Hydrodynamic cavitation-assisted oxidative pretreatment and sequential production of ethanol and xylitol as innovative approaches for sugarcane bagasse biorefineries

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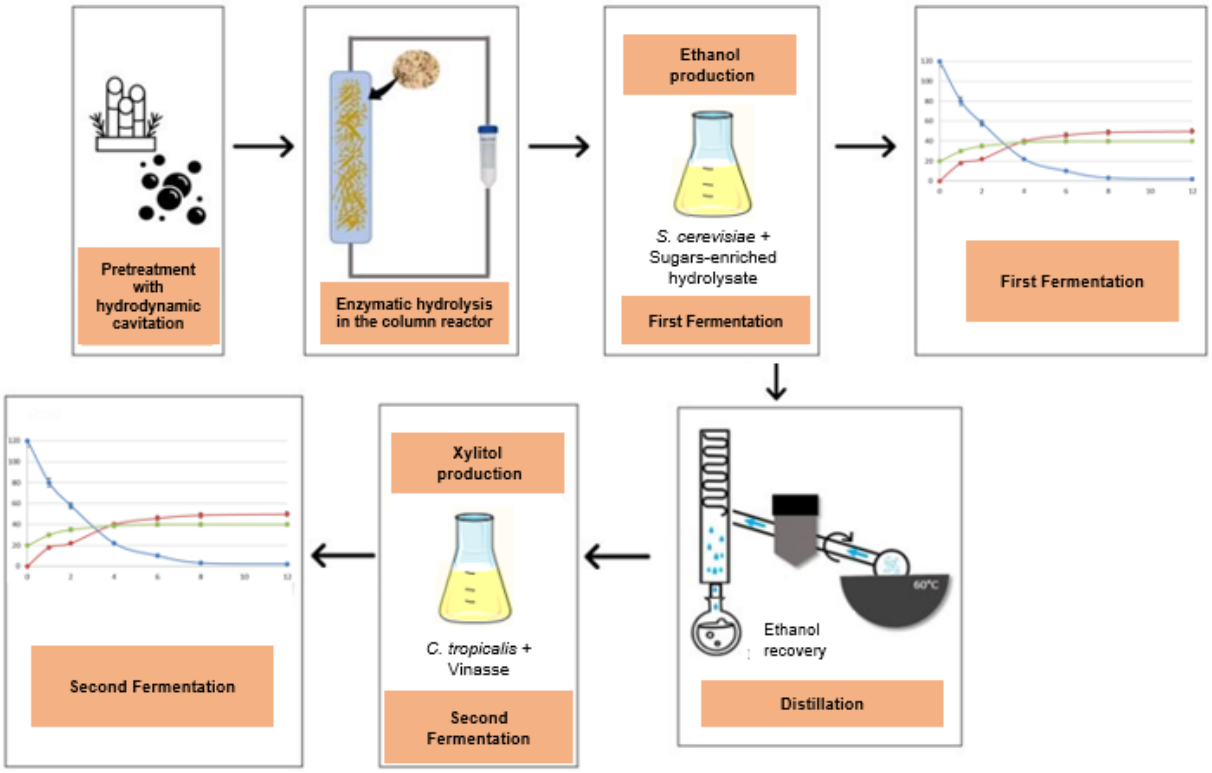
Graphical Abstract



Abstract

In the present work, a new alternative of hydrodynamic cavitation-assisted pretreatment associated with an advanced oxidative process was proposed, together with a new approach to obtain bioproducts in sequential fermentations. Enzymatic hydrolysate of sugarcane bagasse (SCB) was fermented to ethanol by *Saccharomyces cerevisiae*. After distillation of this alcohol, the obtained vinasse was used to produce xylitol by *Candida tropicalis*. Influent variables in pretreatment were evaluated by performing experiments according to a statistical design, and, at optimum conditions (ozone flowrate of 10 mg/ min, and H_2O_2 concentration of 0.61%), about 84% and 78% of glucan and xylan hydrolysis yield were obtained in

enzymatic hydrolysis, respectively. In the sequential fermentations for ethanol and xylitol production, yield values of 0.41 g/g and 0.55 g/g, respectively, were obtained, with corresponding volumetric productivities of 8.33 g/Lh and 0.64 g/Lh, respectively. The proposed strategy was shown as a promising approach for biorefineries, considering the mild conditions of pretreatment and the possibility of high ethanol production using *S. cerevisiae* in a fermentation process similar to that one already available in sucro-alcoholic sector, followed by xylitol production in vinasse-based medium.

Keywords: Second generation biorefinery; Sequential fermentation strategy; Biomass pretreatment; Sugarcane bagasse

1. Introduction

The world has been motivated by environmental concerns to develop research about alternative fuels from renewable sources, aiming to mitigate Greenhouse gas emissions and to reduce the dependence of fossil-based products. Among the available options, second-generation ethanol stands out, with other interesting bioproducts that can be concomitantly produced in biorefineries, creating new and sustainable processes using biomass as raw material [1-3].

Actually, in Brazil, sugarcane bagasse (SCB) is an abundant raw material, obtained as by-product in sugar and alcohol industrial sector [3,4]. The country is responsible for the production of 15 billion liters of ethanol per year, corresponding to an annual generation of 95 million of tons of sugarcane bagasse [4,7].

A limitation for the use of lignocellulosic biomass in biorefineries is its high recalcitrance, which hinders enzymatic digestibility of polysaccharides (mainly cellulose), and, consequently, the release of fermentable sugars to obtain bioproducts [6,8]. Thus, pretreatment is a critical stage for the use of lignocellulosic biomass and can represents almost 30% of the whole process cost [6, 9,10].

Currently emerging pretreatments such as Hydrodynamic Cavitation (HC) have been evaluated, with promising results to overcome the negative points of conventional pretreatments [11, 12]. HC has important advantages, such as low processing time, low reagents requirement, the versatility of operation modes, and easy scale-up [13-15].

Hydrodynamic cavitation has been a strategy to enhance the efficiency of lignocellulosic biomass pretreatment. The HC modifies the lignocellulosic structure due to the high energy released in "hotspots" of cavitation, which can help to generate oxidative radicals to aid the breaking of lignin [12]. HC also results in increase in the surface area and in the porosity of biomass [12, 15, 16]. However, until now there are a relatively small number of papers published about this topic, as reported in recent review articles [12,16,

17]. Thus, some challenges are still to be overcome, aiming to reducing even more the quantity and cost of reagents used together with HC, favoring the viability of the technique.

There are previous works of our research group reported in the literature about HC-assisted pretreatment of biomass, using processes in an alkaline medium [18], or in an alkaline medium containing H_2O_2 [19]. Hilares et al. [18], for example, reported alkaline HC-assisted pretreatment of sugarcane bagasse, obtaining 20 g of total reducing sugars released per 100g of pretreated biomass, by using 3 mol/L of NaOH and 1% of H_2O_2 for 10 min in the pretreatment performed in HC-reactor with an orifice plate with 16 holes of 0.65 mm each one [17]. However, new studies should be performed aiming to reduce the quantity of reagents necessary in the process, and in this way Advanced Oxidative Processes (AOPs) could be an interesting and non-previously reported alternative for HC-assisted pretreatment of biomass.

