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ABSTRACT

Sugarcane straw represents a potential feedstock for cellulosic biorefineries in Brazil since green mechanized harvesting without burning has been adopted. Acid-catalyzed steam explosion pretreatment has been considered a promising method to reduce biomass recalcitrance for enzymatic hydrolysis. However, pseudo-lignin formation during acid pretreatment can significantly reduce the performance of the enzymatic hydrolysis. Bisulfite catalyst may react with pseudo-lignin precursors, mitigating the formation of highly inhibitory humin-like structures. This work aimed to evaluate the pilot-scale pretreatment of sugarcane straw using a combined sulfuric acid- and bisulfite-catalyzed steam explosion process. The influence of different soaking and pretreatment conditions was evaluated considering the glucose and xylose yields obtained during pretreatment and after the enzymatic hydrolysis with Cellic® CTec3 HS. The pretreated materials were characterized using different techniques to elucidate their composition and structure, including X-ray diffraction, FTIR spectroscopy, ¹³C solid-state NMR, confocal laser scanning microscopy, and ¹H NMR relaxometry measurements. A set of acid-catalyzed initial experiments indicated an optimum pretreatment condition at approximately 1.90 severity. The acid- and bisulfite-catalyzed experiments revealed a significant positive effect of the combined higher soaking pHs and bisulfite addition on pretreated solids' accessibility to the enzymes. The pretreated straw obtained with 1.45 soaking pH, 1.65% w/w sodium metabisulfite on biomass, and about 1.90 pretreatment severity resulted in 100% accessibility with the highest achieved hydrolysis yields under the lowest enzyme dosages. Nevertheless, the results evidenced that at lower soaking pHs (~1.20), higher bisulfite dosages are required to mitigate pseudo-lignin formation and benefit the performance of the enzymatic hydrolysis.

Keywords

Sugarcane straw. Steam explosion. Diluted acid. Bisulfite. Cellulosic ethanol.

INTRODUCTION

Brazil is the most significant sugarcane producer in the world, with a cultivated area of 8.2 million hectares [1]. Sugarcane is grown in the southeast and northeast portions of the country, and the state of São Paulo is responsible for 57% of the total cultivated area. In the last few years, the sugarcane harvest system has undergone significant changes, such as a prohibition of burning before harvest and the adoption of the so-called green mechanized harvest [2, 3]. Therefore, a large amount of sugarcane straw is left in the field after harvesting, representing a potential biorefinery feedstock in Brazil [4]. Sugarcane straw comprises dried leaves, fresh leaves, and the top of the sugarcane plant, representing 140 kg of dry matter per ton of sugarcane stalks and 11.7 dry tons of straw per hectare [5]. Recent studies revealed a potential 50% recovery of total available straw, regarding environmental and economic aspects [6]. Structural biomass polysaccharides (such as cellulose and hemicelluloses) may account for ~60% of the dry weight of agricultural residues like corn stover, sugarcane bagasse, and sugarcane straw [7–9]. Although water or acid hydrolysis processes can convert biomass polysaccharides into soluble fermentable sugars (e.g., glucose), these processes present practical limitations considering their industrial application. Therefore, enzyme-catalyzed hydrolysis emerged as a potential option due to the milder reaction conditions and the possibility of achieving theoretical yields [10, 11].

Enzymatic hydrolysis of native cellulosic feedstocks is slow, with low yields [12]. The plant cell wall is a complex intermingling of cross-linked layers that create the resilience of the cell wall (recalcitrance). Overcoming biomass recalcitrance has been considered the main challenge to the commercial production of cellulosic biofuels [13]. Therefore, different pretreatment methods have been developed to improve cellulose accessibility to enzymes [14, 15]. Some essential features must be fulfilled for a successful pretreatment, such as high yields and scale-up feasibility. Steam explosion (a hydrothermal treatment process) has been considered one of the most promising pretreatment methods for industrial applications [11]. Steam explosion consists of heating biomass with saturated steam, followed by a sudden decompression of the pressurized system [16]. Steam penetrates the lignocellulosic material and condensates to form liquid water inside the fibers. After the sudden decompression, water quickly evaporates, generating an explosion inside the fibers [16]. The high temperature and the low pH environment (from acetylated hemicelluloses) lead to hydrolysis of the

polysaccharides, mainly hemicelluloses, and partial transformation of lignin [17]. Steam explosion can be enhanced by adding chemicals as a catalyst. Dilute sulfuric acid has been employed extensively, resulting in almost complete removal of hemicelluloses and higher enzymatic hydrolysis yields (higher solids digestibility) [14].

Regarding the hydrolysis performance, native and modified cell wall lignin have been considered a critical challenge for the enzymatic conversion of pretreated biomass. Lignin migration and redistribution during pretreatment are crucial factors in determining the hydrolysis yield [18]. It has been reported that spherical lignin droplets could be formed on the surface of steam-exploded materials due to the migration of lignins from the cell wall to the bulk liquid phase, followed by surface deposition [19]. Lignin affects the efficiency of enzymatic hydrolysis in three main ways: steric hindrance, nonproductive adsorption, and inhibitory effects of soluble phenolic compounds [18]. Among these factors, nonproductive adsorption is considered the key mechanism of lignin's negative impact on the hydrolysis performance of pretreated materials. Hydrophilic modifications of lignin reduce its inhibitory effect on cellulase enzyme. Sulfonation and carboxylation modifications can increase the hydrophilicity of lignin, improving the enzymatic hydrolysis efficiency [20]. Pretreatment typically promotes the cleavage of original lignin linkages, forming new linkages and changing lignin properties [20, 21]. During the steam explosion, almost simultaneous depolymerization and repolymerization occur in the lignin due to the acidic environment [17, 22, 23]. Recent literature has suggested that a benzylic carbocation is formed in lignin under acidic pretreatment conditions [17, 24]. The cleavage of the β -O-4 linkage starts with protonation and dehydration of the α -OH, resulting in a benzylic carbocation, which subsequently undergoes two productive C–O cleavage events and engages in nonproductive condensation reactions, leading to stable C–C bonds with lignin fragments, increasing the lignin's hydrophobicity [20, 25, 26]. Other nucleophiles, like carbohydrate compounds and extractives, may also add to the benzylium cation [27].

Besides the lignin effect on hydrolysis, polysaccharide-derived humins (pseudo-lignin) can be formed during the steam explosion (mainly the acid-catalyzed process) and deposited onto cellulose, affecting cellulose accessibility and enzyme effectiveness [28, 29]. Sugars dehydration occurs during pretreatment, producing furfural and 5-hydroxymethylfurfural (HMF) from pentoses and hexoses, respectively [12, 30]. Pseudo-lignin arises from the polymerization/condensation reactions of key intermediates derived from furfural and HMF, which can be further degraded into carbon-enriched aromatic structures [19, 31]. Sannigrahi

et al. [32] performed a dilute-acid pretreatment on poplar holocellulose (cellulose and hemicellulose) to investigate the chemistry of pseudo-lignin formation. The structural characterization of the isolated pseudo-lignin revealed that polysaccharide sugars undergo dehydration, rearrangement, aromatization, and condensation reactions under acid pretreatment conditions to form pseudo-lignin [19, 32]. The first strategy to avoid pseudo-lignin formation is minimizing furfural and HMF formation, which can be reached by optimizing pretreatment conditions [33]. Additionally, a recent approach considers using chemical additives capable of suppressing pseudo-lignin formation by stabilizing the precursor species [17, 20, 25, 29, 34]. Carbocation scavengers (reducing agents), like 2-naphthol, are believed to compete with lignin fragments and other nucleophile compounds for the formed benzylic carbocation, preventing lignin repolymerization reactions during steam explosion [24, 34].

Bisulfite salts, like sodium metabisulfite ($\text{Na}_2\text{S}_2\text{O}_5$), are a potential pretreatment chemical to prevent lignin condensation reactions (sulfite pulping) due to their reducing properties [35]. Metabisulfite dissolves readily in water by forming bisulfite ions (HSO_3^-), a reducing agent. Sulfur dioxide (SO_2) and sulfite ions (SO_3^{2-}) are formed from HSO_3^- and the relative concentrations of HSO_3^- , SO_2 , and SO_3^{2-} are determined by the pH of the aqueous solution [27]. Many research studies have investigated using sulfur dioxide, sulfite, and bisulfite as pretreatment catalysts, commonly employing 3-15% w/w catalyst charges on biomass (oven dry basis) [15, 36–39]. The SO_2 -catalyzed steam explosion typically results in good pretreated solids digestibility, but it has limited hemicellulose conversion to monomers during pretreatment [30, 40, 41]. Bura et al. [40] found an optimum condition for the steam explosion of corn stover catalyzed by SO_2 , which included 190°C for 5 min with 3% w/w SO_2 . However, an additional hydrolysis step of the aqueous fraction from the pretreatment was needed to convert soluble oligomers to monomers. Tengborg et al. [42] compared the SO_2 and H_2SO_4 impregnation of spruce softwood on steam pretreatment performance, showing a higher pretreatment yield using the sulfuric acid catalyst, while a superior enzymatic hydrolysis yield with sulfur dioxide impregnation. Similarly, Eklund et al. [43] showed a higher hydrolysis yield of willow wood pretreated with sulfur dioxide compared to sulfuric acid (95% against 82%) based on a wide range of conditions study; however, the highest pretreatment xylose yield was obtained using sulfuric acid instead of sulfur dioxide (80% against 62%). Soderstrom et al. [44] proposed a two-step pretreatment process of spruce

using first sulfuric acid to optimize the hemicellulose conversion, followed by a sulfur dioxide second step to maximize the cellulose accessibility to the enzymatic attack.

This work evaluated the sugarcane straw's steam explosion pretreatment using a combined sulfuric acid- and bisulfite-catalyzed one-step process, aiming to obtain the benefits of an acidic and reductive pretreatment environment. Studying the formation conditions and possibly suppressing pseudo-lignin is crucial for reducing cellulosic enzyme consumption costs, since the formation of pseudo-lignin during pretreatment is a key parameter responsible for the nonproductivity and irreversible binding of enzymes to biomass. Pilot-scale experiments were performed to understand the influence of catalyst charges, soaking pH, pretreatment severity, and straw particle size on the enzymatic hydrolysis of the obtained pretreated materials. The results regarding glucose and xylose (monomers) yields and the enzyme dosages were investigated. Unlike previous literature, this work focused on elucidating the effect of a low dosage of bisulfite on pretreatment, aiming to mitigate the negative impact of pseudo-lignin formation while using techno-economically feasible process conditions.

