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Pilot-scale high-consistency mechanical refining improves enzymatic saccharification of lignocellulosic feedstock

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The natural recalcitrance of lignocellulosic biomass poses a major challenge for biorefineries aiming to convert these materials into fuels, chemicals, and polymers. This study explored the impact of pilot-scale high-consistency mechanical refining on the enzymatic depolymerization of lignocellulosic biomass when associated with low-severity hydrothermal (HPTB) and steam explosion (SEPTB) pretreatments, 180 °C for 15 min and 190 °C for 10 min respectively. Under industrially relevant conditions and using sugarcane bagasse as model lignocellulosic feedstock, we observed that the mechanical refining had a distinct effect on the fiber morphology depending on the pretreatment process. While statistical analyses indicated no correlation between refining parameters and saccharification efficiency for HPTB, an 8% increase in glucose release was observed for SEPTB with mechanical refining. Scanning electron microscopy revealed enhanced fiber morphology and delamination in SEPTB material after mechanical refining, suggesting increased porosity and accessibility for enzymes. Furthermore, distinct chemical compositions were observed between HPTB and SEPTB materials, aligning with the distinct responses to mechanical refining. These findings highlight the potential of combining mechanical refining with steam explosion pretreatment to improve the enzymatic depolymerization of lignocellulosic biomass, advancing the current knowledge on the application and challenges of high-consistency mechanical refining in lignocellulosic biorefineries.

Keywords Sugarcane bagasse, Mechanical refining, Pilot-scale, Enzymatic saccharification, Fiber morphology

The natural recalcitrance of lignocellulosic biomass is a major challenge for biorefineries[1], and a combination of thermochemical processes followed by mechanical refining has been proposed as a promising route to enhance biomass enzymatic saccharification[2–4]. Sugarcane bagasse, a lignocellulosic byproduct of the sugar and ethanol industry, has been widely studied as a potential raw material to produce fermentable sugars in biorefineries. Its abundance makes it a particularly attractive feedstock, with Brazil's 2024/2025 harvest projected to produce 689.8 million tons of sugarcane, generating approximately 230 million tons of bagasse[5]. The efficient utilization of this biomass presents significant economic and environmental opportunities, reinforcing its relevance as a model feedstock.

Mechanical refining is an established technology in the pulp and paper industry and is widely used to improve fiber properties[6,7]. The main refining mechanisms are (i) external fibrillation - creating fibrils on the fiber surface; (ii) internal delamination - swelling of fibers and loosening of internal structures; and (iii) fiber cutting - fiber shortening due to the shear action can be observed in different lengths, depending on the apparatus and refining conditions[8]. Understanding which of these mechanisms provides the best scenario for enzymatic saccharification and the critical fiber properties to be modified by mechanical refining will enable more efficient use of this approach in lignocellulosic biorefineries[9–11].

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According to previous studies[8,11], it is expected that with the implementation of mechanical refining, biomass recalcitrance, and process complexity can be significantly reduced due to the following reasons: (a) increased sugar recovery with reduced enzyme dosages; (b) significant reduction in the pretreatment severity; and (c) ease of integration with any type of thermochemical processes. Moreover, mechanical refining is a commercially proven technology in process streams of approximately 1500 tons per day of biomass. However, the effectiveness of mechanical refining seems to vary mainly depending on biomass type, pretreatment severity, enzyme formulation, and dosage, among other factors[12]. Therefore, understanding the potential of mechanical refining in enhancing enzymatic conversion under industrially relevant conditions for specific biomass conversion routes is strictly necessary to assess its relevance and applicability.

It is particularly promising that mechanical refining might promote efficient biomass saccharification under low severity pretreatment, resulting in less sugar degradation, low amounts of inhibitors during fermentation, and less complex equipment for pretreatment, a true engineering challenge in any lignocellulosic biorefinery. In this context, this work aimed to investigate the effect of high-consistency mechanical refining associated with low severity pretreatments on the enzymatic saccharification of sugarcane bagasse.