Actually, as pointed in a recent review of Prado et. al. [12], AOPs can generate radicals to degrade recalcitrant materials, with benefits such as a low-cost pretreatment in mild conditions, increasing the digestibility of lignocellulosic biomass, and reducing process time and chemical inputs [12,19]. HC association with AOPs using reagents as O_3 and H_2O_2, can favor the formation of free radicals that remove lignin, complementing the quantity of radicals such as HO_2^- and $O_2^{\cdot}$ (Eq. 1 and Eq. 2) that are already formed on-site by HC , increasing the effectiveness of the pretreatment [15,18]. Thus, HC/AOP combined processes could be interesting, resulting in high lignin removal by oxidation, due to the synergy between the two techniques, which produce high quantity of oxidant hydroxyl radicals [15, 19].

$$H_2O_2 \rightarrow HO_2^- + H^+ \qquad \text{Eq.1}$$

$$O_3 + HO^-_2 \rightarrow HO^{\cdot} + O_2^{-\cdot} + O_2 \qquad \text{Eq.2}$$

Besides to consider the pretreatment approach, all fractions of biomass should be taken into account to obtain interesting products, aiming at favoring the utilization of lignocellulosic as raw material in biorefineries [20]. Regarding to carbohydrate fractions, cellulose can be hydrolyzed to obtain glucose, a monomeric sugar directly fermentable by the *S. cerevisiae*. On the other hand, hemicellulose can represent until one-third of biomass, and its hydrolysis results in a xylose-enriched mixture [21]. Xylose can be also used to produce ethanol by genetically modified microorganisms or even by naturally-xylose-fermenting yeasts [22], but other value-added compounds could be produced and result in a more profitable biorefinery. Among options, xylitol is a versatile, and valuable biomolecule, with important applications in chemical,

pharmaceutical, and food sectors [23, 24]. Although currently produced mainly by a chemical process, the xylitol production has been evaluated through bioprocesses and this route has advantages such as the use of comparatively milder conditions, no requirement of extensive xylose purification steps, with low cost of product [23, 25].

Simultaneous ethanol and xylitol production has been studied [22,26], but this alternative could present low productivity for both products, with non-optimized conditions for each one. Karagoz et al. [26], for example, reported simultaneous ethanol and xylitol production using co-culture of two different yeasts (*Scheffersomyces stipitis and Candida tropicalis*), obtaining 20g/L (Yp/s was 0.36) of ethanol in 72h, besides 8 g/ L of xylitol (Yp/s of 0.23).

An interesting approach to produce high concentrations of ethanol and xylitol with high productivities would be the sequential production of these compounds, which could occur in a process similar to that one already used for first generation ethanol. The production of this fuel in Brazilian industry is usually performed by the fermentation of sugarcane juice or molasses, carried out by the yeast *Saccharomyces cerevisiae* [27]. This yeast is the most traditionally used in industrial process, considering its advantages as be resistant to high ethanol concentrations, and production of ethanol from medium with high concentration of sugars in relatively short time (about 8h-12h) [27]. For first generation ethanol from sugarcane, this process is consolidated, involving milling, fermentation, and distillation. Thus, considering the above-mentioned advantages of sugars fermentation with *S. cerevisiae*, a similar process could be advantageous to produce ethanol in glucose-enriched hydrolysates obtained from cellulose of the sugarcane bagasse [26, 27]. Also, in sucro-alcohol Brazilian industry, a great quantity of vinasse is produced in alcohol distillation step. In the country, for each ton of sugarcane, 70 liters of alcohol and 800 to 900 liters of vinasse are produced [28]. If a hydrolysate from cellulose and hemicellulose fractions of sugarcane bagasse was used in alcohol production by *S. cerevisiae*, the xylose is expected to remain in fermented broth, thus remaining in the vinasse obtained in distillation of ethanol. Then, the cultivation of a xylitol producing microorganism in vinasse-based medium would be an interesting approach to integrate 2G ethanol and xylitol production.

This work evaluated innovative approaches for pretreatment of SCB and for ethanol and xylitol production. The pretreatment was performed by combining HC/AOP in a medium added with ozone and H_2O_2. It resulted in biomass enriched in cellulose and hemicellulose, which was hydrolyzed in a column bioreactor [30]. As the resulting hydrolysate had a mixture of sugars, mainly glucose and xylose, an

approach of a sequential production of ethanol and xylitol was proposed and evaluated. *S. cerevisiae* was used to produce ethanol from glucose with high productivity, and, after ethanol distillation, the produced vinasse containing xylose was used for xylitol production by *C. tropicalis.* The flocculent *S. cerevisiae* IR2 strain was used aiming to favor its reuse in the process [31].

2. Material and Methods

2.1 Raw material and chemicals

Ipiranga Agroindustrial LTDA (Descalvado, São Paulo, Brazil) donated the Sugarcane bagasse (SCB). This raw material was dried under sunlight until 10% of moisture, and it was milled until particle size between 0.60 mm and 1.18 mm, which was used for the study.

Commercial cellulases preparation CELLIC CTEC-2 (with 110 FPU/mL) was purchased from Sigma-Aldrich Brasil Ltda. (Cotia, SP, Brazil). Peptone (part number 91079-38-8) and yeast extract (part number Y1625), and other chemicals (all of analytical grade) were also purchased from Sigma-Aldrich Brasil Ltda.

2.2 Hydrodynamic cavitation-assisted oxidative pretreatment

Pretreatment was performed in a HC system, which was described elsewhere [32]. An oxidative pretreatment system was adapted to the cavitation reactor, The Ozonator (Ozone & Life, Waterone, Kansas City- KN, USA) was linked by a pipe to feed O_3 to the HC reator. As shown, the system is composed mainly of a HC-reactor (cavitation zone), in which biomass is placed inside a screen. Liquid medium is recirculated by a 11032.5W centrifuge pump (Grundfos CM 5-3, Bombas Grundfos do Brasil Ltda., São Bernardo do Campo, SP Brazil), generating the cavitation in a 16 holes-orifice plate. A recirculation tank is also present to warrant an enough volume of fluid to be suctioned by the pump.

A 2^2 central composite face-centered design, with triplicate at the center point (Table 1), was used for the pretreatment runs. The results were analyzed aided by Design-expert software v. 13 (Stat-Ease, Inc., Minneapolis, US) and Statistica for Windows (StatSoft, Inc. V.5 Tulsa, OK, USA). The evaluated variables were ozone flow (from 2 to 10 mg/min) and concentration of H_2O_2 in the medium (0 to 1%). The studied range for ozone flow was based on the limits of capacity of available system, and the range for H_2O_2 was determined to be lower than the values used in a previous work of HC-pretreatment without AOP [30].

Response variables were the composition of the pretreated material and the hydrolysis yield of the

carbohydrate fractions of the material, which was evaluated in an enzymatic hydrolysis step carried out in Erlenmeyer flasks according to section 2.3.

All pretreatment runs were carried out with 35 g of bagasse loaded in the system, which was also charged with 3 L of a H_2O_2 solution, in concentrations according to statistical design. The solution was recirculated in the system with a flow of 5 m³/h, and ozone was fed continuously into the reactor cavitation, with air flow in the ozonator (Ozone & slamp; Life, model O&Samp:L 3,0RM) of 0.2 m³/ min and the ozone flow adjusted according to statistical design (total gas flowrate from 12 to 76 mL/min, corresponding to an ozone flow of 2 to 10 mg/min). The pretreatment time was 10 min at 60ºC, with a pressure of 3 atm upstream of the orifice plates. After this step, the pretreated bagasse was separated from the liquid medium by filtration and washed with distilled water. Subsequently, the components (cellulose, hemicellulose, and lignin) of pretreated bagasse were quantified.