MATERIALS AND METHODS

Sugarcane Straw

Sugarcane straw left in the field after sugarcane mechanical harvesting was collected in Campinas, SP, Brazil, in October 2021 for the initial experiments and in September 2022 for the bisulfite-catalyzed experiments (S1-S6 trials). The collected straw was air-dried to about 10% moisture and size-conditioned before use. The straw was milled in a knife mill to a 5.0 ± 0.4 mm average particle size for the acid-catalyzed initial trials. For the bisulfite-catalyzed trials, straw was milled in two stages, first in a knife mill to 7.3 ± 0.6 mm and then in a hammer mill to 3.9 ± 0.4 mm average particle size (see **Fig. S1**). The straw conditioning and milling operations were performed at the Brazilian Biorenewables National Laboratory (LNBR) pilot plant in Campinas, SP, Brazil. The straw particle-size distribution was determined using a machine-sieving procedure employing the standard method ASTM E828-81 [45]. About 70 g of the preconditioned straw was analyzed in triplicate using a sequence of sieve sizes from 0.15 to 50 mm. The average size of the straw particles was determined by considering the weighted average of the mass retained in each sieve and its mean size (the mean of the above and below sieves).

Pretreatment

The pretreatment experiments were conducted using the LNBR's pilot plant infrastructure in Campinas, SP, Brazil (<https://lnbr.cnpem.br/en/facilities>) [46, 47]. The pretreatment process employed in this study was based on Iogen's pretreatment concept, which performs acid impregnation using soaking and dewatering processes before pretreatment [11]. The milled sugarcane straw was subjected to an acid and bisulfite soaking stage before the steam explosion pretreatment. A 350 L stirred tank reactor (jacket-heated pressurized vessel) was used as a soaking tank (Pope Scientific Inc., USA), and a 4% (w/w) consistency straw suspension was impregnated with dilute sulfuric acid and sodium metabisulfite for 20 min at 50°C [48, 49]. Over the preheated water, about 8-12 kg (dry basis) of straw was manually added to the soak tank (~10 min), followed by the sulfuric acid dosage and the metabisulfite addition. The soak tank was kept closed during the soaking time. After the target time, the straw suspension was transferred by gravity to a pressurized Nutsche filter (200 L, communication vessel with the soak tank) and filtered under 1.5 barG pressure (compressed air addition) using a 35 mesh (0.5 mm) metallic filter screen. The Nutsche filter was maintained pressurized while draining the soaked straw until it reached room temperature using cooling water through the filter's jacket (~30 min). The obtained liquid pressate and soaked straw were quantified regarding their mass and insoluble solids content. The soaked straw was conditioned in plastic bags until its use in the pretreatment experiment. The soaking pH was measured after the filtration using the pressate at room temperature and a pH meter calibrated with 1.0 and 4.0 pH standard buffers. **Figs. S2 and S3** show the soaking and pretreatment setup employed during the experiments.

The dewatered soaked straw at about 20% (w/w) insoluble solids (see **Table 1**) was pretreated in a 20 L steam explosion batch reactor made of Hastelloy (Pope Scientific Inc., USA) according to different experiment conditions. A mass of 1.0 kg of the soaked straw (dry basis) was manually added to the preheated reactor per batch through a top 4-inch ball valve, and a 28-barG saturated steam was used to heat the reaction system by direct injection. During the first ~5 sec of steam injection, an on-off vent valve was opened to release the internal reactor's air. To ensure a fast and uniform heating, the steam was injected into the reactor through sixteen orifices distributed around the reactor's body. Therefore, finishing the reaction time, an atmospheric flash tank (cyclone design) received the pretreated material after the reactor's sudden decompression through a blow valve (rapid action on-off ball valve). One, two, and three runnings were performed in series for each pretreatment experiment

during the initial, S1-S4, and S5-S6 trials, respectively (see **Table 1**). The reactor and the flash tank were washed after each pretreatment condition trial, and the washing water was incorporated into the pretreated material to ensure the pretreatment mass balance, resulting in a final pretreated slurry of about 5-8% (w/w) insoluble solids. Finally, the obtained pretreated solids (pulp) were fully collected, quantified, dewatered, and washed before characterization and the enzymatic hydrolysis step. The liquid fraction of the pretreated slurry (C5 stream or hemicellulosic hydrolyzed) was analyzed for sugar monomers and oligomers.

Acid-catalyzed Initial Experiments

During the initial experiments, the soaking stage was carried out with sulfuric acid at 0.67% w/w concentration (solution basis) without bisulfite addition, resulting in a final soaking pH of 1.24 ± 0.02 . A factorial experiment design CCRD (central composite rotational design) was used to evaluate the influence of time and temperature in the pretreatment. Twelve trials were performed to investigate pretreatment conditions ranging between 60-300 sec and 165-205°C (encoded range of -1.41 to +1.41), representing severity values of 1.15-2.33. The CCRD statistical analysis was carried out through Protimiza software (<https://experimental-design.protimiza.com.br>).

Combined Acid- and Bisulfite-catalyzed Experiments (S1-S6 trials)

For the bisulfite-catalyzed experiments, the temperature of 193°C (12.5 barG) was selected based on the initial experiment results. An improvement was made in the reactor's heating control, reducing the time (less than 60 sec) to achieve the pretreatment temperature compared to initial experiments (about 120 sec). Additionally, a new batch of straw was used, different from the initial experiments' straw. A total of six soaking trials (S1 to S6) were performed, changing straw particle size, sulfuric acid dosage, and bisulfite charge. The experiments were separated into two phases: first, the S1-S4 trials, and second, the S5-S6 trials to answer different questions about the influence of soaking conditions on pretreatment and hydrolysis performance. Considering the first phase, the S1-S4 conditions were carried out using a standard sulfuric acid concentration of 0.63% w/w (solution basis). Two different straw particle sizes were used: 7.3 mm for S1 and 3.9 mm for S2 to S4. Sodium metabisulfite was added in two charges, 0.16% and 0.31% w/w (solution basis), during the soaking S3 and S4, respectively. Since bisulfite consumed part of the sulfuric acid during soaking, raising the pH of S3 and S4 soaked straws, a second phase of experiments was performed to evaluate the effect of bisulfite on pretreatment at similar pH conditions (S5-S6 experiments). **Table 1** shows the conditions used during the soaking trials and pretreatment experiments.

Table 1 Experimental conditions used during soaking and steam explosion pretreatment trials.

Sulfuric acid and sodium metabisulfite dosages are based on soaking solution weight.

Condition	Initial Exp.	S1	S2	S3	S4	S5	S6
Straw Size (mm)	5.0	7.3	3.9	3.9	3.9	3.9	3.9
pH	1.24	1.27	1.17	1.36	1.45	1.23	1.19
Sulfuric Acid (%)	0.67	0.63	0.63	0.63	0.63	0.67	0.82
Metabisulfite (%)	0	0	0	0.16	0.31	0	0.26
Soaked Straw Solids (%)	19.9	19.6	18.6	22.9	21.0	22.9	21.6
Pretreat. Time (sec)	60-300	150-250	150-250	150-250	150-250	150	150
Pretreat. Temp. (°C)	165-205	193	193	193	193	193	193
Pretreat. Severity	1.15-2.33	1.87-2.09	1.97-2.19	1.78-2.00	1.69-1.91	1.91	1.95

Performance Calculations

The pretreatment process was analyzed regarding the solids and sugar yields and according to glucan, xylan, and lignin conversions using **Eqs. 1 to 6**. All yield and conversion calculations were performed based on the soaked straw as the start material. The combined severity factor (**Eq. 1**) was used as the basis parameter for the pretreatment analysis [12, 50]. **Eq. 2** was used to calculate the dewatering obtained due to the filtration of the soaked material in the Nutsche filter. **Eq. 3** calculates the catalyst consumption during the pretreatment, considering that the catalyst present in the pressate liquid can be recirculated during the soaking process. The pretreatment insoluble solids yield and the sugars (glucose and xylose) yield were calculated using **Eqs. 4 and 5**, respectively. **Eq. 6** was used to estimate the polysaccharides (glucan or xylan) and lignin conversions.

$$CSF = \log_{10} \left(t \cdot e^{\frac{T-100}{14.75}} \right) - pH \quad (1)$$

CSF is the pretreatment combined severity factor, t is the pretreatment time (min), T is the pretreatment temperature (°C), and pH is the soaking pH.

$$DW = \frac{Pressate}{Suspension} \cdot 100\% \quad (2)$$

DW is the dewatering relation (% w/w), $Pressate$ is the mass of pressate (kg), and $Suspension$ is the mass of soaking suspension (kg).

$$CA_{cons} = \frac{CA \cdot (1 - DW)}{Straw_{raw}} \cdot 100\% \quad (3)$$

CA_{cons} is the catalyst sodium metabisulfite or sulfuric acid consumption (% w/w oven dry biomass), CA is the mass of sodium metabisulfite or sulfuric acid (kg), DW is the dewatering (%), and $Straw_{raw}$ is the dry mass of raw straw (kg).

$$IS_{yield} = \frac{Slurry \cdot IS_{slurry}}{Straw_{soaked}} \cdot 100\% \quad (4)$$

IS_{yield} is the pretreatment insoluble solids yield (% w/w), $Slurry$ is the mass of pretreated slurry (kg), IS_{slurry} is the pretreated slurry insoluble solids (%), and $Straw_{soaked}$ is the dry mass of soaked straw (kg).

$$PT_S_{yield} = \frac{Slurry \cdot (1 - IS_{slurry}) \cdot \frac{Sugar}{\rho}}{Straw_{soaked} \cdot \frac{Carbo}{f} \cdot 1000} \cdot 100\% \quad (5)$$

PT_S_{yield} is the pretreatment sugar (glucose or xylose) yield (%), $Slurry$ is the mass of pretreated slurry (kg), IS_{slurry} is the pretreated slurry insoluble solids (%), $Sugar$ is the C5 stream sugar (glucose or xylose) (g/L), ρ is the liquid density equal to 1.02 (kg/L), $Straw_{soaked}$ is the dry mass of soaked straw (kg), $Carbo$ is the soaked straw glucan or xylan (%), and f is 0.90 for glucan or 0.88 for xylan (g/g).

$$PT_C_{conv} = \left(1 - \frac{IS_{yield} \cdot Carbo_{pulp}}{Carbo_{straw}} \right) \cdot 100\% \quad (6)$$

PT_C_{conv} is the pretreatment glucan, xylan or lignin conversion (%), IS_{yield} is the pretreatment insoluble solids yield (%), $Carbo_{pulp}$ is the pretreated straw glucan, xylan or lignin (%), $Carbo_{straw}$ is the soaked straw glucan, xylan or lignin (%).

Enzymatic Hydrolysis

Standard Protocol

A standard enzymatic hydrolysis protocol was performed using the commercial enzyme Cellic® CTec3 HS from Novozymes (2140 ± 142 BHU activity and 300 ± 20 mg of protein per gram of stock) to evaluate the performance of different pretreatment conditions. A reference protocol from NREL (US National Renewable Energy Laboratory) was used, and the hydrolysis yield was calculated using equations **Eqs. 8-9** [51]. Three enzyme dosages were employed: 45, 90, and 1000 BHU, corresponding to 6.3, 12.6, and 140 mg of protein per gram of cellulose (glucan), the last representing the accessibility assay. The protein content was measured through the bicinchoninic acid (BCA) method. Novozymes (Araucária, PR, Brazil) kindly provided the Cellic® CTec3 HS enzyme and information on enzyme activity and protein content. The BHU activity unit (Biomass Hydrolysis Activity) measures the decay

of cellulose in a substrate consisting of grinded and sieved acid-pretreated corn stover mixed with a fluorescence enhancer [52]. The hydrolysis experiments were carried out with 90% solids washing efficiency (see **Eq. 7**), 10% (w/w) insoluble solids, 96 hours, 52.5°C, and a pH of 4.8 on a 20 g scale using an incubator rotisserie equipment. Regarding the pretreated solids washing, water was added to the pretreated slurry to reduce 90% of xylose in the solids after vacuum filtration.