Results and discussion

Chemical composition of raw and pilot-scale pretreated sugarcane bagasse

The major goal in a pretreatment step is to enable the efficient recovery of carbohydrates and lignin concomitant with the low production of microbial inhibitors and enzyme load. Through the low severity conditions investigated in this work, we pursue to attend these demands, maximizing the hemicellulose recovery and minimizing HMF and furfural formation.

The chemical composition of raw sugarcane bagasse, hydrothermally and steam-exploded pretreated bagasse (Table 1) follows previous work[13], and it is possible to observe significant differences in the contents of the solid fractions when comparing raw and pretreated biomasses. These differences are mainly due to the solubilization of hemicellulose and cellulose, and lignin condensation during the pretreatment reactions. Hemicellulosic polymers were mostly solubilized to C5 oligomers in both pretreatments (hydrothermal and steam explosion), with a minor formation of degradation compounds such as furfural and acetic acid. Whereas for cellulose, as expected, minor solubilization and degradation were observed. Another meaningful change in the lignocellulose structure after the pretreatment is a fragmentation and deposition of lignin on the cellulosic fibers establishing a steric barrier for carbohydrate-active enzymes (CAZymes) that will impact the enzymatic efficiency during the saccharification[14].

It is known that during the pretreatment process at the pilot plant scale, especially for the steam explosion, some compounds are formed and/or volatilized during the reaction, mainly from hemicellulose fraction. For this reason, as previously reported[15,16], it is reasoned that the mass balances could not reach 100%. One of the hypotheses is that the sugars react with some of the degrading compounds (HMF, furfural, carboxylic acids, and others) and lignin fragments, leading to humins formation and other compounds which are rarely reported because of its inherent difficulty for analytical detection[17].

Despite the inherent challenges for the mass balance of pretreated lignocelluloses, satisfactory data were achieved with above 90% of the cellulose and hemicellulose recovery for the hydrothermally pretreated bagasse (HPTB) and at least 75% for the steam-exploded pretreated bagasse (SEPTB) (Fig. 1).

By the data presented in Fig. 1, no significant degradation of the cellulosic fraction was observed. Only traces of HMF were determined, and recovery of this fraction as fibers of approximately 90% for both pretreatments. This is highly desirable, regardless of the biorefinery concept considered, considering that, in the next step, the cellulose will be submitted to enzymatic saccharification to obtain glucose. It is also possible to observe that due to the low severity of the hydrothermal pretreatment, there was practically no release of glucose in the liquid fraction (SS) (Fig. 1A). In contrast, solubilization in a ratio of almost 3:1 (oligo: mono) was observed for the steam explosion (Fig. 1B).

Regarding the hemicellulosic fraction, effective 90 and 70% recovery was possible from hydrothermal and steam explosion pretreatments, respectively. In both conditions, nearly 50% of the hemicellulose is available in the liquid fraction, which offers an opportunity for processes in which stream separation is considered, and the solubilized C5 sugars could then be used in an independent fermentation. It is essential to highlight that, for this scenario, optimization still will be necessary, could be in the pretreatment condition and/or a depolymerization step (acid or enzymatic) to obtain monomeric xylose, since for both conditions evaluated in this work, an oligo: mono ratio of 7:1 (Fig. 1A) and 6:1 (Fig. 1B) were obtained.

By these results, we effectively achieved the expectation of low severity pretreatments, once with severity factors below 4 (3.5 for HPTB and 3.6 for SEPTB), we could solubilize the hemicelluloses with low removal or degradation, following as described[18] that say it starts to solubilize with minimal changes at a severity of 3, and factors greater than 4.7 imply in reduction of hydrolyzed sugars and high inhibitors formation.

