 3.07 to 3.44% (*v/v*), and Shi et al. [38], who pretreated corn straw for 90 min, increasing lignin degradation from 40 to 47.6% by reducing the particle size from 160 to 64 μm . It is therefore suggested that in this study, the ozonation mechanism targeted for the solubilization of lignin could be as important as its degradation, as has been previously observed in other studies [10,21]. Overall, a rise in the ASL fraction with decrease in the hemicellulose content was a clear trend for all the experimental conditions tested, although it did not show a clear relationship with operating conditions ($p > 0.05$).

Table 2. Structural composition and enzymatic hydrolysis performance of un- and pretreated SCB.

Run	Structural Composition (% w/w) ^a				AIL Removed (%) ^b	Enzymatic Hydrolysis	
	ASL	AIL	Cellulose	Hemicellulose		Glucose Yield (%)	Xylose Yield (%)
SCB ^c	3.07 ± 0.22	26.60 ± 1.01	50.59 ± 0.68	18.71 ± 0.99	-	2.12 ± 0.44	0.00
T1	5.91 ± 0.13	19.24 ± 0.79	47.85 ± 0.69	15.63 ± 1.42	27.68 ± 2.98	42.29 ± 0.65	31.91 ± 0.34
T2	5.57 ± 0.36	19.15 ± 0.68	51.43 ± 0.04	14.52 ± 0.07	28.02 ± 2.57	38.41 ± 0.72	37.88 ± 0.68
T3	5.73 ± 0.04	22.23 ± 0.27	47.50 ± 1.37	15.62 ± 0.27	16.44 ± 1.00	44.50 ± 0.90	23.40 ± 0.16
T4	6.03 ± 0.18	23.40 ± 0.84	45.78 ± 0.17	14.47 ± 0.22	12.05 ± 3.15	28.49 ± 0.31	29.07 ± 0.12
T5	6.18 ± 0.18	20.53 ± 0.29	47.24 ± 0.76	13.60 ± 0.62	22.82 ± 1.08	40.57 ± 0.94	30.63 ± 0.22
T6	5.87 ± 0.39	23.73 ± 0.80	46.21 ± 2.22	14.17 ± 2.61	10.78 ± 3.01	33.43 ± 0.21	26.49 ± 0.30
T7	5.59 ± 0.49	21.89 ± 0.21	48.08 ± 0.27	14.74 ± 0.45	17.72 ± 0.77	43.39 ± 0.14	23.96 ± 0.17
T8	5.51 ± 0.41	21.18 ± 1.93	49.22 ± 2.18	15.61 ± 1.61	20.38 ± 2.27	38.18 ± 1.23	30.96 ± 0.55
T9	5.53 ± 0.25	19.50 ± 0.73	50.11 ± 1.03	15.07 ± 0.73	26.29 ± 2.72	45.09 ± 0.31	35.10 ± 0.88
T10	5.91 ± 0.33	24.48 ± 0.50	45.49 ± 2.69	15.25 ± 2.30	7.98 ± 1.89	22.81 ± 1.03	20.81 ± 0.32

Notes. ^a Dry weight basis. ASL: Acid-soluble lignin. AIL: Acid-insoluble lignin. ^b Relative to the AIL content of unpretreated SCB. ^c Average and standard deviation values obtained from the three different particle sizes used.

Mass loss in pretreated SCB varied from about 6 to 17% for an ozone dose of 50 and 100 mg O₃/g SCB, respectively (Figure 2). The loss of mass is attributable to the depolymerization of AIL, ASL, hemicellulose and cellulose in the surface of bagasse due to ozonation [39]. It was found that the higher the ozone dose, the higher the loss of mass ($R^2 = 0.9236$). Thus, the assumption that higher ozone doses induce higher depolymerization of lignocellulosic material, mainly of AIL and hemicellulose, was established.