Performance Calculations

The pretreated solids were water-washed before hydrolysis to achieve a target xylose removal and measured according to **Eq. 7**. The hydrolysis performance was assessed considering glucose and xylose yields calculated by **Eqs. 8 and 9** [53]. The global monomer yield (overall process yield) was computed considering the glucose and xylose released during both pretreatment and hydrolysis (**Eqs. 10 and 11**).

$$W_{effic} = \frac{C5_{stream} \cdot Xylose_{C5}}{Slurry \cdot (1 - IS_{slurry}) \cdot \frac{Xylose_{slurry}}{\rho}} \cdot 100\% \quad (7)$$

W_{effic} is the pretreated straw washing efficiency (% w/w), $C5_{stream}$ is the volume of C5 stream (L), $Xylose_{C5}$ is the C5 stream xylose (g/L), $Slurry$ is the mass of pretreated slurry (kg), IS_{slurry} is the pretreated slurry insoluble solids (%), $Xylose_{slurry}$ is the pretreated slurry xylose (g/L), and ρ is the liquid density (kg/L).

$$Hydr_S_{yield} = \frac{Sugar_{final} - Sugar_{initial}}{Sugar_{max}} \cdot 100\% \quad (8)$$

$Hydr_S_{yield}$ is the hydrolysis sugar (glucose or xylose) yield (%), $Sugar_{final}$ is the final soluble sugar (g/L), $Sugar_{initial}$ is the initial soluble sugar (g/L), and $Sugar_{max}$ is the maximum theoretical sugar (g/L).

$$Sugar_{max} = Carbo \cdot IS \cdot \frac{1000}{f} \cdot \left(\frac{1 + 0.41 \cdot Glucan \cdot IS}{1 - (IS - Glucan \cdot IS)} \right) \quad (9)$$

$Sugar_{max}$ is the maximum theoretical sugar (glucose or xylose) (g/L), $Carbo$ is the pretreated straw glucan or xylan (%), IS is the hydrolysis insoluble solids (%), and $glucan$ is the pretreated straw glucan (%).

$$S_{yield} = (PT_S_{yield} + (1 - PT_C_{conv}) \cdot Hydr_S_{yield}) \cdot \frac{Carbo_{straw}}{f} \cdot 1000 \quad (10)$$

S_{yield} is the global sugar (glucose or xylose) yield (g/kg straw), PT_S_{yield} is the pretreatment sugar yield (%), PT_C_{conv} is the pretreatment carbohydrate conversion (%),

$Hydr_S_{yield}$ is the hydrolysis sugar yield (%), $Carbo_{straw}$ is the soaked straw glucan or xylan (%), f is 0.90 for glucan or 0.88 for xylan (g/g).

$$M_{yield} = \frac{G_{yield} + X_{yield}}{\left(\frac{Glucan_{straw}}{0.9} + \frac{Xylan_{straw}}{0.88}\right) \cdot 1000} \cdot 100\% \quad (11)$$

M_{yield} is the global monomers yield (%), G_{yield} is the global glucose yield (g/kg straw), X_{yield} is the global xylose yield (g/kg straw), $Glucan_{straw}$ is the soaked straw glucan (%), and $Xylan_{straw}$ is the soaked straw xylan (%).

Analytical Methods

Chemical Composition

The pretreatment experiments were evaluated based on a series of analytical procedures aiming to characterize the composition of the obtained pretreated solids and hydrolyzed liquids. Standard protocols from the US National Renewable Energy Laboratory (NREL) (<https://www.nrel.gov/bioenergy/laboratory-analytical-procedures.html>) were used, including methods for the preparation of samples [54], total solids quantification in biomass [55], determination of ash in biomass [56], and for insoluble solids content in pretreated biomass [57]. Determining structural polysaccharides and lignin [58] and liquid fraction process compounds (sugars, acetic acid, furfural, and HMF) [59] were also based on reference methods from NREL. Raw straw was submitted to an ethanol extraction procedure before chemical characterization, whereas the soaked and pretreated straws were not submitted [60].

Pretreated Solids Particle-size

The pretreated solids were characterized regarding their particle-size distribution through a hand-sieving procedure (adapted from the ASTM method [45]) using a 0.5% consistency slurry and a sequence of sieve sizes from 0.15 to 2 mm. Between 5-10 L of the low-consistency slurry were passed through the sieves (in duplicate), followed by excess water addition and horizontal motion to allow all small particles to pass through the openings. The average size of the solids particles was determined by considering the weighted average of the dry mass (105°C dried) retained in each sieve and its mean size (mean of the above and below sieves).

X-ray Diffraction (XRD)

To evaluate crystallinity indices (CrIs) of the pretreated solids, X-ray diffraction (XRD) experiments were performed at room temperature on a Rigaku Rotaflex X-ray diffractometer (RU200B model; Tokyo, Japan), using monochromatic $CuK\alpha$ radiation ($\lambda = 1.54 \text{ \AA}$). The XRD equipment was set to operate at 40 kV and 15 mA, and 2θ scans were

measured from 5 to 50° in 0.05° steps with an X-ray exposure of 15 s per step. The Segal empirical method was applied to derive the CrIs [61]. Samples were measured in duplicate, and the CrIs were calculated through the individual diffraction peaks extracted using a curve-fitting process from the experimental diffraction scans. The peak fitting program (PeakFit; www.systat.com) assumed Gaussian functions for each peak [37, 62].

FTIR Spectroscopy

Pretreated straws were analyzed by Fourier Transform Infrared (FTIR) spectroscopy using a spectrophotometer Shimadzu IRAffinity-1S. The pellets were prepared by mixing the sample with KBr in a 1:100 ratio (sample: KBr), and the spectra were recorded in the region of 4000–400 cm^{-1} with 40 scans at $\pm 2 \text{ cm}^{-1}$ resolution and baseline corrected [63, 64].

^{13}C Solid-state NMR Spectroscopy

^{13}C solid-state nuclear magnetic resonance (^{13}C ssNMR) experiments were performed using a Bruker Avance 3 Spectrometer operating at 100.5 MHz and 400.0 MHz for ^{13}C and ^1H , respectively. A double resonance MAS probe of 4 mm was used with a frequency stability superior to 1 Hz. A ramped ^1H – ^{13}C cross-polarization under magic angle spinning (CPMAS) was performed as an excitation method with an RF amplitude increment of 80–100% during 1 ms of contact time. A high-power dipolar decoupling of 70 kHz (^{13}C CPMAS-DEC) was applied. Typical ^{13}C and ^1H 90° pulse lengths of 3.5 and 3.7 μs were used, respectively. All measurements were performed using 2 s recycle delays [37, 65].

Confocal Laser Scanning Microscopy (CLSM)

The CLSM technique was used to map the lignin distribution in the pretreated solids and to obtain information on its molecular arrangement within the cell wall by measuring the lignin autofluorescence emission spectra and its fluorescence decay times [66, 67]. Confocal microscopic analyses were conducted using a Zeiss LSM 780 confocal microscope (Jena, Germany) with a 405 nm diode laser and a Coherent Chameleon laser (Tisapphire) as a two-photon excitation source. The pretreated materials were dispersed in water, and the images were obtained by averaging three independent scans using a Plan-Apochromat objective lens (20x, water immersion, numerical aperture 1.4). The images were obtained as an average of three independent geometric scans, optimally divided into 1024 x 1024 pixels, resulting in a lateral resolution of about 400 nm. Each image pixel contained information on a fluorescence spectrum between 400 and 700 nm. The color in the images results from an RGB color model decomposition of the measured spectrum for each image pixel in the visible region [62, 68].

¹H NMR Relaxometry Measurements

The raw and pretreated straws (as prepared for chemical composition analysis) were soaked with ultrapure water and kept at a 600 mmHg vacuum overnight, followed by excess water removal before analysis [37]. The ¹H NMR relaxometry measurements (time-domain low-field NMR) were performed on a Bruker Minispec MQ-20 spectrometer with a 0.5 T magnetic field (¹H Larmor frequency of 20 MHz). A Carr-Purcell-Meiboom-Gill (CPMG) sequence was used with 30,000 echo acquisitions, echo times of 100 μs, and recycle delays of 10 s. A numerical Inverse Laplace Transform (ILT) procedure was used to obtain the distributions of the transverse relaxation time T_2 [69]. The water confinement in a porous medium affects the transverse relaxation of the ¹H nuclear spins due to the interaction with the pore surface. In the fast diffusion regime and absence of unbounded liquid, the T_2 is given by the equation: $1/T_2 = \rho_2 S/V$, where ρ_2 is the surface relaxivity, S is the pore surface, and V is the pore volume [70]. The distribution of pore sizes and differences in the water mobility within the pores result in a multiexponential decay of the CPMG signal (distribution of T_2 times) directly related to the pore size distribution [37, 62].

RESULTS AND DISCUSSION

Acid-catalyzed Initial Experiments

The knife-milled straw (5.0 mm average particle size) was soaked with a sulfuric acid solution of 0.67% w/w concentration and 4% w/w consistency, resulting in a solution pH of 1.24 ± 0.02 at the end of the soaking time, representing an acid consumption of 2.92% (**Eq. 3**) based on dry biomass weight. Two soaking batches were performed to deliver enough material for the pretreatment experiments. The soaked straw suspension was filtered with a dewatering relation (**Eq. 2**) equal to 82.0% and the obtained straw, from the two batches, was mixed, delivering an average soaked straw insoluble solids of $19.9 \pm 0.2\%$. Due to low molecular weight solubilization (e.g., extractives), straw lost 11.7% (w/w) of its initial mass, increasing the glucan, xylan, and lignin composition after the acid-soaking stage. The acetyl content decreased from 34.0 ± 0.1 mg/g in the raw straw to 16.1 ± 0.1 mg/g in the soaked straw. The raw straw chemical composition of glucan, xylan, and lignin (soluble and insoluble) was 336.1 ± 5.7 mg/g, 220.5 ± 9.6 mg/g, and 221.8 ± 1.9 mg/g, respectively. The extractive content was 61.1 ± 3.7 mg/g. In comparison, the soaked straw presented 354.6 ± 8.8 mg/g of glucan, 228.1 ± 11.0 mg/g of xylan, and 248.3 ± 6.9 mg/g of lignin. Moreover, the ash content in the raw and soaked straws was 50.7 ± 0.3 mg/g and 44.6 ± 0.2 mg/g, respectively. See **Table S1** for a complete description of the soaking data.