At optimized conditions, which was indicated by an empiric mathematical model composed after statistical analysis, experiments were performed in triplicate to confirm the prediction of the model. Also, control experiments were performed under those conditions: a. Without orifice plates in the cavitation system (control without HC), using the same recirculation flow in the pump; b. Without ozone (only with air inlet from ozonation system) nor H_2O_2 in the cavitation system (only HC). Additionally, a control was carried out with the enzymatic hydrolysis of untreated biomass performed according to section 2.3.

2.3 Enzymatic Hydrolysis in Erlenmeyer flasks to evaluate the pretreatment

This step, using the pretreated SCB, was carried out in Erlenmeyer flasks (125mL) with 5% of solid loading in a citrate buffer solution (50 mM) with pH 4.8. The enzymatic hydrolysis was performed employing Cellic® CTec2 (Novozymes Latin America Ltda., Brazil), a commercial cellulases enzyme blend, with a loading of 20 FPU g^{-1} of dry pretreated SCB. Reaction was performed at 50°C and 200 rpm for 24h. Concentrations of hexoses and pentoses released in hydrolysis were analyzed by high performance liquid chromatography (HPLC). Hydrolysis yields were calculated as Equations 3 and 4 [33].

$$Glucan\ hydrolysis\ yield(\%) = \frac{(G*0.9*V)}{(CC*M)} *100\% \quad \text{Eq. 3}$$

$$Xylan\ hydrolysis\ yield(\%) = \frac{(Xyl*0.88*V)}{(CC*M)} * 100\% \quad \text{Eq. 4}$$

Where:

G = concentration of glucose g /L released from glucan in enzymatic process

Xyl = concentration of xylose g/ L released from xylan in enzymatic process

V = total volume of reactional medium

CC = percentage of glucan or xylan in raw SCB

M = mass of SCB used in hydrolysis enzymatic

2.4 Enzymatic hydrolysis in column reactor

Column enzymatic hydrolysis process was performed as described by [30], with 21% of solid loading (40 g of SCB, total liquid 190 mL). The reactor consisted of one column with 30 mm of internal diameter. Liquid medium was composed by a citrate buffer solution (50 mM) with pH 4.8, and Cellic® CTec2 (Novozymes Latin America Ltda., Brazil), using 20 FPU /g of dried pretreated biomass. Following, hydrolysate was vacuum concentrated at 50°C in a Rotary Evaporator. Then, concentrated hydrolysate was used in sequential fermentation experiments.

2.5 Sequential production of ethanol and xylitol by *Saccharomyces cerevisiae* IR2 and *Candida tropicalis* UFMGX12.

2.5.1 Microorganism, inoculum preparation and cells immobilization

S. cerevisiae IR2 and *C. tropicalis* UFMGX12 were obtained from stock cultures available in the Sustainable Bioproducts Laboratory (LBIOS) at the Engineering School of Lorena–University of Sao Paulo, Brazil. Inoculum was prepared in 250 mL Erlenmeyer flasks containing 100 mL of medium, the cultivation carried out in a BIO CB SSB rotary shaker (ERETZBIO, São Paulo, Brazil), for 24 hours, 150 rpm, at 30ºC.

For the yeast *S. cerevisiae* IR2, the medium was composed of 50 g/L of glucose, 10 g/L of bacteriological peptone and 10 g/L of yeast extract. For *C. tropicalis* yeast, the medium was composed of 60 g/L xylose, 20 g/L yeast extract, 2 g/L NH_4SO_4, 0.1 g/L $CaCl_2.2H_2O$. For both fermentations, with initial pH adjusted to 5.5.

Subsequently, both cells were recovered by centrifugation at 5000g for 10 min, washed and suspended in water (distilled) to obtain a suspension with high cell density, which was used as an inoculum in order to obtain 20 g/L and 1 g/L of initial cell concentration in the fermentation process performed with *S. cerevisiae* and *C. tropicalis*, respectively.

2.5.2 Ethanol and xylitol sequential production

198 The sequential production was performed using two processes, the first fermentation aimed ethanol
199 production by *S. cerevisiae* in a medium formulated with concentrated hydrolysate (containing 150 g/L of
200 glucose, 60 g/ L of xylose), supplemented with 10 g/ L of peptone, and 10 g/ L of yeast extract (conditions
201 according to Ramos et. al. [31]. The process was performed in a rotary shaker in 125mL Erlenmeyer flasks
202 loaded with 75mL of medium, with agitation of 150 rpm, at 30°C for 24h. After the process, cells were
203 separated from medium by decantation, followed by centrifugation at 5000g for 10 min.
204 Then, ethanol was removed from the fermented broth by a vacuum rotary evaporator at 55°C for
205 6h. The complete removal of ethanol was verified by analysis of samples by HPLC. Afterwards, the
206 resulting liquid mixture (vinasse) containing xylose was fermented by *C. tropicalis* to obtain xylitol. This
207 second fermentation was performed in a rotary shaker in 50 mL flasks loaded with 25mL of medium
208 containing: vinasse (60 g/L of xylose), supplemented with 20 g /L of yeast extract, 2 g/ L NH_4SO_4 and 0,1
209 g/L $CaCl_2.2H_2O$, according to De Arruda et. al. [34]. Medium was adjusted to initial pH of 5.5 with aqueous
210 solution of NaOH 0.1 mol/L, and fermentations were performed with agitation of 150 rpm, at 30°C for 48h.
211 For both fermentations, periodic samples were withdrawn from the medium for analysis of sugars and
212 products.
213 For fermentation process, yield values (Yp/s) were calculated considering the ratio between the
214 concentrations of obtained product and consumed substrate. Efficiency values were calculated as the ratio
215 between the obtained and the theoretical values of Yp/s, which are 0.51 g/g for ethanol and 0.917 g/g for
216 xylitol [35]. Productivity values (Qp) were calculated by dividing the concentration of obtained product by
217 the time of fermentation.
218
219 **2.6 Analytical methods**
220 **2.6.1. Humidity**
221 The sugarcane bagasse moisture was determined by using an infrared balance Mark M163 (BEL
222 Engineering, Piracicaba-SP).
223 **2.6.2. Compositional characterization of bagasse**
224 The components of *in natura* and pretreated bagasse were quantified in cellulose, hemicellulose,
225 lignin, ashes, and extractives, following the methodology of Mesquita, Ferraz, and Aguiar [36]. Solid
226 recovery was calculated by the ratio between the dry mass of the material after pretreatment and before
227 pretreatment (Eq.5).

$$\%Solid\ rec. = \left[\frac{m\ SCB\ pret.}{msCBstart}\right] \times 100 \quad \text{Eq. 5}$$

Where:

Solid rec = Solid recovery, in percent (%)

m SCB pret. = dry mass of the material after pretreatment (g)

m SCB start = dry mass of the material before pretreatment (g)

2.6.3. Analysis of sugars, acetic acid, ethanol, and xylitol

These compounds were analyzed by HPLC on Agilent Technology 1200 series chromatograph (Agillent Technologies 1200, New York, USA). Before analysis, liquid phase was filtered in Sep Pak C18 filters and analyzed in chromatograph, according to Antunes et. al. [20].

2.6.4. Biomass concentration

For the experiments using *S. cerevisiae*, after cultivation, all the medium volume was centrifuged at 3000 g for 20 min, cells were washed two times with distilled water, and the biomass concentration was measured considering the dry mass obtained after drying cells in an oven at 105ºC.