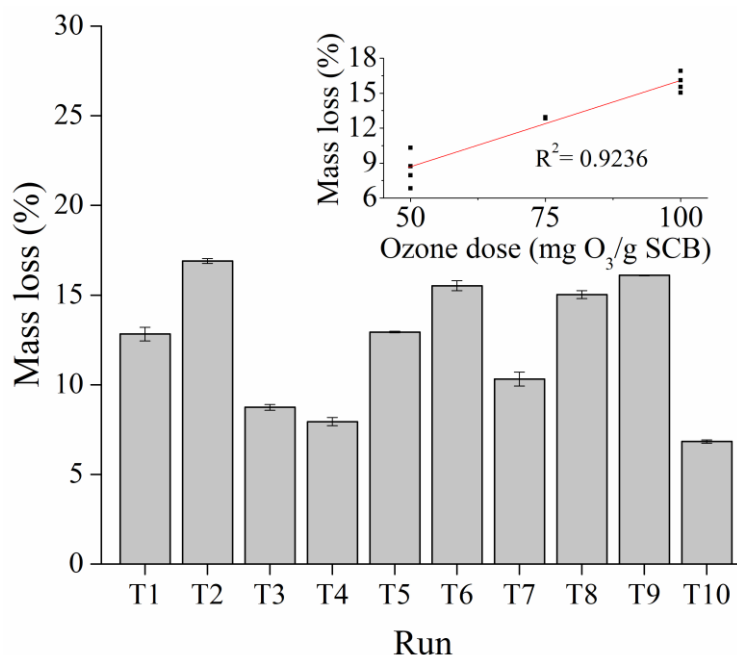


Figure 2. Mass loss of sugarcane bagasse (SCB) after the application of ozone pretreatment in different experimental conditions (inset figure shows the loss of mass at the different applied ozone doses).

3.2. FTIR Analysis

Un- and pretreated SCB were analyzed by FTIR to validate structural changes (Figure 3a). To illustrate this, runs T3 and T7 and unpretreated SCB were selected based on their higher sugars released to expended ozone ratio, as is discussed in Section 3.4. Absorption intensities in the near infrared spectra are mainly related with cellulose, hemicellulose and lignin fractions. The 1030 cm⁻¹ band corresponds to glycosidic bond vibration due to polysaccharides [13], whereas the bands at 895–898 and 1160 cm⁻¹ correspond to amorphous cellulose and crystalline cellulose, respectively [40,41]. The variation of vibrational signals at 1240–1270 cm⁻¹ is related to β -ether bonds in lignin and carbohydrates [40,42]. Bands at 1506 cm⁻¹ were distinguished due to the presence of double bonds of aromatic rings at 1729–1733 cm⁻¹ because of C=O bonds of hemicellulose [43] and at 3300 cm⁻¹ for hydrogen bonds (carboxylic acid O–H) in lignin [6]. FTIR, therefore, confirmed that the ozone pretreatment had an effect on the structure of the SCB.

3.3. XRD Analysis

Crystalline and amorphous fractions of un- and pretreated SCB were detected in the XRD analysis (Figure 3b). Runs T3 and T7 and unpretreated SCB were also chosen. Crystalline cellulose I was identified in both the un- and pretreated SCB [44], with the 002 peak at 22.5° in 2 θ and the 101 peak at 15° in 2 θ [45], while the amorphous region was observed at 18° in 2 θ [46]. After ozone pretreatment, the 002 and 101 peak intensities increased and the amorphous region decreased because of hemicellulose and lignin degradation [46], increasing the I_{cr} from 50 to 57%. The highest I_{cr} values were obtained in runs T6 and T10, using 1000 μ m particle size and 60% moisture. Overall, it has been suggested the increase in crystallinity could limit enzymatic hydrolysis [47], but in other cases, it could improve

enzymatic susceptibility, depending on the lignin content of material [39]. Although a correlation between I_{cr} and glucose yield was not found ($p > 0.05$), which agrees with Benko et al. [48], it seems that enzymatic hydrolyzability could be enhanced via combined reduction in the lignin and hemicellulose content, which is related to an increase in the I_{cr} index and not merely the reduction in the crystallinity of cellulose [21].