Table S2 shows the pretreatment and hydrolysis data obtained during the initial experiments as a function of steam explosion severity. All calculations regarding the straw component conversion during pretreatment were performed based on the soaked straw as the start material instead of raw straw, therefore disregarding chemical changes due to the soaking stage. The xylan conversion ranged from 70 to 90% when severity was increased from 1.04 to 2.33. The xylose yield presented a maximum value near 1.75 severity, reaching approximately 70% yield, and xylo-oligomers were observed under all severity conditions. Regarding the glucose release, the glucose yield and glucan conversion achieved maximum values of about 12% and 16%, respectively, at 2.33 severity. The formation of furfural and HMF grew substantially after the 2.00 severity condition, achieving about 11 and 3 g/kg of initial soaked straw (oven dry basis), respectively. The results revealed a relatively high residual xylan even at elevated severities; at 2.33 severity, the solids xylan was nearly 40 mg/g, representing about 10% of the original xylan (90% conversion). Glucan and lignin content increased as expected due to xylan solubilization. The pretreated solids' lignin increased from about 290 to 340 mg/g when the severity changed from 1.04 to 2.33. The pulp's glucan reached a maximum of about 520 mg/g at ~1.90 severity, decreasing at higher severities. Regarding the hydrolysis performance, the glucose yield was improved at higher severity levels. With 12.6 mg/gCel enzyme dosage, the yield achieved 80% at about 2.00 severity. Moreover, a significant yield increase was observed from 6.3 to 12.6 mg/gCel enzyme dosage. However, the pretreated straw accessibility (140 mg protein/gCel) was relatively low, achieving about 85%.

Fig. 1 shows the global yield of the acid-catalyzed initial experiments. **Fig. 1A** revealed an optimum global yield reached nearly 1.90 severity, representing about 70% and 80% yield for 6.3 and 12.6 mg/gCel enzyme dosage, respectively. **Fig. 1B** shows the CCRD contour plot; different pretreatment time and temperature combinations can achieve optimum global monomer yields above 75%. Typically, lower residence times are required as the steam explosion temperature increases, and maximum yields can be reached, for example, with 185°C/300 sec or 195°C/150 sec combinations. Considering industrial applications, lower residence times may favor techno-economic aspects of the pretreatment scale-up [11]. For the bisulfite-catalyzed experiments, the temperature of 193°C (12.5 barG) and a 150-250 sec time range were selected as optimum pretreatment conditions. **Eq. 12** shows the obtained global yield regression model. All model coefficients are statistically significant (p -value < 0.05), and according to ANOVA (Analysis of Variance) analysis (**Table S3**), the regression model

adequately fits the experimental results with an R^2 of 99.40% and a p-value of 0.0000. Y is the global monomers yield (%), x_1 is the pretreatment time, and x_2 is the pretreatment temperature (x_1 and x_2 range from -1.41 to +1.41).

$$Y = 78.87 + 2.35x_1 - 1.37x_1^2 + 3.30x_2 - 4.19x_2^2 - 3.83x_1x_2 \quad (12)$$

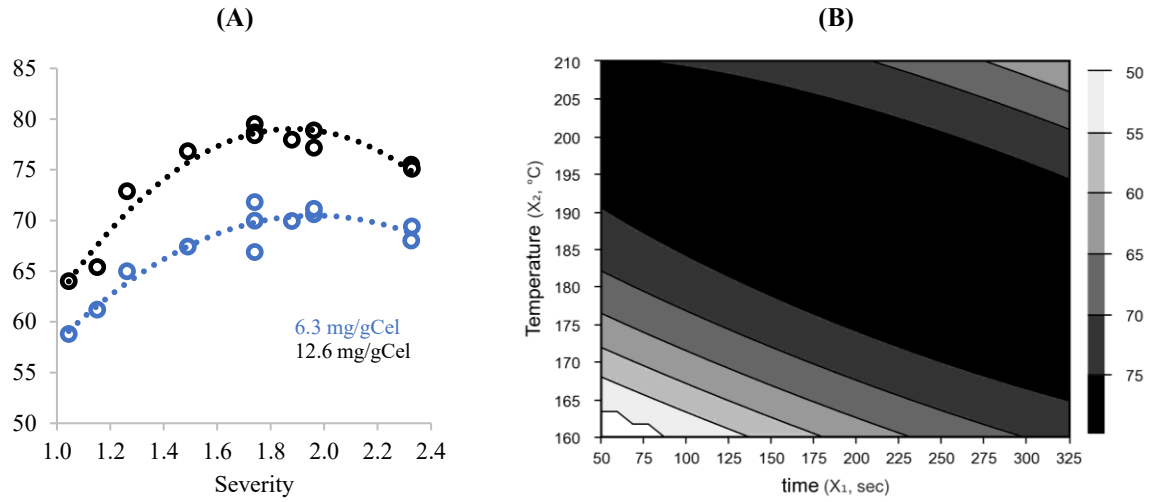


Fig. 1 Global monomers yield (%) of the acid-catalyzed initial experiments. (A) global yield as a function of pretreatment severity for two enzyme dosages. (B) Global yield contour plot for different pretreatment times and temperatures using 12.6 mg protein/gCel of Cellic CTec3 HS enzyme dosage.

Considering the X-ray diffraction (XRD) results, crystallinity index (CrI) first increases following the xylan conversion and cellulose enrichment (XRD CrI is not the absolute cellulose crystallinity, as pretreated biomass has amorphous components). Above the severity of 2.00, the CrI is substantially reduced, suggesting an absolute reduction in the cellulose crystallinity. Kang et al. [71] evaluated the SO_2 -catalyzed steam explosion of *Loblolly* pine and showed that there exists a critical severity point beyond which the degradation of crystalline cellulose dominates the process rather than the opening of a tight biomass substructure. **Table S2** shows that, although the CrI reduction, a significant improvement in hydrolysis yield was not observed over 2.00 severity, probably due to the increase in pseudo-lignin formation, finally leading to a plateau in the hydrolysis yield between 2.00 and 2.33 severity. **Fig. S4** shows that the lignin conversion decreases gradually from about 23% to 21% following the severity increasing from 1.2 to 2.0, and when the severity reaches 2.3, the lignin conversion quickly decreases to about 18%. Cellulase enzyme adsorption onto cellulose is thought to increase with decreasing cellulose crystallinity,

indicating enhanced cellulose accessibility [28]. On the other hand, pseudo-lignin significantly impacts cellulose accessibility and enzyme effectiveness because it acts as a physical barrier and causes enzyme inactivation [34, 72]. Kumar et al. [28] investigated the lignin conversion during the hydrothermal pretreatment of corn stover. The authors showed that the amount of lignin converted increases initially and, after a maximum point, decreases with the extent of xylan solubilization due to xylan degradation to humins (increasing Klason lignin). Previous research also showed that lignin melting and relocation occur during steam explosion and dilute-acid pretreatments [12, 50]. Lignin and pseudo-lignin droplets deposited on cellulose may form a boundary layer, limiting the hydrolysis performance [28].

Combined Acid- and Bisulfite-catalyzed Experiments (S1-S6 trials)

S1-S4 Trials

Due to the limited accessibility obtained during the initial experiments (~85%), a new batch of straw was used (from a new collection), and an improvement in the reactor's heating ramp was implemented, leading to less than 60 sec to reach the pretreatment temperature of 193°C. Regarding the chemical composition of the raw straw used, in the 3.9 mm particle size straw, the glucan and xylan content were 365.1 ± 11.1 mg/g and 222.8 ± 6.3 mg/g, respectively. The 7.3 mm straw presented 342.9 ± 8.2 mg/g of glucan and 210.0 ± 5.4 mg/g of xylan. The ash content in the 7.3 and 3.9 mm straws was 50.4 ± 0.7 mg/g and 33.7 ± 0.2 mg/g, respectively. Enriching the 3.9 mm straw polysaccharides compared to the 7.3 mm straw composition may be partially related to reducing straw ash during milling (due to straw manipulation). During the bisulfite-catalyzed experiments, the dewatering stage of the soaked slurry typically rendered a dewatering relation of about 77-80% (w/w) and resulted in 6-11% (w/w) solids solubilization due to low molecular weight release from straw (e.g., non-structural components). Therefore, the glucan, xylan, and lignin contents increased in the S1-S4 straws after soaking, similar to those described in the initial experiments. The S1-S4 soaked straw compositions ranged between 347.3-379.1 mg/g for glucan, 211.5-234.3 mg/g for xylan, and 267.1-275.2 mg/g for lignin. The consumption of sulfuric acid and sodium metabisulfite was calculated considering the mass balance of the catalyst impregnated in the soaked material (according to **Eq. 3**). The resulting consumption was in the range of 3.1-3.7% (w/w) and 0.85-1.65% (w/w) for sulfuric acid and sodium metabisulfite (dry biomass basis), respectively. **Table 1** shows the conditions of the different soaking trials, including S1 to S4 experiments. The soaked-straw insoluble solids ranged from 18.6 to 22.9%. The sulfuric acid charge was the same for S1-S4 experiments; however, the final pH values changed between

1.17 and 1.45 due to differences in straw ash content (S1 and S2) and sodium metabisulfite dosages (S2, S3, and S4). See **Table S1** for a complete description of the soaking data of S1-S4 trials.

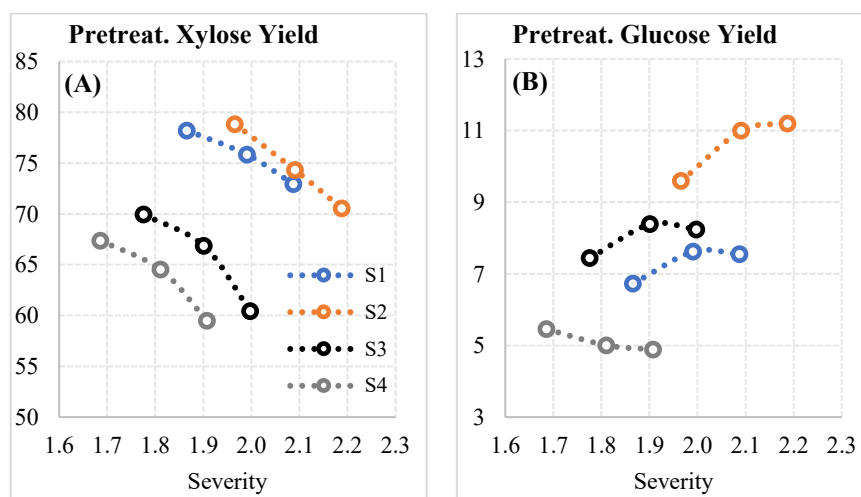


Fig. 2 Pretreatment yields of the combined acid- and bisulfite-catalyzed experiments as a function of severity for different soaking conditions (S1-S4). (A) xylose yield (%) and (B) glucose yield (%).