For experiments using *C. tropicalis*, periodic samples were removed from medium, and the yeast concentration was measured by turbidimetry [20].

2.6.5. Structural analysis of sugarcane bagasse by Fourier Transform Infrared Spectroscopy (FTIR)

The Fourier Transform Infrared Spectroscopy (FTIR) was performed in an equipment Shimadzu IR Prestige-21(Shimadzu, Kyoto, Japan). Samples were previously prepared in potassium bromide (KBr) pellets pressed at 50 kN. Spectra were collected in transmittance mode, with 64 scans, 4 cm^{-1} resolution and a spectral range from 4500 to 400 cm^{-1}. The samples analyzed were SCB *in natura*, and SCB pretreated with HC-assisted oxidative process in the optimized conditions.

2.6.6. Structural analysis of sugarcane bagasse

Different structures in SCB surfaces were observed using LEO44Oi Electron Microscopy/Oxford (Cambridge, England), as described by [20]. Crystallinity index (CrI) in raw and pretreated SCB was determined by X-ray diffraction (XRD), according to Kumar et. al. [2]. The crystallinity was scanned over the range of 2θ = 5-50° and the crystallinity index (CrI) was calculated using Eq.6 [2].

$$CrI(\%) = \left[\frac{(I\ crystalline - Iamorphous)}{Icrystalline}\right] \times 100 \quad \text{Eq. 6}$$

Where:

I crystalline= Intensity at 22.3°

I amorphous = Intensity at 16.1°

2.6.7. Chemical oxygen demand (COD), Biologic oxygen demand (BOD) and Total Organic Carbon (TOC) analysis

Vinasse obtained from ethanol production and the fermented broth obtained after xylitol production (after cells removal by centrifugation at 5000 g for 10 min) were characterized regarding to COD, BOD and TOC.

The methodologies used for COD and BOD determination were adapted from Standard Methods [36].

For COD determination, samples were previously diluted 10 times and NaOH (0.1 mol/L) was used to adjust the pH to 7.0. BOD5 assay was performed at 50°C for 5 days. It was measured by the difference in demand of oxygen before and after the incubation period, with deionized water previously saturated with compressed air. Dissolved oxygen was measured in samples using HANNA INSTRUMENTS®Modelo (New York, USA).

TOC determinations were performed in a total organic carbon analyzer (Shimadzu, TOC-VCPH model, Kyoto, Japan). Two calibration curves were constructed: the first for low carbon content, ranging from 0 to 100 mg/L, and the second for high content, ranging from 100 to 1000 mg/L of carbon. All samples were previously diluted up to 10 times and pH was corrected (between 2 and 3), in order to ensure total acid digestion prior to combustion. The equipment used was programmed to perform two measurements for the samples, and the analytical control was performed as a function of coefficient of variation (CV), aiming to a value less than 3% (otherwise, another measurement was performed).

3. Results and Discussion

3.1 HC-assisted AOP Pretreatment effect on enzymatic hydrolysis of SCB

SCB was pretreated under different conditions using AOP assisted by HC process, and the result of pretreatment was evaluated in biomass composition and in enzymatic hydrolysis of its cellulose and hemicellulose fractions.

Untreated biomass had 40% of glucan, 26% of hemicellulose (24% of xylan, 1% of arabinan, and 1% of acetyl), 25% of lignin, besides and 1% of ash and 3% extractives. Table 1 presents the results of the statistical design carried out for evaluating the effects of the variables O_3 flow (2 to 10 mg/min) and H_2O_2 concentration (0% to 1%) on the yields of enzymatic hydrolysis of glucan and of xylan, and on biomass composition and removal of its compositional fractions.

As shown in Table 1, the pretreatment resulted in modification on the SCB composition, rendering it as a material enriched in cellulose and with lower content of lignin. Indeed, the lignin was the main fraction removed by the pretreatment (removal of 42-56%), followed by xylan (28-35%), while glucan removal was lower than18%.

Indeed, the hydrodynamic cavitation effect on the pretreatment of lignocellulosic biomass has been reported in literature [11, 15, 30], and the chemical and physical action of cavitation provides benefits such as mild conditions for oxidative degradation, and reduction of lignin fraction of biomass under short reaction period [19]. The HC phenomenon results in high energy released into the medium, which contributes for generation of oxidative radicals [12, 17]. Besides, the addition of oxidizing agents (ozone and H_2O_2) further contributes to process efficiency due to the high oxidative power (2.08V, 1.78V of ozone and H_2O_2, respectively). HC and oxidizing agents synergically act on lignocellulosic material, breaking down lignin molecules [12, 38]. HC effect is also associated with high-speed microjets action and shockwave generation due to violent cavity collapse. This improves cellulose and hemicellulose enzymatic conversion into fermentable sugars, once the specific surface area grows and total pore and micropore volumes increase [14, 32].

As observed in Table 1, the compositional modification resulted in different values of glucan and xylan hydrolysis. The highest yield values (84% for glucan and 78% for xylan) were observed using ozone (O_3) flow of 10 mg/ min and 1% of H_2O_2 in the medium.

The obtained data were statistically analyzed, and empiric models (Eq. 3 and 4) were composed to explain the experimental behavior of the variables glucan and xylan hydrolysis yield. ANOVA

(Supplementary material) was performed for the models, checking they were significant (*p-value*<0.05), although also with significant lack of fit (*p-value*<0.05). Both models were reduced by removing non-significant terms (except if required for model hierarchy), and R^2 values were 95% and 83% for the models of glucan and xylan hydrolysis yield, respectively. Even with significant lack of fit, the models were used to trace response surfaces and for process optimization, considering their significance at 95% confidence level and, mainly for glucan hydrolysis yield, the high value of R^2. As following shown, those models were able to predict results at optimized conditions.

Thus, based on the composed models (Equations 7 and 8), the response surfaces were traced (Figure 1). The enzymatic hydrolysis of the carbohydrate fractions of the sugarcane bagasse pretreated by the AOP process assisted by hydrodynamic cavitation were linearly dependent on the input flow of ozone in the cavitation reactor, with higher hydrolysis yield obtained using greater ozone flow. Regarding H_2O_2 concentration, the dependence of glucan and xylan hydrolysis yield was quadratic.

$$\text{Glucan hydrolysis yield (\%)} = 22.48 + 0.46A + 103.93B - 81.22B^2 \quad R^2 = 95\% \quad \text{Eq. 7}$$

$$\text{Xylan hydrolysis yield (\%)} = 21.41 + 0.41A + 96.47B - 82.34B^2 \quad R^2 = 83\% \quad \text{Eq. 8}$$

Where: A is ozone flow; B is H_2O_2 concentration

The models were then used to optimize the response variables. The maximization was performed using the Design-expert software-specific tool. The optimized conditions were obtained according to the numerical tool of the Design Expert software, in order to maximize both the hydrolysis of glucan and xylan. The conditions obtained were: 10 mg/min of ozone and 0.61 % of H_2O_2.

Experiments were performed in triplicate under these conditions to confirm the models and the yield of glucan hydrolysis predicted by the model, of (90.6± 6.9) % (average value ± 95% confidence interval), was confirmed by the experimental result obtained, (84.1 ± 4.1) % (average value ± standard deviation). The yield of xylan hydrolysis predicted by the model, (80.9 ± 12.2) % (average value ± 95% confidence level), was also confirmed by the experimental result obtained (72.0 ± 1.5) % (average value ± standard deviation).