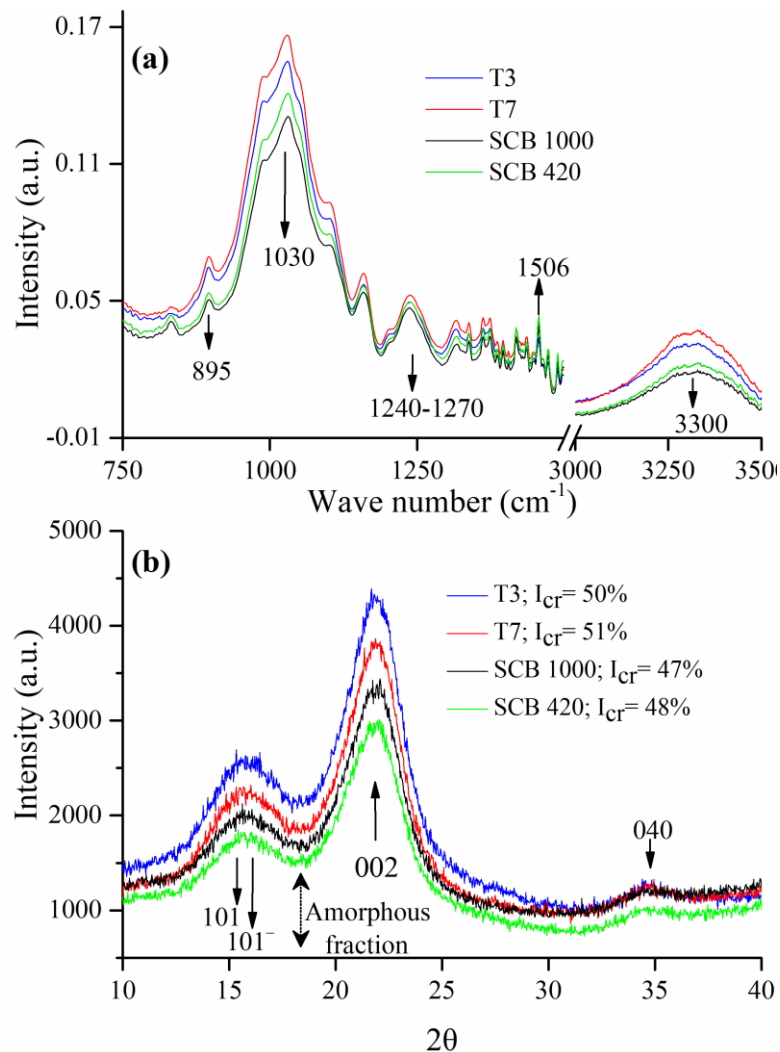


Figure 3. (a) Fourier Transformed Infrared (FTIR) spectra and (b) X-ray diffractograms and I_{cr} for un- and pretreated SCB samples. Footnote: SCB 1000 and SCB 420 correspond to unpretreated materials with particle sizes of 1000 and 420 μm , respectively.

3.4. Effect of Ozonation Conditions on Consumed Ozone

Figure 4 displays the ozone consumption efficiencies recorded for all the experimental conditions, which ranged from 86 to 99%. The highest ozone dose tested (100 mg O₃/g SCB), used in pretreatments T2, T6, T8 and T9, led to slightly lower ozone consumption efficiencies (89–93%). Pretreatments T3 and T7 presented higher efficiency in the consumption of ozone—98 and 99%, respectively, both achieved at the shortest ozone dose (50 mg O₃/g SCB) and particle size (420 μm). By contrast, the lowest ozone consumption efficiency was obtained in runs T4 (86%) and T10 (88%), which were conducted at 50 mg O₃/g SCB ozone dose and 1000 μm particle size. Hence, under the conditions tested, smaller particle sizes in conjunction with lower ozone doses sustained the highest ozone consumption efficiency. It has been reported that the amount of ozone absorbed in the bagasse could be enhanced by increasing the exposed surface of the material and employing a suitable moisture content (>30%) [10,13]. Li et al. [49]

reported an ozone consumption efficiency from 28 to 69% using maize stover samples with A_w values from 0.71 to 0.80, a particle size of 50 to 841 μm , a moisture content from 30 to 75% w/w , and an ozone dose of 360 mg O_3/g SCB. In another study, Barros et al. [12] ozonated SCB with a dose of 153–612 mg O_3/g SCB, obtaining ozone consumption efficiencies of 51–64.5%. Regarding the amount of ozone expended per gram of dry SCB, which is regarded as the most important variable of ozonation from an economical viewpoint [4], the results showed ozone consumption values ranging from 43.2 to 99.8 mg O_3/g SCB, with achieved yields for runs T3 and T7 of 49.4 and 49.6 mg O_3/g SCB, respectively; these yields are lower than those reported earlier [12,14].