The S1-S4 steam explosion pretreatments resulted in an insoluble solids yield between 57.9-64.4% (**Eq. 4**). **Fig. 2** shows the behavior of xylose and glucose yields during the pretreatment experiments. The conditions evaluated resulted in a wide range of sugar yields, about 60-80% for xylose (**Fig. 2A**) and about 5-11% for glucose (**Fig. 2B**). **Table S4** provides all data obtained with S1-S4 pretreatment trials. S1 and S2 soaked straws resulted in excellent xylan conversions above 95%, indicating the effectiveness of the steam explosion trials, with a severity of ~2.00 and pH below 1.30. S3 and S4 materials presented lower polysaccharide conversions, especially the S4 straw, with a soaking pH of 1.45. The xylose-yield results achieved superior values compared to the initial experiments, reaching about 80% against 70% in the best cases. No xylo-oligomers were found in the pretreated liquid fractions (C5 streams) from S1 and S2 materials. The results indicated that the soaking pH critically influences their yield and conversion. Lower pHs produced higher xylose and glucose yields. An interesting behavior was observed considering the pH effect; each pH value resulted in a specific yield curve with different optimum severity values. Therefore, similar severities do not produce comparable results; for example, the xylose yield of the S2 1.97 severity (pH of 1.17) was equal to 78.8%, whereas the S3 2.00 severity (pH of 1.36) resulted in a 60.4%

yield. Regarding the pretreatment time, increasing the residence time from 150 to 250 sec reduced the xylose yield in all cases, indicating that the yield curves were all in their descending region. The effect of straw particle size (S1 and S2 materials) was more significant for glucose than for xylose yield. At 2.09 severity, the 3.9 mm (S2) and 7.3 mm (S1) straws resulted in 11.0 and 7.5% glucose yield, respectively. The furfural and HMF content in the pretreated slurry were between 4.9-11.4 g/kg and 1.2-2.8 g/kg of initial soaked straw, respectively.

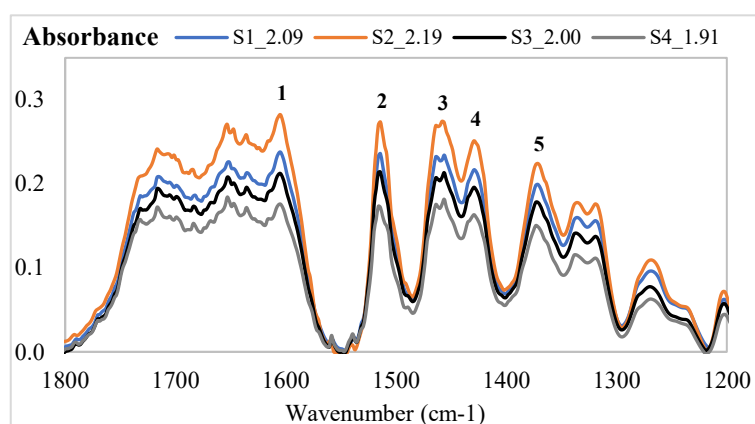


Fig. 3 FTIR spectra of the S1-S4 pretreated straws obtained during the combined acid- and bisulfite-catalyzed experiments. The bands between 1300 and 1600 cm^{-1} reflect a major contribution from the aromatic skeletal vibrations of lignin.

The pretreated materials were characterized by different techniques to investigate the main factors affecting the enzymatic hydrolysis performance. **Fig. 3** shows the FTIR spectra (baseline corrected) of the S1-S4 materials pretreated with 250 seconds of residence time. The FTIR analysis was used to investigate straw structural modifications caused by different pretreatment conditions [73]. The FTIR analysis indicated a similarity of the S1-S4 bands, although there are significant differences in the peaks' intensities, especially between S2 and S4 materials. The bands between 1300 and 1600 cm^{-1} reflect a major contribution from the aromatic skeletal vibrations of lignin. The peaks at $\sim 1600 \text{ cm}^{-1}$ (peak 1) and 1510 cm^{-1} (peak 2) represent lignin bands arising from C=C aromatic ring stretch. The bands at $\sim 1460 \text{ cm}^{-1}$ (peak 3) and $\sim 1425 \text{ cm}^{-1}$ (peak 4) are also assigned to aromatic C=C bonds [31]. Besides the lignin structure, peaks 1 to 4 can be attributed to the C=C furan ring stretch due to the pseudo-lignin structure (derived from furfural and HMF) [21, 31]. The band at 1360 cm^{-1} (peak 5) is assigned to aliphatic C-H rocking [73]. These bands are strongly enhanced in the S2

pretreated straw due to a relative increase in lignin content and pseudo-lignin formation (see **Table 2**) [74]. The straw pretreated under the S4 conditions, with the highest pH and bisulfite values, resulted in the lowest intensity bands among S1-S4 materials, revealing a limited modification in their structure compared to other straws. The FTIR spectra correlate well with the chemical composition of the pretreated solids. Considering the S2 and S4 materials, the S2 composition in glucan (562.8 ± 2.2 mg/g) and lignin (376.5 ± 6.0 mg/g) is considerably higher than the glucan (504.5 ± 1.9 mg/g) and lignin (328.4 ± 1.0 mg/g) in the S4 straw, following more intense bands in the S2 spectra.

Regarding the NMR spectroscopy results, **Fig. S5** shows the ^{13}C solid-state NMR spectra of the raw and pretreated straws S1-S6. All spectra were normalized based on the 31-36 ppm peaks [65]. The ^{13}C CPMAS-DEC NMR experiments have been considered a valuable tool to identify lignocellulosic biomass's chemical and structural change due to pretreatment [75]. The most intense peaks are concentrated in the 50-120 ppm region, mainly attributed to cellulose carbons and with contributions from hemicellulose and lignin signals [76]. **Fig. S5 A and B** showed a significant reduction in carbon nuclei in cellulose and hemicellulose signal intensities of S1-S6 materials compared to raw straw. The resonances corresponding to the acetyl (peak 1) and carboxyl groups in hemicellulose (peak 17) were drastically reduced in all pretreated materials. Moreover, the signal at 102 ppm (peak 9, a shoulder on peak 10), attributed to C1 of hemicellulose, also decreased after pretreatment, evidencing an extensive hydrolysis of the hemicellulose. **Fig. S5 A** results evidence the higher signal intensities of polysaccharide carbons (peaks 3-8 and 10) in the S3-S4 materials compared to S1-S2 pretreated straws, following the lower glucan and xylan conversions of the S3-S4 pretreatment conditions (see **Table S4**). Regarding lignin, ^{13}C ssNMR signals from lignin are relatively broader due to the chemically complex and disordered lignin structure [37, 75]. The aromatic lignin signals are spread out in the 110-170 ppm spectral region (peaks 11 to 16), while the signal of methoxy groups of lignin appears at 56 ppm (peak 2). The peaks 11 (115 ppm) and 12 (128-132 ppm) represent C5 and C1 of guaiacyl units in lignin. The C1 of syringyl units appears at 133-138 ppm (peak 13), whereas peaks 14 and 15 (143-153 ppm) typically represent aromatic lignin signals. Peak 16 is attributed to carboxyl groups of lignin. According to **Fig. S5**, the S1-S4 spectra revealed lower lignin signals compared to the raw straw spectrum, following the partial lignin conversion commonly observed during dilute-acid pretreatments. The peak 2 intensity (methoxy groups) was significantly reduced after all S1-S6 steam explosion pretreatments.

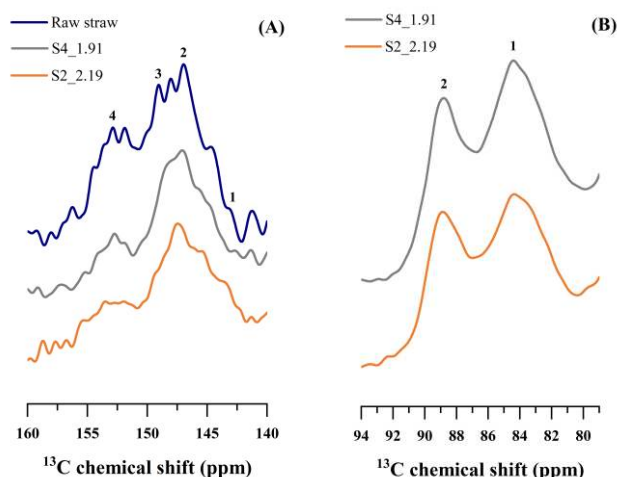


Fig. 4 ^{13}C ssNMR (CPMAS-DEC) spectra of the raw straw and pretreated straws S2 and S4. (A) 140-160 ppm region characteristic of aromatic C-O bonds and (B) 79-94 ppm region with peaks assigned to the C4 in cellulose.

Fig. 4 shows the ^{13}C ssNMR spectra of the raw and pretreated straws S2 and S4, considering the 140-160 ppm region (**Fig. 4A**) characteristic of aromatic C-O bonds and the 79-94 ppm region (**Fig. 4B**) with peaks assigned to the C4 in cellulose. **Fig. 4A** shows a significant change in lignin signals due to S2 and S4 pretreatment conditions. Peak 1 (143 ppm) is assigned to C4 of guaiacyl units, peak 2 (147 ppm) is mainly due to non-ether-linked C3 and C5 of syringyl and C3 of guaiacyl units, peak 3 (149 ppm) is attributed to ether-linked C4 of syringyl and guaiacyl units (β -O-4 aryl ether linkage), and peak 4 (153 ppm) is primarily related to ether-linked C3 and C5 of syringyl and C3 of guaiacyl units. The signal intensity at 153 ppm (peak 4) and 147-149 ppm (2-3 peaks) progressively decreased from raw straw to S4 and S2 pretreated straws, indicating lignin depolymerization. More alkoxy-substituted aromatics may be converted into hydroxyl-substituted species under S2 pretreatment conditions, indicating cleavage of an increasing number of aryl-ether bonds [77]. Similarly, Kobayashi et al. [76] studied the influence of dilute-acid pretreatment on ^{13}C ssNMR spectra of poplar wood, showing that the ether-linked C5 of lignin moieties are cleaved as the treatment severity increases, following the 153 ppm signal intensity decrease. Interestingly, although ^{13}C ssNMR spectra in **Fig. 4A** evidenced a lignin reduction from S4 to S2 materials, the resulting lignin content (based on Klason lignin) under the S2 condition was considerably higher than the S4 pretreatment condition (see **Table 2**), suggesting a higher formation of pseudo-lignin in the S2 pretreated material. The FTIR spectra in **Fig. 3** also indicated higher lignin content in accordance with the Klason lignin, suggesting these results

are in decorrence of pseudo-lignin formation. Regarding **Fig. 4B**, peaks 1 and 2 were used to determine the cellulose crystallinity index (CrI) of the S2 and S4 materials, different from the X-ray diffraction results described earlier (**Fig. S4**) that represent the CrI of the pretreated solids and suffer the influence of lignin. The cellulose CrI was determined by separating the C4 region of the spectra into crystalline (peak 2) and amorphous (peak 1) peaks and calculating by dividing the area of the crystalline peak (87 to 93 ppm) by the total area assigned to the C4 peak (80 to 93 ppm) [65, 78]. The S2 2.19 severity material presented a cellulose CrI of 35.4%, and the S4 1.91 severity solids showed a similar result of 34.6%. Similarly, Bernardinelli et al. [65] studied the influence of a two-stage pretreatment (dilute acid followed by dilute alkali) of sugarcane bagasse on the cellulose CrI measured by ^{13}C MultiCP ssNMR. The authors showed that all the bagasse samples were nearly constant with CrI $\sim 45\%$, suggesting that the cellulose crystallinity was not considerably changed by the pretreatment and delignification employed. The cellulose CrI results indicated that other pretreated solids characteristics were more important in determining the straw digestibility of the S2 and S4 materials, which follows **Table 2** data discussion below (XRD results and S1-S4 solids properties).