Control experiments were also performed in triplicate under the optimized conditions, as section 2.2. The first control used ozone inlet (10 mg/min), and H_2O_2 (0.61%) in the HC-reactor at the same recirculation flow, but without an HC-generator device (orifice plates). Thus, in this case, only the effects

oxidizing agents were present. The second control considered only the HC effect (without oxidizing agents). As shown in Figure 2, the beneficial effect of using a combined AOP-HC system was shown, considering the enzymatic hydrolysis yields of glucan and xylan were lower than 50% and 40%, respectively, in the control experiments. Anyway, in all cases, the pretreated biomass resulted in hydrolysis yields higher than those ones observed for untreated biomass, which corresponded to 18.27±0.18 % for glucan, and 8.53±0.24 % for xylan (results were presented as average±standard deviation).

In a previous work, Teran Hilares et al. [20] used 1 mol/L of NaOH and 1% of H_2O_2 for alkaline HC-assisted pretreatment of sugarcane bagasse, reporting a hydrolysis yield of 89 % of glucan and 78 % of xylan in biomass pretreated for 10 min. Although those results were slightly superior to the reported in the present manuscript, it is important to note we used lower quantity of H_2O_2 (0.61%) and no NaOH. Instead, ozone was used, which has been reported as a low-cost reagent [23].

3.1.1. Changes in biomass due to the pretreatment: FTIR, XRD, and SEM

In order to verify changes in biomass along pretreatment, FTIR, X-ray diffraction (XRD) and Scanning electron microscopy (SEM) were performed for *in natura* and for biomass pretreated under optimized conditions.

The samples of *in natura* and pretreated SCB presented some similar bands when analyzed by FTIR (Figure 3). As also observed by Cai et al. [39] and Yan et al. [40], the FTIR spectrum showed bands at 3337-3432 cm^{-1} due to -OH symmetrical and asymmetrical vibration, at 2946-2895 cm^{-1} due to groups CH_2 and CH_3, and at 1031 cm^{-1} due to C–O, C═C and C–C═O elongations. They are assigned to the three fractions of biomass and are present in the spectra of both analyzed samples. In pretreated material, it was observed an accentuation in the peak 1746 cm^{-1}, referring to the free ester carboxyl of the hemicellulose fractions, the accentuation of the -OH band in the plane at 1375-1444 cm^{-1}, referring to cellulose and hemicellulose, and the disappearance of 1258 cm^{-1} peak, referring to ═O–C–O–C stretching vibrations of lignin. Also, in pretreated material occurred an accentuation of the 900 cm^{-1} peaks, referring to glycosidic bonds, in 1643-1512 cm^{-1} peak, referring to aromatic structure modification, and in 845 cm^{-1} peak, which demonstrated the C-H structure after aromatic deformation. Those phenomenona can be related to the effects of oxidative pre-treatment on the SCB, since removal of lignin explain the accentuation of characteristic peaks of glycosidic and alteration on aromatic structure fractions, also explaining the disappearance of peaks of lignin fraction.

From the X-ray analysis, *in natura* SCB showed CrI of 39%, while pretreated SCB with HC-assisted oxidative process under optimized conditions presented CrI of 57%. Indeed, the sugar's fractions and lignin content of the biomass modify the CrI of lignocellulosic material [41-42]. In the present work, the CrI of the pretreated material was higher compared to *in natura* SCB. Amorphous compounds, as lignin and hemicellulose, help decreasing the crystallinity of the material. Depending on the effect of the pretreatment, these compounds can be removed, increasing the total crystallinity of the material. However, since part of the present cellulose is a crystalline polymer, the pretreatments usually reduce this specific polysaccharide's CrI, while the material's CrI is magnified [42].

Subsequently, the raw and pretreated sugarcane bagasse were looked over by scanning electron microscopy (SEM), in order to study the changes provoked by the pretreatment in the morphology of the structures (Figure 4). The images of raw sugarcane bagasse revealed the fiber surface is more homogeneous compared to the pretreated material. The structure of raw SCB is mainly covered by lignin and extractives, which is characteristic of *in natura* agricultural residues. Images of pretreated sugarcane bagasse morphology confirmed the disruption of the material due to a high removal of lignin and extractives [43].

3.2 Sequential Fermentation production of ethanol and xylitol

Enzymatic hydrolysis was carried out in column reactor using high solid loading (21%). This is an interesting approach to obtain high sugars concentration in the hydrolysate, as previously reported [30, 31]. After 48h of process, a hydrolysate with 60 g/L of glucose and 30 g/L of xylose was obtained. After this step, the hydrolysate was vacuum concentrated until reach 120 g/L and 60 g/L of glucose and xylose, respectively, aimed to the further fermentation assays.

The ethanol production process was performed with an initial cell concentration of 20 g/L, an approach usual in Brazilian sucro-alcohol industry [44], adequate to reduce the fermentation time, thus increasing the productivity.

As shown in Figure 5A, after 6 hours of process, the glucose consumption had achieved 90%, and ethanol concentration attained about 50g/L, corresponding to a Yp/s of 0.41 g/g (fermentation efficiency of 82.1%) and Qp of 8.33 g/Lh. This value of Yp/s was similar to the reported by Ramos et. al. [31]. Those authors used the same yeast strain, *Saccharomyces cerevisiae* IR2, with cultivation in a bubble column bioreactor using medium based on hydrolysate enzymatically obtained from sugarcane bagasse pretreated by alkaline sulfite method. However, the maximum Qp values observed by those authors (1.58 g/Lh) were

lower than the obtained in the present work.

As also can be seem in Figure 5A, microbial cells also increased, reaching 40 g/L in 4h of fermentation. Actually, ethanol production is reported as a growth-associated product [45-46]. After the process, cells were separated from fermented broth by decantation, an advantageous of using the strain IR2 of *S. cerevisiae*, which is flocculent [31, 35], a characteristic of this strain that favors biorefineries due to its ease separation and reuse [30]. Even so, aiming to warranty a free cell broth, an additional centrifugation was performed before distillation of ethanol.

Subsequently, ethanol was then separated from medium by distillation in a rotary evaporator. After removal, the residual liquid mixture remaining in the system (vinasse) presented great quantity of xylose (about 60 g/L), feasible for use as carbon source in fermentation process for value-added product obtaining, i.e., xylitol production. In this case, besides xylitol production, this process is an interesting approach to reduce the high organic loading of vinasse, considered as a problem for industry with environmental concerns [35].

Thus, Figure 5B presents xylitol production fermentation profile, showing that in 36h of the process the consumption of xylose had already achieved 84%. Furthermore, in 48h of process, maximum of 32 g/ L of xylitol was observed, corresponding to Yp/s of 0.55 g/g (60% of fermentation efficiency), and Qp of 0.64 g/ Lh.

The obtained results were similar or superior to other reported works by using different systems for xylitol production. Antunes et. al. [46] evaluated the production of xylitol by *Candida tropicalis* UFMGBX12 in agitated Erlenmeyer flasks by using acid sugarcane bagasse hydrolysate. In that work, the fermentation was performed with a medium composed of 30g /L of xylose, 10g/L of yeast extract, 0.4g/L of magnesium sulfate and 5g/L of KH_2PO_4, and the process was conducted under 30°C and agitation of 200rpm. The authors reported production of 12g/ L of xylitol after 96h of process, showing Yp/s of 0.61 g/ g, and Qp of 0.12g/Lh. In another work, De Arruda et. al. [34] studied xylitol production by *Candida guilliermondii* FTI 20037 in 2.4L stirred tank reactor (450 rpm of agitation and 0.7 vvm of aeration) by using medium based on sugarcane bagasse hemicellulosic hydrolysate. The fermentation was conducted under 30°C and initial pH of 5.65g/L of xylose and supplementation with 2.0 g/L of ammonium sulfate, 0.1g/L of calcium chloride, and 10g/L of rice bran extract. The authors reported values of 0.55 g/g of Yp/s, efficiency of 60%, and Qp of 0.31g/Lh, with 35 g/L xylitol production in 84h. It is worth to mention no previous work was found reporting the production of xylitol as a sequence of ethanol production in a

438 medium based on enzymatic hydrolysate of the cellulosic and hemicellulosic fractions of sugarcane
439 bagasse.