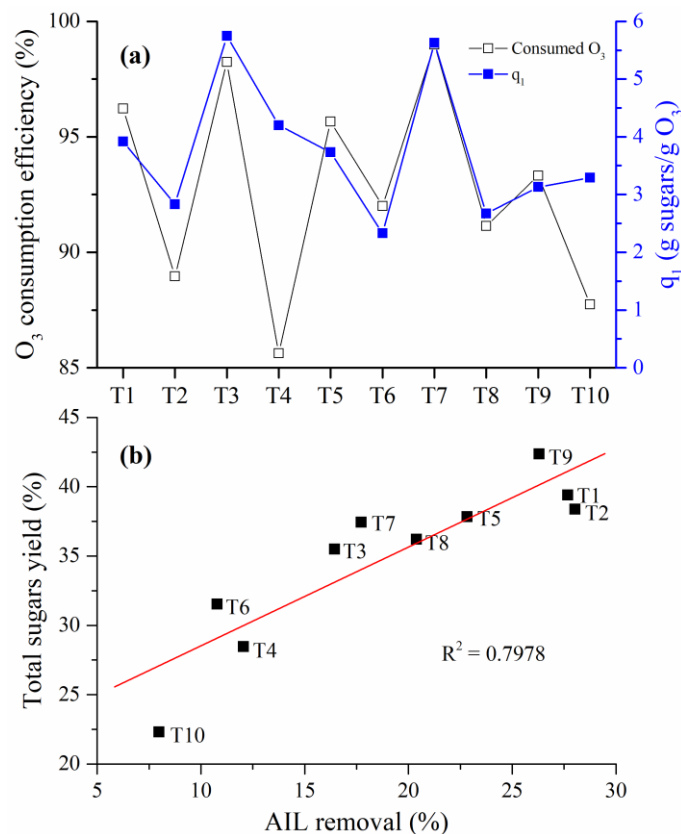


Figure 4. (a) Ozone consumption efficiencies and sugars released to expended ozone ratio (q_1) at different experimental conditions. Note: The amount of sugars is the sum of glucose and xylose contents. (b) Relationship between total sugars yield (glucose and xylose) and AIL removed.

The ANOVA analysis indicated that particle size and ozone dose were significant variables (data not shown) on the consumed ozone ($p < 0.05$, $R^2 = 0.87$), while, in the range of values studied, the moisture content did not show any significant effect (data not shown). However, to confirm the importance of moisture content in ozone pretreatment, an extra run was performed using dried bagasse (4–6% w/w moisture content) with a particle size of 420 μm and a dose of 75 mg O_3/g SCB. The results showed that there were no structural changes in the content of cellulose, hemicellulose, AIL and ASL compared to the non-ozonated SCB, while the consumed ozone was only 11%, which is in agreement with that reported by Benko et al. [37]. This reaffirms the fact that moisture is necessary to boost the diffusion of ozone from the gas phase to the surface material, allowing it to react mostly with lignin. In this study, the A_w level of samples of SCB previously moistened to 30, 45 and 60% w/w moisture was determined to be 0.99–1.0, which did not show any significant difference among the different moisture contents and particle sizes tested. As expected, such measured A_w levels were higher than those of dry, non-ozonated SCB samples, which were found between 0.45 and 0.55. The high levels of A_w observed could be explained by the prolonged water impregnation time of 18 h used

herein during the conditioning of SCB, which could allow water to be absorbed and distributed inside pores in a homogeneous way. The high A_w may enhance the diffusion of ozone through the matrix bagasse and, consequently, the ozone consumed; however, more studies are necessary to provide conclusive evidence.

3.5. Effect of Ozone Pretreatment on Sugars Release Yields

Ozonation-enzymatic saccharification is a promising cascade pretreatment approach for exploiting lignocellulosic biomass via biotech-based valorization processes. Table 2 shows the glucose and xylose yields obtained after ozone pretreatment and enzymatic hydrolysis. The highest sugar yields were 45.09% for glucose and 37.88% for xylose, obtained in run T9 with the lowest particle size (420 μm), the highest moisture content (60% w/w) and the highest ozone dose (100 mg O_3/g SCB). Higher glucose yields were obtained when the smallest particle size was used (runs T3, T7, T9), except for the run T2, although similar values were also achieved in runs T3 and T7 with glucose and xylose yields of 44.5 and 43.39%, respectively. The increase in sugar release yields was attributed to the disrupted structure of the material by ozonation (as confirmed previously the modifications observed on the amorphous and crystalline fractions of the SCB by FTIR vibrational and XRD analyses), making the matrix of bagasse more susceptible to further enzymatic hydrolysis. The lowest yields of glucose and xylose were 22.81 and 20.81%, respectively, corresponding to the run T10 (1000 μm particle size, 60% w/w moisture content, 50 mg O_3/g SCB ozone dose). As the particle size of bagasse increased, the glucose yield slightly decreased owing to the decreased surface area which was exposed to the attack of ozone. It should be considered, however, that an energy and economic burden could be imposed by the obtention of finely ground powders during the physical pretreatment of biomass.