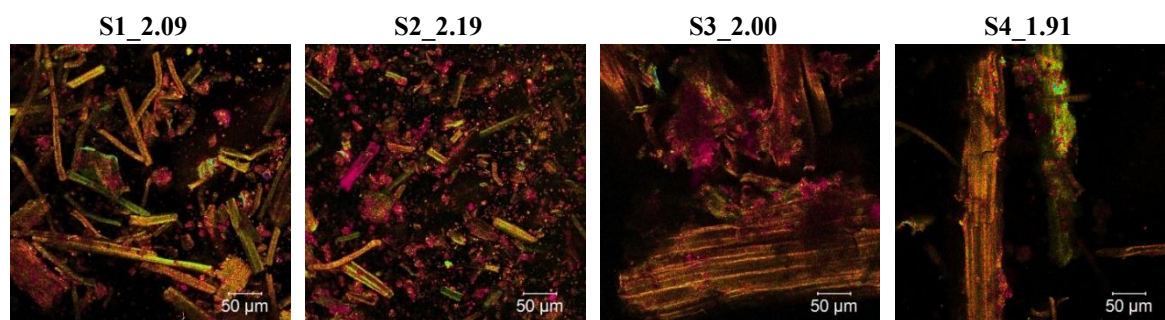


Fig. 5 Confocal laser scanning microscopy (CLSM) images of the S1-S4 pretreated straws of the combined acid- and bisulfite-catalyzed experiments.

Fig. 5 shows the obtained confocal microscopy (CLSM) images of the S1-S4 pretreated straws. The CLSM technique using two-photon excitation of lignin autofluorescence is a powerful tool to characterize lignin organization, aggregation, and interactions in native or treated biomass [67]. CLSM imaging allows the chemical mapping of chromophores, like lignin, on the various sample layers, both on the surface and inner regions, by adjusting the focal plane [66]. For this work, the confocal plane was adjusted to obtain information from the surface of the straw fibers. Raw biomass mainly emits in a blue

spectrum (400–500 nm), and longer wavelengths (lower energy emissions) are commonly associated with the formation of complex molecular aggregates (red spectrum). **Fig. 5** reveals structural and compositional changes suffered by the soaked S1-S4 straws after the steam explosion pretreatment. Initially, CLSM images evidenced the differences in the straw particle size between S1-S2 and S3-S4 materials (in accordance with the pretreated solids particle-size in **Table 2**). S1-S2 solids' structure was highly deconstructed in comparison to S3-S4 materials. S1-S2 pretreated solids were obtained with a soaking pH of 1.17-1.27, whereas S3-S4 solids were pretreated under 1.36-1.45 pH. The soaking pH was more decisive than the residence time (150-250 sec) in reducing the particle size and deconstructing the straw (not shown). Major differences between the S1-S4 materials were observed due to different soaking conditions instead of pretreatment severity. Additionally, CLSM images showed that lower pH conditions lead to an aromatic structure (lignin and pseudo-lignin) being distributed on the fiber surface (reddish particles). Under S2 pretreatment conditions, there is a strong tendency to form lignin agglomerates. Otherwise, the S4 solids, obtained at the highest pH value and bisulfite charge, presented a predominantly green and yellow spectrum, indicating a clean fiber surface from aromatic fragments. Finally, **Fig. 5** highlights the reallocation of lignin in the straw structure caused by steam explosion and corroborates with complementary analytical methods (e.g., FTIR and lignin content) as evidence for lignin condensation and pseudo-lignin formation.

Xing et al. [79] performed a steam explosion followed by an alkaline sulfite treatment to improve the enzymatic hydrolysis of bamboo. The surface of bamboo was analyzed by energy-dispersive spectroscopy, revealing the changes in O/C elemental composition due to pretreatment. The steam-exploded material presented an O/C ratio of 0.44, whereas the alkaline sulfite-treated bamboo resulted in an O/C ratio of 0.37, indicating a lower surface lignin. The enzymatic hydrolysis of the pretreated bamboo showed a substantially higher performance of the steam explosion plus alkaline sulfite pretreatment compared to the standard steam explosion method. Zhao et al. [80] studied the inhibiting mechanism of pseudo-lignin on the enzymatic digestibility of cellulose by surface plasmon resonance. The authors showed that adsorbed cellulase on pseudo-lignin is more easily dissociated when pseudo-lignin is lower in aliphatic hydroxyl content, molecular weight, and hydrophobicity. Other pretreatment studies proposed the use of different chemical additives to mitigate the negative effect of pseudo-lignin on hydrolysis. He et al. [26] investigated the effects of dilute-acid pretreatment of bamboo with 4% w/w 2-naphthol-7-sulfonate addition (oven dry

biomass) on enzymatic hydrolysis performance. The surface properties of the pretreated materials suggested that the introduced sulfonic acid group increased the surface hydrophilicity while decreasing the lignin content. The residual lignin analysis indicated that sulfonic acid modification promoted lignin depolymerization, maximizing the cellulose hydrolysis yields. Additionally, Tang et al. [81] carried out a SO₂-catalyzed steam pretreatment of poplar wood; the authors showed a positive effect of sulfur dioxide dosage on enzymatic digestibility of the obtained materials, which increased from about 40% to 60% and 70% for the non-catalyzed treatment, the 3% SO₂ and 5% SO₂ charges (dry biomass basis), respectively. Similar to these studies, the S3-S4 materials showed that lower fiber surface lignin resulted in superior hydrolysis performance as described in **Table 2** and **Fig. 6** data.

Table 2 Pretreatment and hydrolysis results of the combined acid- and bisulfite-catalyzed experiments (S1-S4 trials).

Soaking	Pretreat. Severity	Accessib. (%)	Glucose Yield % (6.3 mg)	Pulp Xylan (mg/g)	Pulp Size (mm)	CrI (%)	Pulp Lignin (mg/g)	Lignin Conv. (%)
S1	1.87	92.9±0.8	78.9±2.8	22.8±2.1	1.65±0.06	34.1±1.1	342.5±7.7	20.6±0.6
	1.99	95.1±3.0	75.3±2.0	18.7±0.7	1.63±0.07	38.8±0.9	341.7±0.5	22.3±0.0
	2.09	92.8±2.2	74.5±1.9	18.1±0.7	1.53±0.05	36.0±1.1	347.7±2.3	22.8±0.2
S2	1.97	91.6±0.7	77.2±1.9	18.2±0.3	1.41±0.10	36.1±2.1	362.9±2.1	18.1±0.1
	2.09	92.4±1.2	74.4±2.0	17.0±0.3	1.28±0.08	39.7±1.0	362.3±0.3	21.3±0.0
	2.19	92.5±1.0	71.9±1.8	15.3±0.1	1.21±0.12	40.5±0.9	376.5±7.0	19.1±0.4
S3	1.78	94.0±1.2	74.6±0.5	25.3±1.0	1.33±0.08	37.4±2.0	331.9±5.1	28.9±0.4
	1.90	96.1±1.5	75.4±0.4	25.2±0.3	1.20±0.11	38.2±1.3	337.6±3.7	28.7±0.3
	2.00	96.3±1.5	77.8±0.2	22.1±0.3	1.20±0.06	39.0±2.2	337.7±5.4	27.7±0.4
S4	1.69	98.0±1.1	76.4±1.7	36.0±3.5	1.51±0.10	44.0±0.8	323.6±7.0	23.5±0.5
	1.81	100.9±1.1	79.7±0.9	34.3±1.1	1.71±0.13	42.4±1.1	314.4±6.9	24.3±0.7
	1.91	99.6±0.8	82.5±1.6	32.9±2.6	1.57±0.09	40.4±2.3	328.4±0.8	20.8±0.1

Table 2 shows some properties of the S1-S4 pretreated solids (pulp), including the accessibility, enzymatic digestibility (6.3 mg/gCel enzyme dosage), xylan content, average solids size, crystallinity index (XRD), and lignin content and conversion. See **Table S4** for a complete data description. The results revealed a complex correlation between the hydrolysis yield and pulp properties, showing that different solid attributes can influence the enzymatic hydrolysis of the pretreated materials. Considering the enzymatic hydrolysis performance, the results showed a highly positive effect of metabisulfite dosage with higher soaking pHs on accessibility (S3 and S4 materials). At 0.31% w/w metabisulfite dosage (solution basis) with a pH of 1.45 (S4 condition), the accessibility was virtually 100%, even at relatively low

severities between 1.69-1.91. Like pretreatment yield (**Fig. 2**), the soaking pH highly influences the hydrolysis accessibility. However, different from pretreatment, the higher the pH and bisulfite, the higher the accessibility. Typically, better results are obtained during the enzymatic hydrolysis as the pretreatment severity is higher, as shown by the initial experiments (see **Table S3**). However, the combination of higher pHs and bisulfite addition resulted in a substantial improvement in accessibility.

Concerning the solids' particle size, size reduction was not a decisive condition for obtaining high yields, as evidenced by S4 hydrolysis results, which achieved the highest glucose yields even with the highest pulp's particle size. However, smaller particle sizes contributed to improving hydrolysis under low enzyme dosages, as revealed by the performance change between S1 and S2 straws when the enzyme dosage was reduced to 6.3 mg/gCel. The S1 material (1.53-1.65 mm particle size) performed better during hydrolysis at 12.6 mg/gCel enzyme dosage, while the S2 (1.21-1.41 mm particle size) was slightly superior at 6.3 mg/gCel dosage. The S1 trial at 1.99 severity resulted in $75.3 \pm 2.0\%$ glucose yield, whereas the S2 trial at 1.97 severity resulted in $77.2 \pm 1.9\%$ using 6.3 mg/gCel of CTec3 HS. Interestingly, although the S1 1.99 severity condition reached better pulp's indicators ($95.1 \pm 3.0\%$ of accessibility, 341.7 ± 0.5 mg/g of lignin content, and $22.3 \pm 0.0\%$ of lignin conversion) versus the S2 1.97 severity condition ($91.6 \pm 0.7\%$ of accessibility, 362.9 ± 2.1 mg/g of lignin content, and $18.1 \pm 0.1\%$ of lignin conversion), the smaller particle size of the S2 material was more determinant in benefiting low enzyme dosage hydrolysis. MacAskill et al. [82] performed a steam explosion pretreatment of *P. radiata* sawdust and evaluated the enzymatic digestibility of the pretreated materials using the Cellic CTec2 enzyme, showing an increase in the digestibility as a function of pretreatment severity. However, when the pretreated materials were ball-milled to the same particle size of 30-40 μm , the digestibility decreased with higher pretreatment severities, suggesting that particle size is a major determinant of accessibility, although enzyme inhibition represents a more significant factor during the hydrolysis of similar particle size pretreated materials.