440 As the hydrolysate used in the present work was obtained by enzymatic route, no inhibitors are
441 expected in the medium to interfere in the evaluated fermentative processes [47-50]. Besides, as the
442 performance of the xylitol process was similar or superior compared to other reported works, the results
443 indicate no possible metabolites of *S. cerevisiae* from ethanol fermentation remained in the vinasse in an
444 amount enough to impair the xylitol production.

445 An additional consideration is related to the quality of generated wastewater. In the usual Brazilian
446 ethanol production process, organic loading of vinasse can offer environmental concerns. Actually, there is
447 a high volume of vinasse generated as residue of the first generation ethanol distillation. It can be used for
448 fertigation, but its high concentration on the soil by evaporation can cause problems on subsoils [29].
449 Indeed, sugarcane vinasse causes alterations in the properties of soils and biota in general [50, 28]. Taking
450 this into account, an evaluation of organic loading of wastewater produced in the second generation
451 sequential process proposed in the present work was performed. In this way, the values of COD, BOD5,
452 and TOC were measured in vinasse before and after xylitol production process (cell free fermented broth).
453 The results indicated high organic loading in the vinasse after first fermentation, but great reduction after
454 xylitol fermentation (Table 2). TOC values were reduced in 91%, while COD and BOD5 values were
455 reduced, respectively, in 93% and 97%. BOD5/COD was 0.50 in vinasse and was 0.87 in cells-free
456 fermented broth, indicating the increase in biodegradability after xylitol production. Moreover, the
457 fermented broth after xylitol production would have more reduction in organic loading after downstream
458 of the product.

459

460 **4. Conclusion**

461 The proposed innovative method with AOP-HC-assisted pretreatment of sugarcane bagasse was
462 shown as promising. The influential variables in pretreatment were evaluated, and the optimized conditions
463 corresponded to ozone flowrate of 10 mg/min and 0.61 % of H_2O_2 in the medium, with glucan and xylan
464 hydrolysis yield values of 84% and 72%, respectively. The hydrolysate was used to produce ethanol and
465 xylitol, with a proposal of a sequential production process using *S. cerevisiae* and *C. tropicalis.* 50 g/ L of
466 ethanol were produced in 8h of fermentation, and an ethanol yield of 0.41 g/g was obtained. After
467 distillation of the produced alcohol, the vinasse-based medium resulted in xylitol production of 32 g/L,

corresponding to yield and productivity of 0.55 g/g and 0.64 g/Lh, respectively. The proposed strategies have potential to be used in processes similar to those ones of current first-generation ethanol industries in Brazil, with high production of ethanol by *S. cerevisiae* from glucose present in hydrolysate, followed by a high production of xylitol by *C. tropicalis* from xylose remaining in vinasses. Future works should consider economic aspects of the HC-assisted technology, also considering economic and environmental sustainability of a biorefinery using the proposed approach.

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Statements & Declarations

Consent for publication: All authors agreed to publish the content.

Competing interests: The authors declare no competing interests.

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Figure captions

Figure 1 – Response surface for the yield of enzymatic hydrolysis of glucan (A) and for the yield of enzymatic hydrolysis of xylan (B), as a function of H_2O_2 concentration and ozone flow used in HC-assisted oxidative pretreatment of sugarcane bagasse

Figure 2 – Enzymatic hydrolysis yield obtained using sugarcane bagasse pretreated by AOP-HC-assisted process at optimized conditions (10 mg/min ozone inlet, and 0.61 % H_2O_2), and obtained in control experiments performed under similar conditions, but without HC generation device (AOP without HC) and without addition of ozone nor H_2O_2 (HC without AOP). Results are average of triplicates and are shown as average ± standard deviation (error bars).

Figure 3 – FTIR spectrum of sugarcane bagasse *in natura* and pretreated by AOP-HC-assisted process under optimized conditions (10 mg/min ozone inlet, and 0.61 % H_2O_2)

Figure 4 – SEM images of sugarcane bagasse *in natura* and pretreated by AOP-HC-assisted process under optimized conditions (10 mg/min ozone inlet, and 0.61 % H_2O_2): *in natura* (left) and pretreated (right)

Figure 5 – Sugar consumption and fermentation kinetic profile for (A) Ethanol with up to 82% of efficiency, and (B) xylitol production. Results are average of triplicates and are shown as average ± standard deviation (error bars).

678 **Figure 1**

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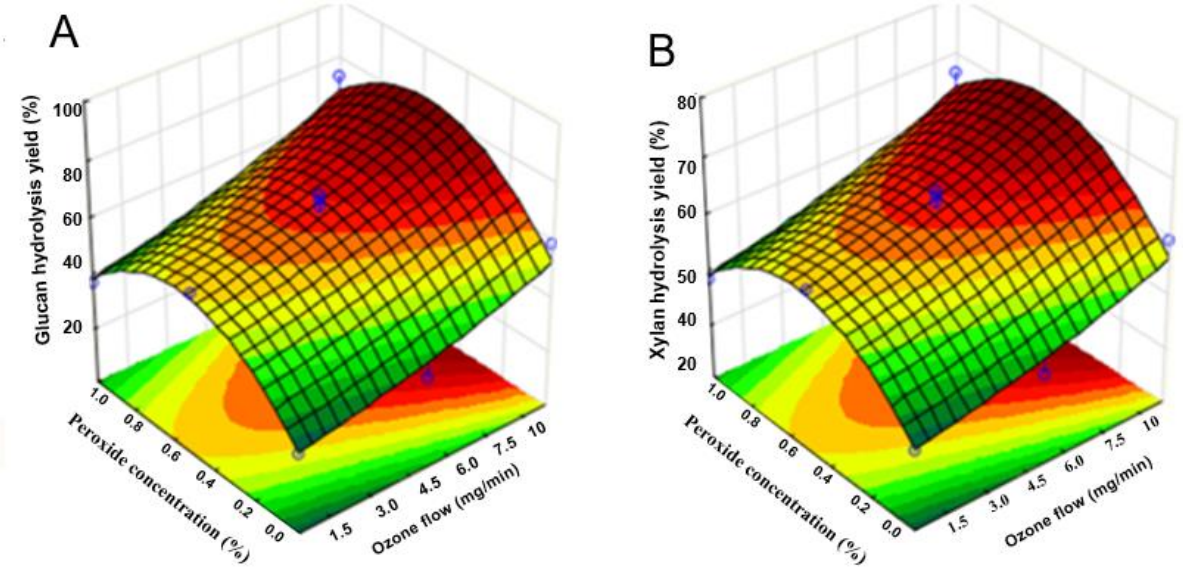