Table 3 summarizes a comparison of the sugars yield of ozonated SCB obtained in this work with other data previously reported in the literature. Eqra et al. [15] reported an increment in the sugars yield from 20% (corresponding to the untreated material) to 67% when they ozonated the SCB at 10 g/L of ozone for 210 min. Barrera-Martínez et al. [6] obtained a sugars yield of 13.43% using an ozone dose of 54 mg O_3/g SCB and 120 min ozone reaction. Travaini et al. [4] obtained a higher glucose yield of 77.55% with ozone doses up to 222 mg O_3/g SCB applied for 150 min. Perrone et al. [11] increased the glucose yield from 3 to 16% with a reaction time of 60 min and a dose of 96 mg O_3/g SCB. The glucose yield of 45% obtained in the present work represents an acceptable result, taking into consideration the short reaction time of 30 min and the low ozone dose of 50 mg O_3/g SCB used.

Interestingly, ANOVA analysis showed that the moisture content did not impact the glucose and xylose yields of ozonated enzymatically hydrolyzed SCB, confirming the results discussed in Section 3.4. The effect of particle size on the glucose yield proved to be statistically significant ($p < 0.05$), which is in accordance with the study reported by Eqra et al. [15], but disagrees with the findings of Travaini et al. [4] who concluded that ozone concentration is the most important parameter for sugar release. A significant effect of ozone dose on the xylose yield was also found. By contrast, there were no significant interaction effects among the three factors. Figure 5 shows the estimated response surfaces for (a) glucose and (b) xylose yields, illustrating that higher glucose yields could be obtained at 420 μm and 50 mg O_3/g SCB, while higher xylose yields could be reached at 420 μm and 100 mg O_3/g SCB. Response surfaces showed different curvature effects; particularly, the curvature is located at the lowest ozone dose and the largest particle size levels for glucose yield, while for xylose yield, it is at the highest ozone dose and the smallest particle size levels, suggesting that curvature effects should be considered to accurately predict the process.

Sugar release as the total sugars yield (as the sum of glucose and xylose) is influenced by the delignification degree [5]— in this study, specifically, the total sugar yield tended to increase when AIL removal increased (Figure 4b), with a determination coefficient (R^2) of 0.7978, thus proving the effectiveness of the ozone pretreatment for reducing the recalcitrance in bagasse while making it more susceptible to enzymatic hydrolysis, especially considering the short reaction time of 30 min.

Table 3. Comparison of ozonation and enzymatic hydrolysis parameters on reported sugarcane bagasse ozone pretreatments.

Ozonation Parameters	Best Result Parameters	Sugars Yields (%)	q_1 (g sugars/g O ₃)	g sugars/g SCB	Relative Cost for Ton of SCB	Reference
0.5–1.5 L/min, 42–222 mg O ₃ /g SCB, <1250–<4760 µm, 30–70% moisture, 38–180 min, 24 g SCB	<1.25 µm, 50% moisture, 222 mg O ₃ /g SCB, 150 min	77.55 GY	2.28 sugars	0.24	USD 153.48 (150 min)	[4]
0.2–0.4 L/min, 4.3–54 mg O ₃ /g SCB, 250–420 µm, 50% moisture, 15–120 min, 1 g SCB	120 min	13.43 SY	1.26 sugars ^a	-	USD 37.53 (120 min)	[6]
32 mg O ₃ /min, 96 mg O ₃ /g SCB ^a , <3000 µm, 33% moisture, 60 min, 20 g SCB	-	16 GY	0.69 glucose ^a 0.93 sugars ^a	0.089	USD 66.72 (60 min)	[11]
204 mg O ₃ /L, 0.5 L/min, ~153–612 mg O ₃ /g SCB ^a , ~20–40 µm, 60% moisture, 30–120 min, 20 g SCB	120 min	63.22 GY	0.5 glucose ^a 0.55 sugars ^a	0.335	USD 425.34 (120 min)	[12]
10 g O ₃ /L, <1000–5000 µm, 40–60% moisture content, 90–270 min	1000 µm, 50% moisture, 210 min	67 SY	-	-	-	[15]
31–62 mg O ₃ /L, 1.5 L/min, 50–100 mg O ₃ /g SCB, 420–1000 µm, 30–60% moisture, 30 min, 28 g SCB	420 µm, 100 mg O ₃ /g SCB, 60% moisture	44.5 GY 35.5 SY	4.74 glucose, 5.75 sugars	0.29	USD 34.75 (30 min)	Present work

Notes: ^a Values calculated based on the reported information. GY: Glucose yield. SY: Sugars yield, as the sum of the absolute amounts of glucose and xylose released after enzymatic hydrolysis divided by the sum of the absolute amounts of glucose and xylose contained in the untreated SCB.