No direct correlation was observed between the crystallinity index and the enzymatic digestibility of the S1–S4 materials. Zhang et al. [83] studied the correlations between sugar conversion and recalcitrant structures after a steam explosion pretreatment of corncob. The results showed that the product of CrI and lignin content correlates negatively with sugar conversion even better than that of CrI or lignin content alone, suggesting a combined effect of the solids' properties on recalcitrance. Considering the S2 and S4 pretreated straws, the S2

materials presented CrI in the range of 36.1-40.5% and lignin content of 362.3-376.5 mg/g, whereas the S4 materials showed CrI values between 40.4-44.0% and lignin content of 314.4-328.4 mg/g, suggesting that the lower level of lignin content allowed the S4 pulps to achieve the highest hydrolysis yields. Regarding lignin, **Table 2** shows that the combination of higher pretreatment pHs and bisulfite addition provided the lowest pseudo-lignin formation (lower pulp lignin content and higher lignin conversion). As described in the initial experiments section (see **Fig. S4**), lower lignin conversions combined with low levels of pulp xylan can indicate pseudo-lignin formation (Klason lignin mass increase), which decreases cellulose accessibility and enzyme effectiveness. Foody et al. [53] combined sulfuric acid and sulfurous acid catalysts during a dilute-acid pretreatment study using wheat straw as feedstock. The pretreatment was performed under a standard pH of 2.0 and 180°C for 15 min. Using exclusively sulfuric acid at 2.4% w/w on biomass led to 19.6% of lignin solubilization, whereas under the combined 1.2% sulfuric acid and 4.0% sulfurous acid, the conversion reached 29.7%. Moreover, 8.0% w/w sulfurous acid on biomass (oven dry basis) led to 41.0% lignin solubilization. Ultimately, the authors showed that the pretreated straw obtained under an optimum condition of 6% w/w sulfurous acid and pH of 1.1 at 195°C for 5 min resulted in excellent hydrolysis yields. Finally, the pulp's properties of **Table 2** evidenced that the main factors affecting the hydrolysis performance with Cellic® CTec3 HS enzyme are related to the extension of the biomass modifications, including the reduction of the solids' particle size and the formation of pseudo-lignin, which respond to the major effects on hydrolysis yields.

Fig. 6 shows the hydrolysis and global yields of the S2 (without bisulfite) and S4 (with bisulfite) trials with two different enzyme dosages, 6.3 and 12.6 mg protein/gCel. At 12.6 mg/gCel dosage, glucose yield behaves similarly to accessibility results regarding the effect of bisulfite and pH (see **Table 2**), with pretreatment severity producing a small impact on yield, indicating the achievement of a hydrolysis plateau. Otherwise, an interesting hydrolysis behavior was observed concerning the effect of severity on yield when the enzyme dosage was reduced to 6.3 mg/gCel. For the S2 material, a significant yield reduction was observed as a function of severity, whereas for the S4 material, higher severities favored glucose yield. The S4 material, pretreated at 1.91 severity, achieved about 83% of glucose yield during hydrolysis with 6.3 mg protein/gCel dosage, showing the excellent result provided by the S4 pretreatment condition combined with Cellic® CTec3 HS enzyme. McMillan et al. [84] compared four different cellulase enzymes using a dilute-acid pretreated corn stover substrate obtained under steam explosion conditions, also similar to S1-S2 experiments, with 30%

solids and a pH of 1.20 (sulfuric acid) at 190°C for about 1 min. Reaching 85% hydrolysis glucose yield on washed pretreated stover required enzyme dosages of 13-40 mg protein (BCA assay) per gram of cellulose with 10% insoluble solids and 168 h of hydrolysis. Regarding the total released sugars (glucose and xylose) during the pretreatment and hydrolysis, **Fig. 6B** shows the global monomer yields of the S2 and S4 materials. Due to the low pretreatment xylose yield, the S4 pretreated straw resulted in low global yields compared to S2 pulp. At 12.6 mg/gCel enzyme dosage, the S4 pulps resulted in nearly 85% global yield at 1.69-1.81 severity, even with low pretreatment yields (see **Fig. 2A**). At 6.3 mg/gCel enzyme dosage, the highest global yields were obtained through the S2 material, resulting in about 80% yield after the steam explosion at nearly 1.90 severity. This result means that 80% of the theoretical potential of glucose and xylose, based on the soaked straw composition, was converted into glucose and xylose. Ultimately, although having the lowest accessibility, the S2 pretreated solids provided the best global yields due to the highest pretreatment yield.

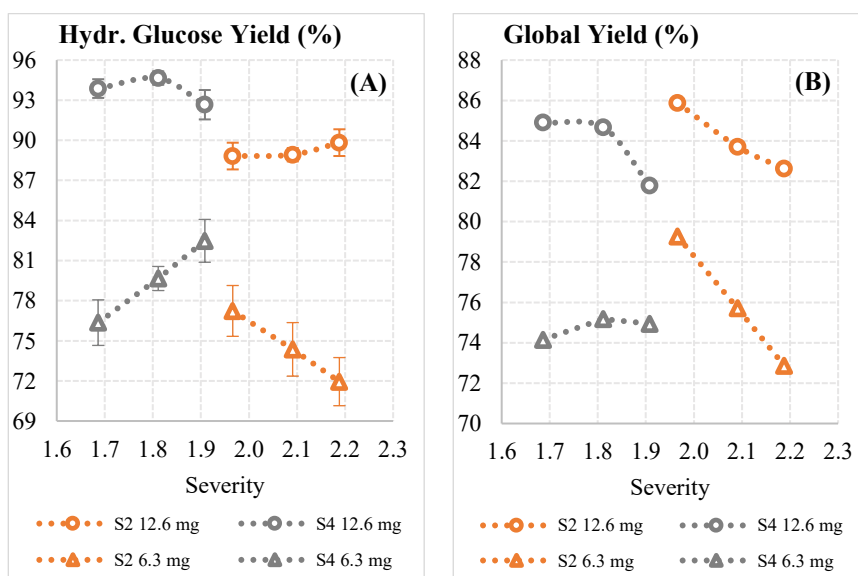


Fig. 6 Hydrolysis glucose yield (A) and global yield (B) of the combined acid- and bisulfite-catalyzed experiments (S2 and S4 materials) for different pretreatment severities and enzyme dosages.

Fig. 7 shows the T_2 distribution times from the ^1H NMR relaxometry measurements (normalized signal intensities) of the raw straw, S2 (2.19 severity), and S4 (1.91 severity) pretreated straws. **Fig. S6** shows the obtained decay of the CPMG signals. T_2 distribution times result from the biomass-solvent interactions, depending on both the physical and

chemical environment experienced by the solvent, with more solvent constraints resulting in shorter T_2 relaxation curves [85]. The nature and strength of the association between the probe molecule (water) and the cell wall are directly related to the ultrastructural and chemical state of the biomass [86, 87]. Lignocellulosic biomass has three characteristic interstitial scales (pores), resulting in a T_2 profile with three components that encode the length scales of the pores and their occupancy by the enclosed fluid [37]. The components associated with each interstitial scale are indicated in **Fig. 7** by the peaks 1, 2, and 3 corresponding to the microfibril surface, cell wall, and lumen, respectively [62]. Water molecules in the different biomass structures show T_2 distributions ranging from longer to shorter components of T_2 ; briefly, the smaller the pore, the shorter the T_2 . Longer T_2 components were associated with higher-mobility water molecules located in the core of the lumen. Shorter T_2 components were associated with water molecules with lower mobility, sorbed at the cell wall around the lumens. Moreover, the water molecules in shorter T_2 components had limited mobility due to the hydrophilic interactions with the microfibril surface [87, 88]. Because the wettability of the pores is also expected to be affected by chemical modifications on the pore surface, the increase in the amount of water on a given interstitial scale can be correlated with the rise of both the material porosity and chemical affinity of the surface to water [62, 89].

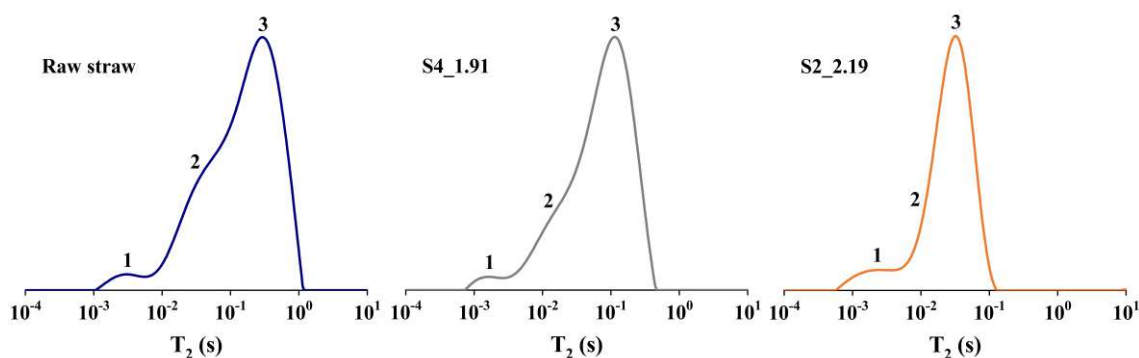


Fig. 7 T_2 ^1H NMR relaxometry measurements (normalized signal intensities) of the raw and pretreated straws obtained under the S2 and S4 pretreatment conditions.

The obtained T_2 relaxation times shown in **Fig. 7** were analyzed to assess straw structural changes due to S1-S4 pretreatment conditions. T_2 distributions revealed different profiles among the raw straw and the S4 and S2 materials with two principal characteristics: first, the expressive reduction of the component associated with cell wall interstitial scale (peak 2) and, second, a significant decrease in the T_2 values from the raw straw to S4 and S2

pretreated straws (following this sequence). The relative area of peak 2 was gradually reduced from raw straw to S4 and then to S2 material, suggesting a progressive decrease in the cell wall pore size and/or water affinity. Additionally, the component with longer T_2 (peak 3) shifted its central T_2 value from 0.2967 s for the raw straw to 0.1162 s for the S4 material and 0.0326 s for the S2 material, indicating lower pore sizes of the S2 pretreated straw. Literature provides a series of studies evaluating the influence of different pretreatment methods and conditions on T_2 relaxation times, some of which are discussed below to elucidate the present work results.