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699 **Figure 2**

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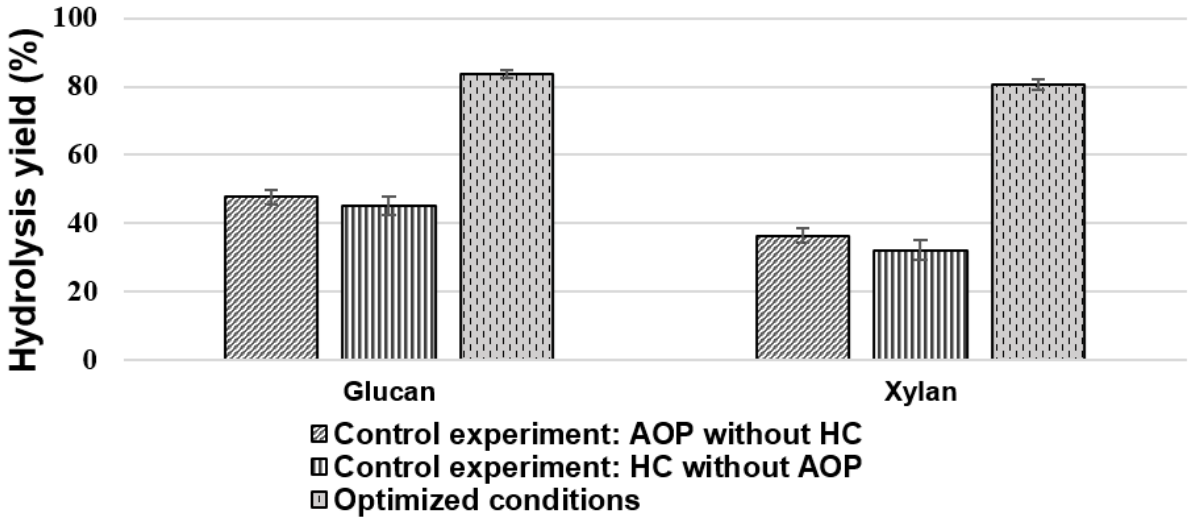

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721 **Figure 3**

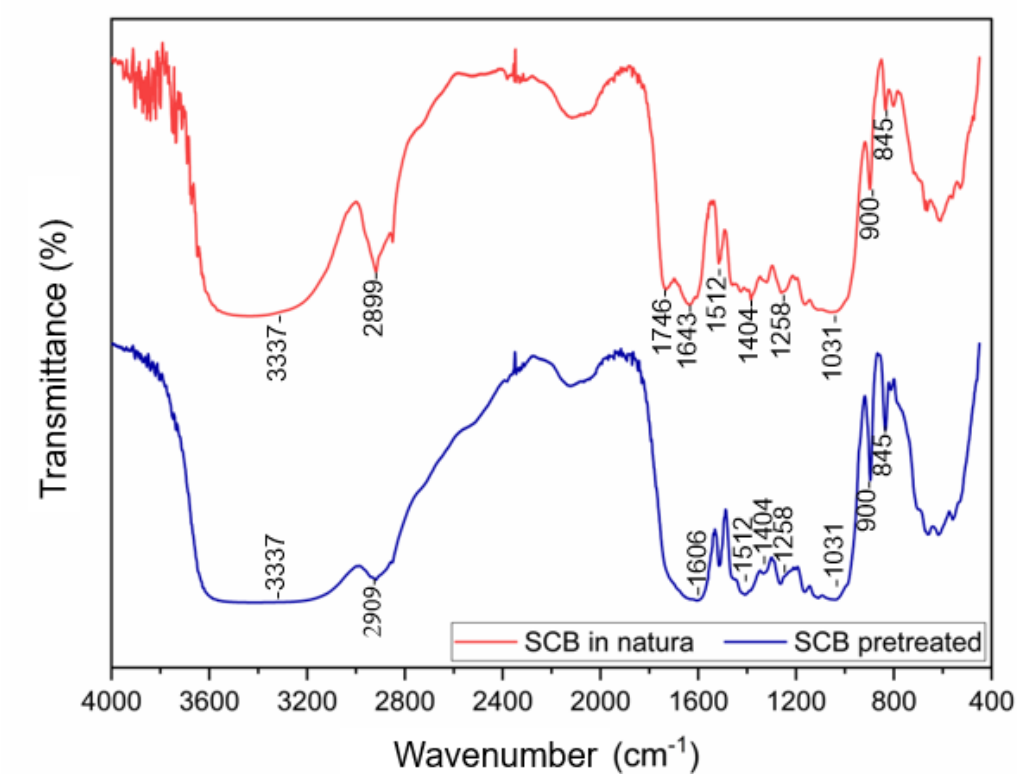

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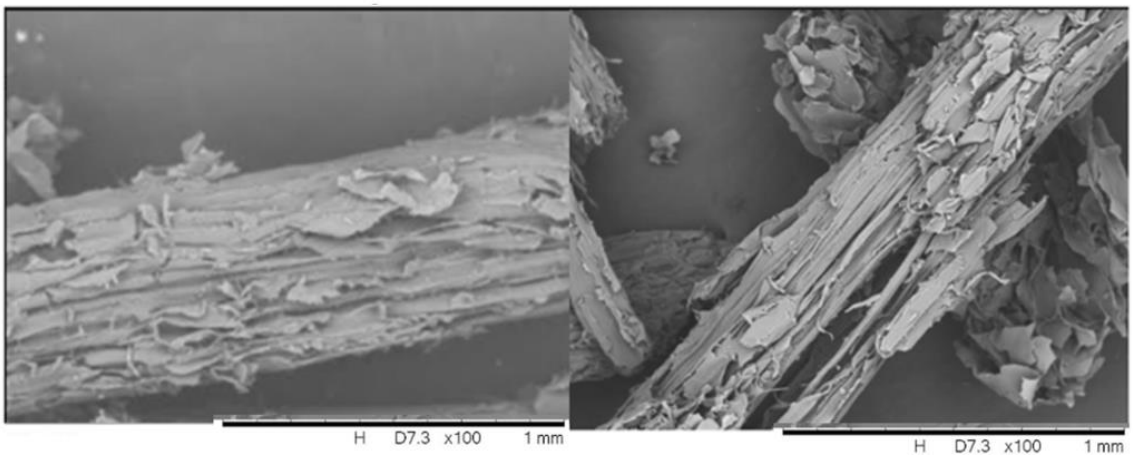
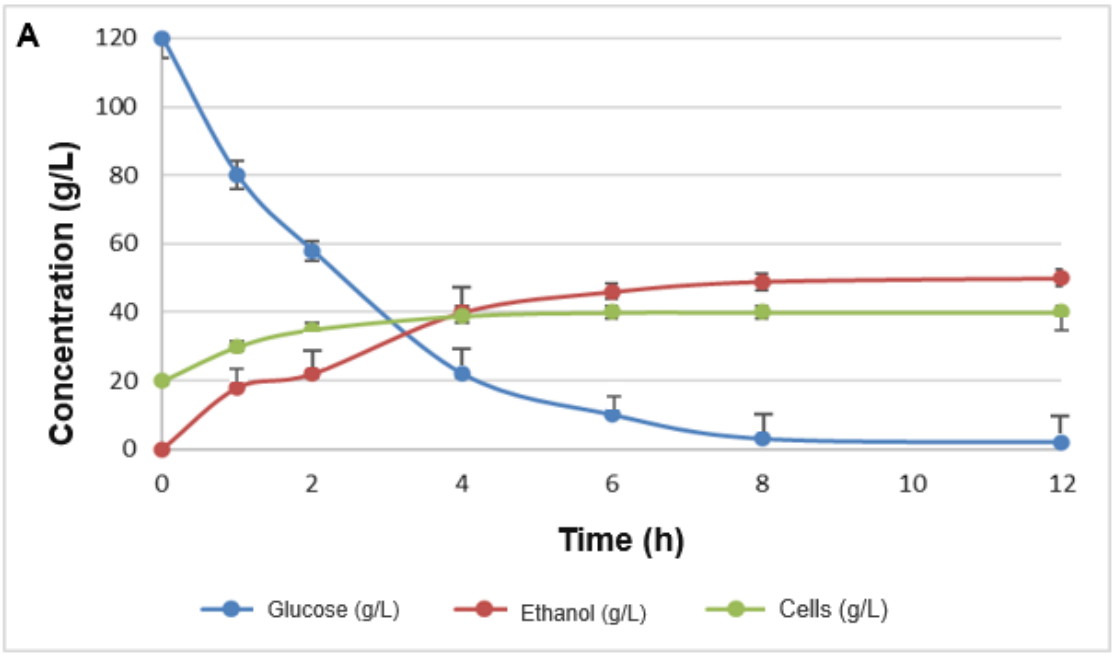
Figure 4