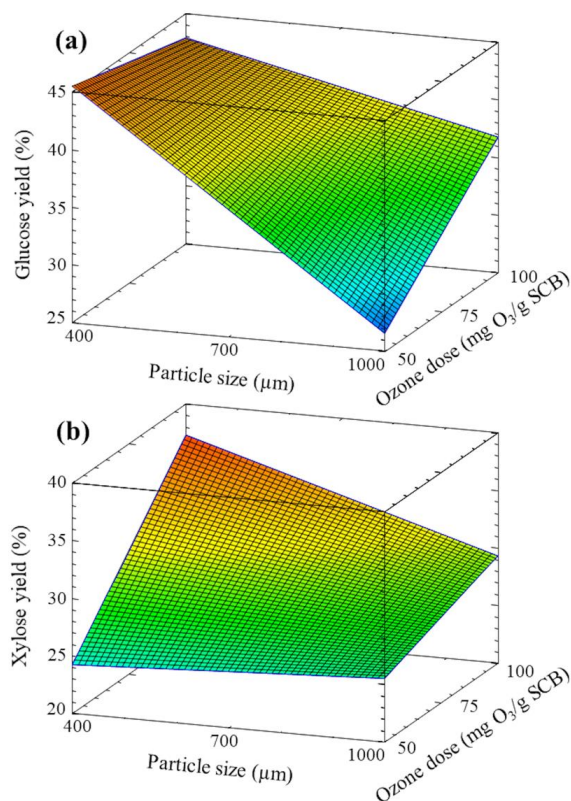


Figure 5. Estimated response surfaces for (a) glucose and (b) xylose yields at 45% *w/w* moisture content.

Figure 4a shows q_1 for each run, with yields varying from 2.33 to 5.75 g sugars/g O₃. The highest q_1 values were obtained for T3 and T7, with 5.75 and 5.63 g sugars/g O₃, respectively, which, interestingly, correspond to the maximum consumed ozone percentages achieved with a particle size of 420 μm and an ozone dose of 50 mg O₃/g SCB, suggesting that the application of low ozone doses in combination with an expanded surface area of the material could allow a better balance between the release of sugars (particularly of glucose, as shown by the surface response in Figure 5a) and the ozone expended. Indeed, the ANOVA analysis indicated that ozone dose was the most influential factor on sugar release, followed by the particle size ($p < 0.05$).

Table 3 shows sugars released/ozone consumption ratios (q_1) previously reported in literature regarding the ozonation of SCB. The achieved q_1 in this study was found to be higher compared to those from previous studies, which, in most cases, employed high ozone doses and ozone concentrations and long reaction times. For instance, Barros et al. [12] pretreated sugarcane bagasse with a dose of 612 mg O₃/g SCB, obtaining a quotient of 0.55 g sugars/g O₃. After the ozonation of bagasse for 120 min, Barrera-Martínez et al. [6] obtained 1.26 g sugars/g O₃, whereas Travaini et al. [4], using a dose up to 222 mg O₃/g SCB, reported a yield of 2.28 g sugars/g O₃. It should be noted that higher glucose yields have been obtained compared to the values herein reported, depending on the ozonation conditions; nevertheless, in such cases, a high amount of expended ozone was observed. In this study, a yield of 0.29 g sugars (as the sum of glucose and xylose) per gram of dry SCB was achieved, which is comparable to the results reported by Travaini et al. [4] and Barros et al. [12]. Taken together, the results obtained here proved the feasibility of minimizing ozone input while maximizing product yield, a fact that has been claimed as essential to achieve a positive energy balance of lignocellulosic biomass-based biorefineries [1]. Indeed, the total ozone generated in this work was recorded in the range of 1.4 to 2.8 g, depending on the applied ozone dose. Considering that 10 kWh are required to produce 1 kg ozone [20], and the current average price of electricity for industrial sector in the US is USD 0.0695/kWh [50], the costs to generate the ozone applied in this study were estimated at

USD 0.09–0.19. Thus, the relative cost for ozonation of one ton of SCB was estimated at USD 34.7, which might represent a further step towards the implementation of ozonation in SCB biorefineries.