Bianchini et al. [87] showed that the intermediate T_2 component (cell wall component) was significantly reduced after steam explosion pretreatment of sugarcane bagasse. Otherwise, bagasse pretreated by a hydrothermal pretreatment resulted in a preserved cell wall interstitial scale compared to raw bagasse. Foston and Ragauskas [90] studied the structural changes of *Populus* wood after a dilute-acid pretreatment using ^1H NMR relaxometry measurements; the results revealed that the increase in pore size during pretreatment was due to existing pores' expansion rather than generating new pores. Weiss et al. [91] performed an SO_2 -catalyzed steam explosion study of spruce wood and showed that T_2 relaxation times decreased significantly between raw and pretreated materials and as a function of pretreatment severity. For the higher severity condition, only one major peak was observed in the T_2 distribution times, suggesting a reduction in the number of different water constraint environments in the pretreated material compared to raw biomass. The authors showed that increased water constraint (resulting from increased pretreatment severities) correlates with improved biomass digestibility and may be a consequence of increasing smaller pores (higher specific surface area). Other literature references have also investigated the influence of delignified pretreatments on T_2 distribution, showing interesting results regarding T_2 data interpretation. Kane et al. [37] studied the T_2 relaxation times of pretreated sugarcane bagasse obtained under different sulfonation conditions, showing a significant increase in the cell wall interstitial scale component after 71% delignification compared to untreated bagasse. Williams et al. [85] showed that T_2 relaxation times of corn stover and switchgrass pretreated by alkaline hydrogen peroxide correlate with differences in hydrolysis yields of materials due to pretreatment. The results suggested that pretreated solids with more constrained water (lower T_2 times) had greater cellulose accessibility to the enzyme and resulted in higher hydrolysis yields.

The literature results evidenced that different pretreatment methods provide specific biomass features. The steam explosion effect on biomass structure involves cell wall fragmentation, and not only the chemical conversion of cell wall components. The primary mechanism of steam explosion in deconstructing biomass is the cell wall disintegration due to the explosive discharge decompression of the reaction system, leading to a reduction in the solids particle size (see **Table 2**) and increasing specific surface area [92]. Ultimately, the present work results showed that S4 1.91 severity pretreated straw was significantly more accessible to Cellic® CTec3 HS than S2 2.19 severity material. The S1-S4 trial results showed that the pretreated solids digestibility is a complex function of biomass structural and chemical properties, which depends on the pretreatment method, process conditions, and also the enzyme cocktail characteristics. Although S2 material presented smaller particle sizes than S4 material, **Fig. 7** results evidenced that severe steam explosion may be detrimental to the cell wall interstitial scale, negatively affecting the accessibility of enzymes to cellulose. Confocal images (**Fig. 5**) showed the S2 solids impregnated by an aromatic structure that may respond to the reduced cell wall interstitial water affinity (e.g., increased hydrophobicity) and the lower effectiveness of Cellic® CTec3 HS on S2 pretreated material. Finally, the results suggest that lignin modifications and pseudo-lignin formation that occurred during the S2 pretreatment can be the principal reason for the lower accessibility of S2 material compared to the S4 pretreated straw.

S5-S6 Trials

S5 and S6 soaking and steam explosion experiments were carried out to evaluate the effect of sodium metabisulfite addition with a soaking pH target of about 1.20, aiming to achieve a higher global monomer yield, improving both pretreatment and hydrolysis yields. The combination of higher soaking pHs and the addition of metabisulfite favored the hydrolysis yield using the Cellic CTec3 HS enzyme; however, it represents a poor condition considering the release of xylose during pretreatment, harming the global yield. The metabisulfite charge of 0.26% w/w (**Table 1**) was selected to perform the S6 soaking, and an additional sulfuric acid dosage was added (total acid of 0.82% w/w) after the metabisulfite addition, reducing the pH value to 1.19. The S5 trial was used as a comparison reference to the S6 experiment, and S5 conditions included 0.67% w/w acid dosage and a final soaking pH of 1.23 (see **Table S1** for details of the soaking data). The pretreatment experiments were performed in a standard condition of 193°C for 150 sec with three running batches in series, resulting in pretreatment severities of 1.91 and 1.95 for the S5 and S6 materials, respectively.

The pretreated solids composition in glucan and xylan was 549.5 ± 4.1 mg/g and 18.5 ± 0.6 mg/g for the S5 material, respectively. The S6 pretreated straw presented 537.9 ± 3.5 mg/g of glucan content and 14.8 ± 0.3 mg/g of xylan composition. The pretreatment xylose and glucose yields were similar between the S5 and S6 experiments; the S5 trial resulted in 72.6 ± 1.3 % and 7.5 ± 0.1 % of xylose and glucose yields, respectively, and the S6 trial reached 71.6 ± 1.4 % of xylose yield and 7.7 ± 0.1 % of glucose yield (see **Table S5** for details of the pretreatment data). The obtained pretreated solids were submitted to a hydrolysis dose-response evaluation regarding the enzyme dosage and the resulting glucose yield. The enzymatic hydrolysis was performed with 13% w/w insoluble solids and 70% washing efficiency, keeping other conditions according to the standard hydrolysis protocol. A lower washing efficiency typically requires higher enzyme dosages to produce satisfactory sugar yields [84]. However, there is an economic balance between the enzyme consumption and the investment and operational costs related to the additional water added to wash the pretreated solids to 90% efficiency. Therefore, the 70% washing value was selected for the hydrolysis S5-S6 protocol, aiming for realistic process conditions.

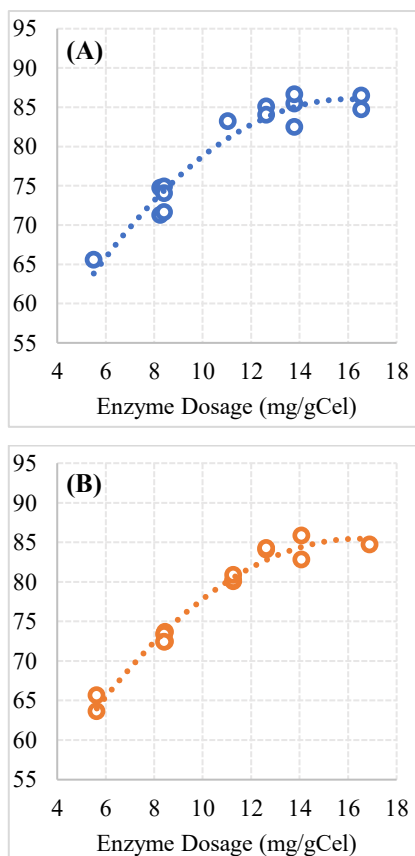


Fig. 8 Hydrolysis glucose yield of the S5 (A) and S6 (B) pretreated straws as a function of Cellic CTec3 HS dosage in mg protein (BCA assay) per g of cellulose.

Fig. 8 shows the performance of the S5 and S6 materials during hydrolysis with an enzyme dosage between about 5-17 mg protein per gram of cellulose (40-120 BHU/gCel). The obtained glucose yields were similar for both pretreated solids, concerning the yield curve behavior and the maximum yield achieved (about 85%). See **Table S5** for details of the hydrolysis data. The hydrolysis results of S5-S6 materials led to a non-conclusive effect of metabisulfite on hydrolysis performance under the pretreatment conditions employed with pH of ~1.20. Not enough bisulfite was probably added (1.19% w/w based on dry biomass weight) to react with the amount of pseudo-lignin precursors formed during pretreatment at about 1.20 soaking pH. The ^{13}C ssNMR spectra of S5-S6 materials (**Fig. S5 B**) show that both pretreated solids are very similar in chemical composition, suggesting limited alterations caused by the metabisulfite addition. This work aimed to investigate the effect of low bisulfite dosages on straw pretreatment and hydrolysis performances. Adding a higher bisulfite dosage depends on a techno-economic analysis, considering the benefits of enzyme reduction and the challenges and costs of using additional SO_2 .

CONCLUSIONS

This work used a pilot-scale system to evaluate the steam explosion pretreatment of the sugarcane straw using a combined acid- and bisulfite-catalyzed condition to mitigate pseudo-lignin formation and improve straw digestibility. The initial acid-catalyzed experiments (without bisulfite addition) showed that the pretreatment xylose yield achieved a maximum at ~1.80 severity, and the hydrolysis glucose yield reached a plateau after about 2.00 severity, leading to an optimum global yield at ~1.90 severity. The initial experiments showed that the lignin conversion readily achieved ~24% (based on initial lignin) under low severity conditions; nevertheless, the lignin conversion decreased as a function of severity, reaching ~18% at 2.33 severity, indicating a significant pseudo-lignin formation.

During the combined acid- and bisulfite-catalyzed experiments, six soaking and pretreatment trials (S1-S6 experiments) were carried out to investigate the influence of different process conditions on straw digestibility using Cellic® CTec3 HS enzyme. The S1-S4 trials showed that the soaking pH greatly influences steam explosion performance. Interestingly, distinct pHs produced different yield curves as a function of pretreatment severity. The xylose and glucose pretreatment yields significantly differed under equal severity conditions but with distinct pH values. Xylose yields of 75-80% were obtained when

the soaking pH was below about 1.30 (S1-S2 conditions). Regarding the hydrolysis performance, the results showed that the particle-size reduction from 7.3 to 3.9 mm was not a decisive condition for obtaining high yields; however, lower solid particle sizes contributed to improving hydrolysis under low enzyme dosages. Considering the pretreated straw accessibility, better results were obtained under the lowest pretreatment severities due to the combination of bisulfite addition and higher soaking pHs. Combining 1.65% w/w sodium metabisulfite on biomass and soaking pH of 1.45 (S4 condition) resulted in ~100% accessibility and reached the highest hydrolysis yields at low enzyme dosages. The S4 1.91 severity condition resulted in about 83% hydrolysis glucose yield with 6.3 mg protein/gCel enzyme dosage.

The S1-S4 hydrolysis results suggested that lignin modifications and pseudo-lignin formation were the main factors affecting the enzyme effectiveness. The chemical composition analysis showed higher lignin conversions under S3-S4 conditions, which include soaking pHs of 1.36-1.45 and 0.85-1.65% sodium metabisulfite charges (biomass basis). Confocal images showed that lower soaking pHs resulted in an aromatic structure deposition on the straw surface. Under S2 pretreatment conditions, there was a strong tendency to form lignin agglomerates. Otherwise, the S4 material presented a predominantly green and yellow spectrum, indicating a clean fiber surface from aromatic fragments. The ¹H NMR relaxometry measurements showed that severe steam explosion may be detrimental to the cell wall interstitial scale, negatively affecting the accessibility of enzymes to cellulose. Finally, the S5-S6 trials showed that at soaking pHs of about 1.20, the sodium metabisulfite dosage may be higher than those used (1.19% w/w on biomass) to mitigate pseudo-lignin formation and benefit hydrolysis performance. Since pH values below 1.30 are desired to improve xylan to xylose conversion during pretreatment, additional study is needed to evaluate the feasibility of using higher bisulfite charges to improve the hydrolysis yield at this pH level.

STATEMENTS & DECLARATIONS

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Author Contributions

MBWS and ARG contributed to the study's conception and design. MBWS performed the pretreatment experiments and wrote the original draft. FEGG performed the confocal microscopy analysis. ERDeA and RHSG performed the ^{13}C NMR and ^1H NMR measurements. VOAP and IP performed the X-ray diffraction analysis. All authors reviewed and approved the final manuscript.

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Data availability

All data generated or analysed during this study are included in this published article and its supplementary information file (Figures S1-S6 and Tables S1-S5).

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

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