Mechanical refining evaluation

The effect of mechanical refining notably varies depending on biomass type, pretreatment severity, and enzyme formulation and dosage. Previous studies show that refining benefits are limited by biomass recalcitrance and total conversion. It has been reported that high severity pretreatments associated with mechanical refining do not improve enzymatic saccharification[8]. Moreover, high severity pretreatments led to extensive production of harmful inhibitors for the fermentation and might compromise the C5 fraction for biological conversion. Therefore, in this study, we investigated the combination of two types of low severity pretreatments and high-consistency mechanical refining at an industrially relevant environment (pilot plant) to obtain fermentable sugars (glucose) by the enzymatic saccharification of the pretreated sugarcane bagasse.

Sample	Severity	Insoluble solids (%)	Cellulose (%, IS)	Hemicellulose (%, IS)	Lignin (%, IS)	Ash (%, IS)	Extractive (%, IS)	Soluble solids (%)	Glucose mono (%, SS)[a]	Glucose Oligo[b] (%, SS)[a]	HMF (%, SS)[a]	Xylose mono (%, SS)[a]	Xylose oligo[b] (%, SS)[a]	Furfural (%, SS)[a]	Acetic acid (%, SS)[a]
SCB	n.a.	n.a.	42.1 ± 0.3	30.5 ± 0.1	24.4 ± 1.1	1.2 ± 0.1	3.51 ± 0.08	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
HPTB	3.53	63.2 ± 0.8	60.1 ± 0.8	15.1 ± 0.2	24.5 ± 1.3	1.1 ± 0.1	n.a.	36.8 ± 0.8	0.1	4.5	0.2	6.2	39.5	0.6	2.4
SEPTB	3.65	65.6 ± 0.1	59.9 ± 1.0	9.2 ± 0.3	28.3 ± 0.6	2.8 ± 0.1	n.a.	34.4 ± 0.1	0.2	6.9	0.6	12.5	29.9	1.6	3.7

Table 1. Compositional analysis of raw sugarcane bagasse (SCB), HPTB – hydrothermally pretreated Bagasse (180 °C for 15 min) and SEPTB – steam-exploded pretreated Bagasse (190 °C for 10 min) based on the insoluble solids content (IS) and soluble solids content (SS). *n.a.* not applied; [a]These concentrations are from single assays; [b]Oligo concentration was determined by the difference of sugar amount after the liquor laboratory acid hydrolysis from the previous.

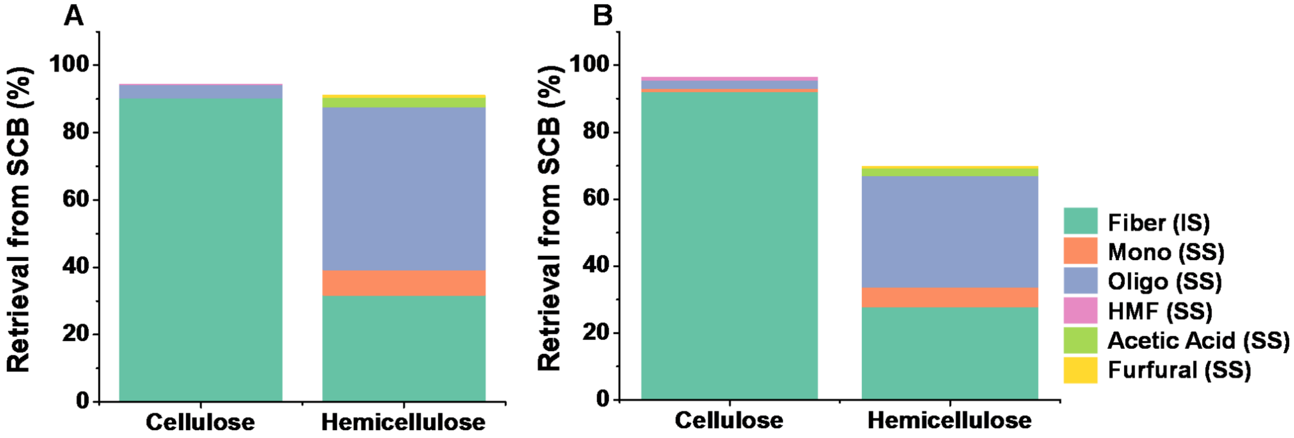

Fig. 1. Mass balance after pilot-scale pretreatment based on the insoluble solids (IS) and soluble (SS) content. (**A**) hydrothermally pretreated bagasse (180 °C for 15 min); (**B**) steam explosion pretreated bagasse (190 °C for 10 min). Fiber is the solid fraction, mono is the glucose or xylose released, and oligo is the oligomeric fraction of glucose or xylose released.