Regarding the generation of potential inhibitory compounds, formic, acetic and oxalic acids were detected in all the pretreatment tests. Particularly, formic, acetic and oxalic acid concentrations ranged from 0.89 to 2.97, 6.68 to 12.86 and 41.78 to 46.58 mg/g SCB, respectively. The presence of these organic acids is related to the lignocellulosic material degradation due to ozone reaction [14]. Travaini et al. [14] determined 38.3 mg/g SCB of formic acid and 48.6 mg/g SCB of acetic acid with 110–210 mg O₃/g SCB, whereas Benko et al. [37], in ozonated wood chips, attained 52 mg/g biomass of formic acid, 6.6 mg/g biomass of acetic acid and 8 mg/g biomass of oxalic acid with 612–3060 mg O₃/g biomass. Finally, neither furfural nor 5-hydroximetilfurfural were detected in this work, which is in agreement with Perrone et al. [11] and Travani et al. [14].

3.6. CLSM

The SCB was observed by CLSM in an attempt to illustrate structural changes suffered after the integrated ozonation-enzymatic hydrolysis pretreatment. After visual inspection, the observed changes were found to be similar regardless of the run tested; thus, SCB with a particle size of 710 µm was chosen as a representative sample. Figure 6 shows the CLSM images for (a) unpretreated SCB, (b) pretreated SCB and (c) pretreated enzymatically hydrolyzed SCB. Unpretreated SCB showed an unreacted and integral physical structure, while the ozonated fibers displayed a disruption in their physical structure, even inside of the vascular bundles, as a consequence of the ozonation. In general, these changes were attributed to the hemicellulose degradation and lignin solubilization. Finally, the physical effects provoked by the ozonation followed by the enzymatic hydrolysis could be more easily observed in the fibers, which showed a smooth and washed surface due to the lignin solubilization and subsequent release of sugars by the action of enzymes.

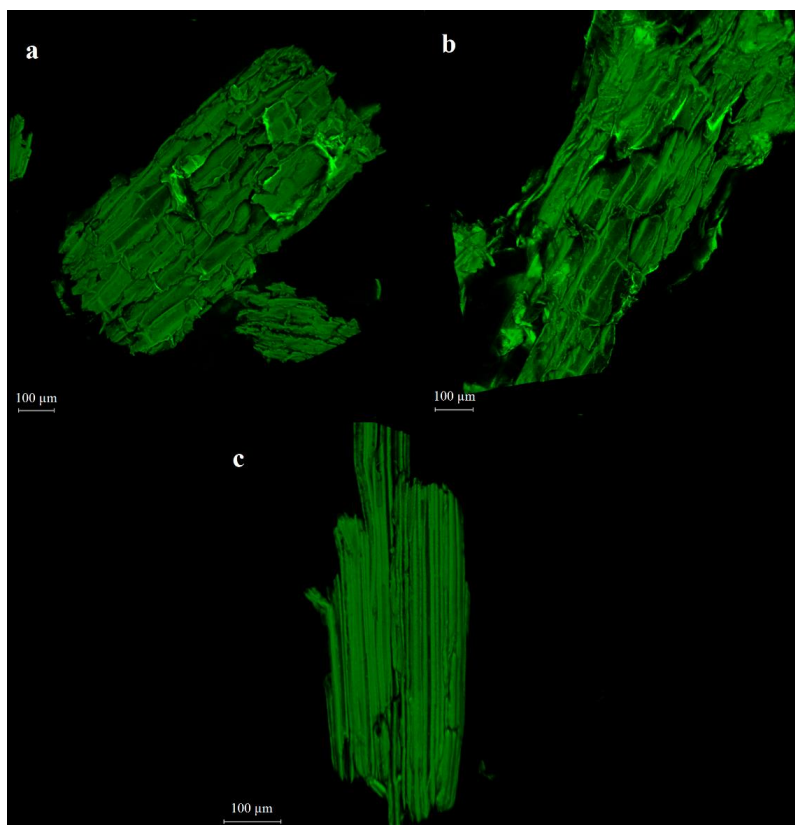


Figure 6. Confocal laser scanning microscopy image of SCB (710 µm particle size) for (a) unpretreated SCB, (b) pretreated SCB and (c) pretreated enzymatically hydrolyzed SCB.

3.7. Mass Balance

A mass balance of the ozonation of SCB is presented in Figure 7. The balance, based on 1 g of SCB, took into account the total solids content, the relative biomass loss, and the changes in the content of cellulose, hemicellulose, AIL and ASL in the un- and pretreated bagasse, as well as the total sugars released after the enzymatic hydrolysis. The amount of 1 g of SCB contained 0.97 g of total solids and 0.5 g of cellulose. To figure out the changes in mass balance after ozonation, the run T3 was selected owing to its maximum sugars released/ozone consumption ratio (q_1). After ozone pretreatment, the total solids content decreased from 0.97 g to 0.88 g, corresponding to a biomass loss of 8.7%. As discussed in Section 3.1, the loss of mass was mainly explained by the degradation of hemicellulose and AIL. Regarding the cellulose fraction, it suffered a slight degradation from 0.5 to 0.47 g. After enzymatic hydrolysis, a bagasse recovery of 0.7 g was achieved in the solid fraction, with cellulose and hemicellulose contents of 0.25 and 0.11 g, respectively. The mass balance also considered the resulting liquid fraction after enzymatic hydrolysis, in which the release of glucose and xylose into the hydrolyzed liquor was 0.24 and 0.05 g, respectively.