762 **Figure 5**



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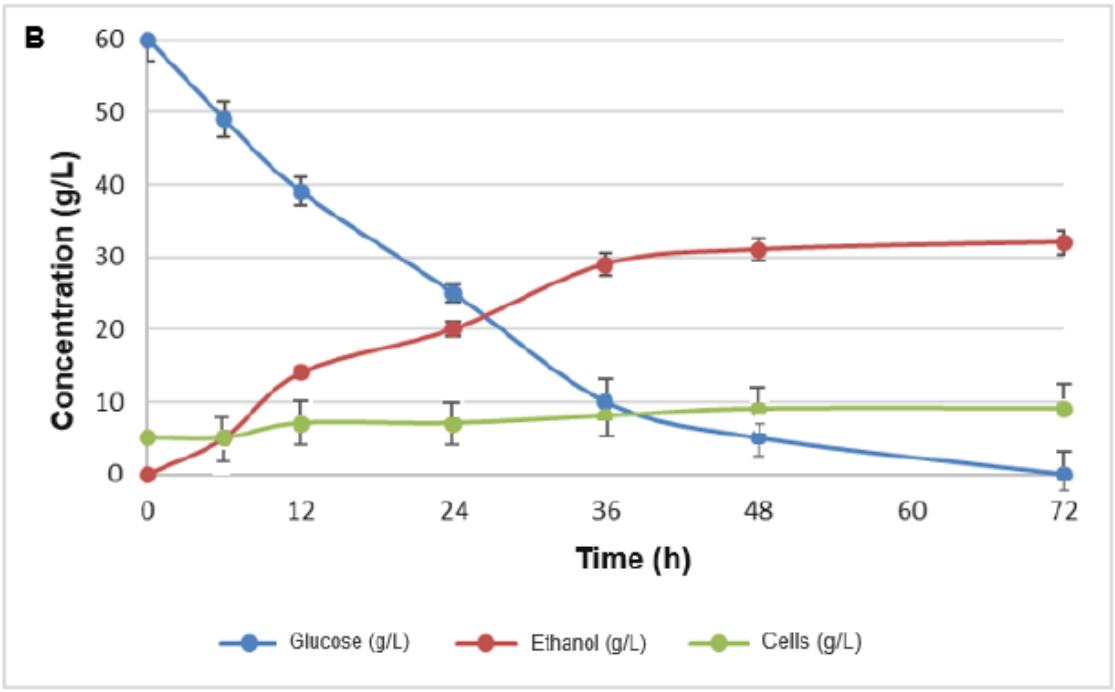

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Table 1- Composition and removal of sugarcane bagasse pretreated by HC-assisted advanced oxidative process technology according to a 2^2 face-centered design performed to evaluate the influence the influence of important variables in the process. Results of bagasse composition, removal of biomass components and hydrolysis yield were presented as average±standard deviation of the analytical results.

Run	Conditions (values in parentheses)		Solids Recovery (%)	Bagasse Composition			Removal			Hydrolysis Yield	
	Ozone (2 - 10 mg/min)	H_2O_2/ (0 - 1 %)		Glucan (%)	Xylan (%)	Lignin (%)	Glucan (%)	Xylan (%)	Lignin (%)	Glucan (%)	Xylan (%)
1	2 (-1)	0 (-1)	71.3	54.08 ±0.11	24.08 ±0.21	17.34±0.01	3.60± 0.11	28.46± 0.11	50.54 ± 0.01	30.31± 0.10	28.21 ± 0.01
2	10 (+1)	0 (-1)	70.0	55.01±0.01	24.01±0.01	19.01±0.01	3.75 ± 0.12	29.97± 0.12	46.77 ± 0.11	58.60± 0.10	57.95± 0.01
3	2 (-1)	1 (+1)	71.0	54.82±0.21	24.82±0.21	19.31±0.01	2.67 ± 0.13	26.57± 0.13	45.15 ± 0.01	50.80± 0.01	36.45± 0.11
4	10 (+1)	1 (+1)	67.0	59.32±0.01	23.32±0.11	19.82±0.01	0.64± 0.10	34.90± 0.14	46.88± 0.10	83.84± 0.01	78.02± 0.01
5	2(-1)	0.5 (0)	72.0	45.76±0.01	22.76±0.14	18.31±0.01	17.6 ± 0.11	31.72± 0.15	47.25 ± 0.12	54.90± 0.11	58.92± 0.10
6	10 (+1)	0.5 (0)	64.0	57.06 ±0.11	27.06 ±0.01	17.47±0.01	8.70 ± 0.11	27.84± 0.11	55.27 ± 0.13	81.87± 0.12	66.60± 0.11
7	6 (0)	0 (-1)	72.0	52.7 ±0.11	22.7 ±0.11	15.29±0.01	5.14 ± 0.10	31.90± 0.11	55.96 ± 0.01	39.24± 0.12	32.38± 0.12
8	6 (0)	1 (+1)	73.0	51.40±0.01	21.40±0.13	19.79±0.01	6.19± 0.12	34.91± 0.05	42.18± 0.01	61.63± 0.12	46.46± 0.12
9	6 (0)	0.5 (0)	72.0	52.7 ±0.21	23.7 ±0.21	17.76±0.01	5.14 ± 0.13	28.90± 0.05	48.85 ± 0.10	79.26± 0.13	72.09± 0.13
10	6 (0)	0.5 (0)	72.0	52.40±0.31	23.40±0.12	17.71±0.01	5.68 ± 0.13	29.80± 0.05	48.99 ± 0.10	78.76± 0.13	68.23± 0.13
11	6 (0)	0.5 (0)	72.0	52.40 ±0.11	23.40 ±0.20	17.15±0.01	5.68 ± 0.13	29.80± 0.05	50.60 ± 0.10	77.09± 0.13	69.98± 0.13

Table 2- Chemical oxygen demand (COD) and Total organic carbon (TOC) of vinasse obtained from ethanol distillation, and of the cells-free fermented broth of xylitol fermentation

Inputs	**COD (g L^{-1})**	**TOC (g L^{-1})**	**BOD (g L^{-1})**
Vinasse of First Fermentation	351.0 ± 0.50	179.0 ± 0.01	179.1 ± 0.91
Cell-free Fermented Broth	16.4± 0.05	15.64 ± 0.26	14.1 ± 1.0