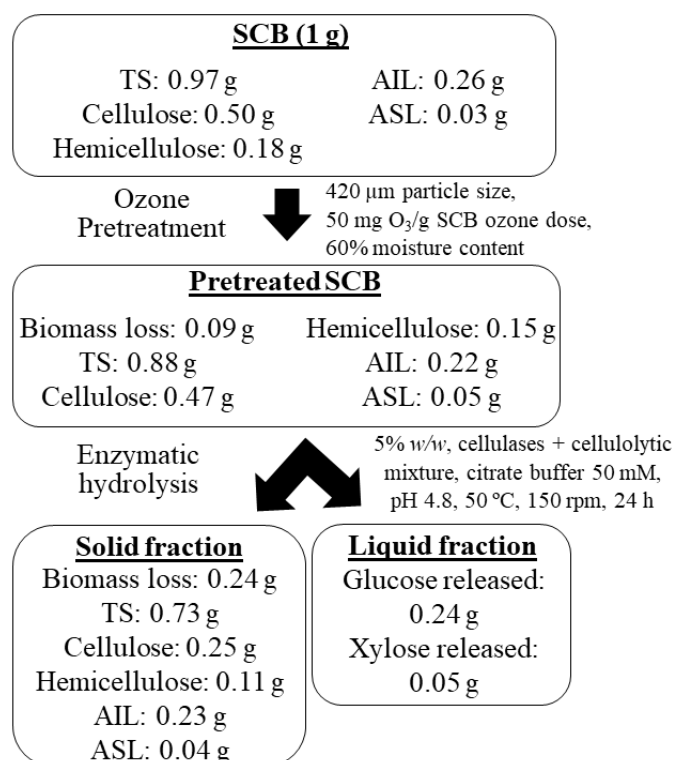


Figure 7. Mass balance for the ozonation of sugarcane bagasse (SCB) at an applied ozone input of 50 mg O₃/g SCB, a particle size of 420 μm, 60% moisture and 30 min exposure time.

4. Conclusions

The combined effects of three key ozonation parameters, namely, particle size, ozone dose, and moisture content on structural-morphological characteristics, ozone consumption and sugars release during enzymatic hydrolysis were investigated using SCB as a lignocellulosic feedstock. The results showed that ozonation decreased the recalcitrance of SCB, clearly enhancing its enzymatic susceptibility, regardless of the conditions tested, and even using a short ozone pretreatment time of 30 min. Particularly, a marked decrease in AIL (from 26.6 to 19.15% *w/w*) was observed at higher ozone doses (100 mg O₃/g SCB) which also were associated to higher mass loss (up to 17%), while a rise in ASL (from 3.07 up to 6.03%) was found irrespective of the experimental variations studied. Cellulose and hemicellulose content varied from 50.59 down to 46.21% *w/w* and from 18.71% down to 13.6% *w/w*,

respectively, depending on conditions. The observed structural changes after ozonation were verified by FTIR and XRD analyses, highlighting that, under the conditions tested, the ozonation mechanism mainly targeted the partial degradation of lignin and hemicellulose, thus increasing the crystallinity index compare to the non-ozonated material. Analyzing ozonation and enzymatic hydrolysis together, higher ozone consumption efficiencies (98%) were attained at 420 μm , 50 mg O_3/g SCB and 60% moisture, deriving a more efficient ozone utilization (49.4 mg O_3/g SCB) and sugars released/ozone consumption ratio (5.75 g sugars/g O_3). Under such conditions, a yield of 0.29 g sugars/g SCB was achieved with an associated preliminary cost of ozonation of USD 34.75/ton of SCB.

Author Contributions: D.R.O.-L.: investigation, formal analysis, writing—original draft preparation; O.G.-D.: writing—review and editing; R.V.-R.: review and editing; A.L.-L.: conceiving the idea; E.L.-B.: conceptualization, supervision, writing—review and editing, project administration, funding acquisition. All authors have read and agreed to the published version of the manuscript.

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