The optimal conditions for GW and DR were initially investigated using HPTB and the CCRD approach according to the equipment's operational limits. The results presented in Fig. 2 highlight that the maximum cellulose conversion into glucose was 50% in 72 h, below conversion levels observed in the literature for pretreatments with higher severity[15,19]. Notably, all combinations of mechanical refining with HPTB resulted in higher cellulose conversion levels into glucose. However, the statistical analysis indicates that such values are at the same magnitude as the average of the central point, precluding to infer a clear correlation between saccharification efficiency and gap width or rotation, which was confirmed by the non-significative regression coefficients (R^2) obtained for all the mathematical models in Table 2 and ANOVA. Regardless that no mathematical model could be determined to optimize the mechanical refining process, the positive impact of this additional step on saccharification can be observed, principally after 24 h of reaction (Fig. 2), ranging from 5 to 15% of higher conversion rates.

In addition, we investigated the effect of mechanical refining on another low severity pretreatment by steam explosion, which is currently employed at a commercial scale in lignocellulose biorefineries (Fig. 3). Interestingly, the mechanical refining had a more pronounced effect on the saccharification efficiency of steam-exploded pretreated bagasse (SEPTB) compared to HPTB, increasing in 8% its efficiency. It is notable that this gain in the saccharification productivity is observed even at the first hours, and the tendency was maintained throughout the 72 h of reaction, which was not observed for HPTB.

It indicates that the low severity hydrothermal pretreatment was ineffective in reducing biomass recalcitrance, even though we are working in the range where the benefits of mechanical refining should be significant, as proposed at the laboratory scale[20]. They showed a dependence between the pretreatment severity and enzyme loading. Where the refining was limited by recalcitrance when pretreated biomass achieved a sugar yield of 40% and by total conversion at 80%. Nevertheless, an additional 15% increase in biomass yield was observed when it was 60% before refining. These results suggest the dependence of the refining reactivity on the biomass type and pretreatment while raising the question: does it pay off adding the refining step to the sugar release process?

Most previous works supporting this route at a pilot scale[9,10,21] were executed with hardwood and corn stoves, and the equipment used allowed a minimum gap width of 0.05 mm (20 times lower than the applied in this study). At the same time, few works have investigated the refining with pretreated sugarcane bagasse, generally at a lab scale and low consistency[22,23]. In the present work, we evaluated the mechanical refining performance at the pilot scale and high consistency associated with the hydrothermal and steam explosion pretreatment. Since the increase in cellulose conversion was higher when the pretreatments were compared, being 20% instead of the 8% achieved after the refining process with the SEPTB, further research must be conducted to answer the proposed question to determine the relations of refining reactivity to the pretreatment and biomass source.

In order to understand the distinct effects of mechanical refining on both pretreated materials, scanning electron microscopy (SEM) was applied to evaluate morphological characteristics (Fig. 4). The micrographs show exposed flattened and cylindrical fibers, common to the morphology of sugarcane bagasse for both pretreatments (Fig. 4A and C). However, severe fibers opening and delamination are only evident in the steam-exploded material and after its mechanical refining, which also enables access to the fiber tracheid, supportive with saccharification assays (Fig. 4C and D). As noticed in Fig. 4B, the refined HPTB did not exhibit equivalent morphology, reinforcing the concept that refining reactivity is linked to the pretreatment severity. Such microscopic analysis indicates that the improvement in enzymatic saccharification efficiency by the high-consistency mechanical refining is associated with the exposure of the cell wall fibrils and delamination, likely reflected in higher porosity and swellability of the pretreated biomass.

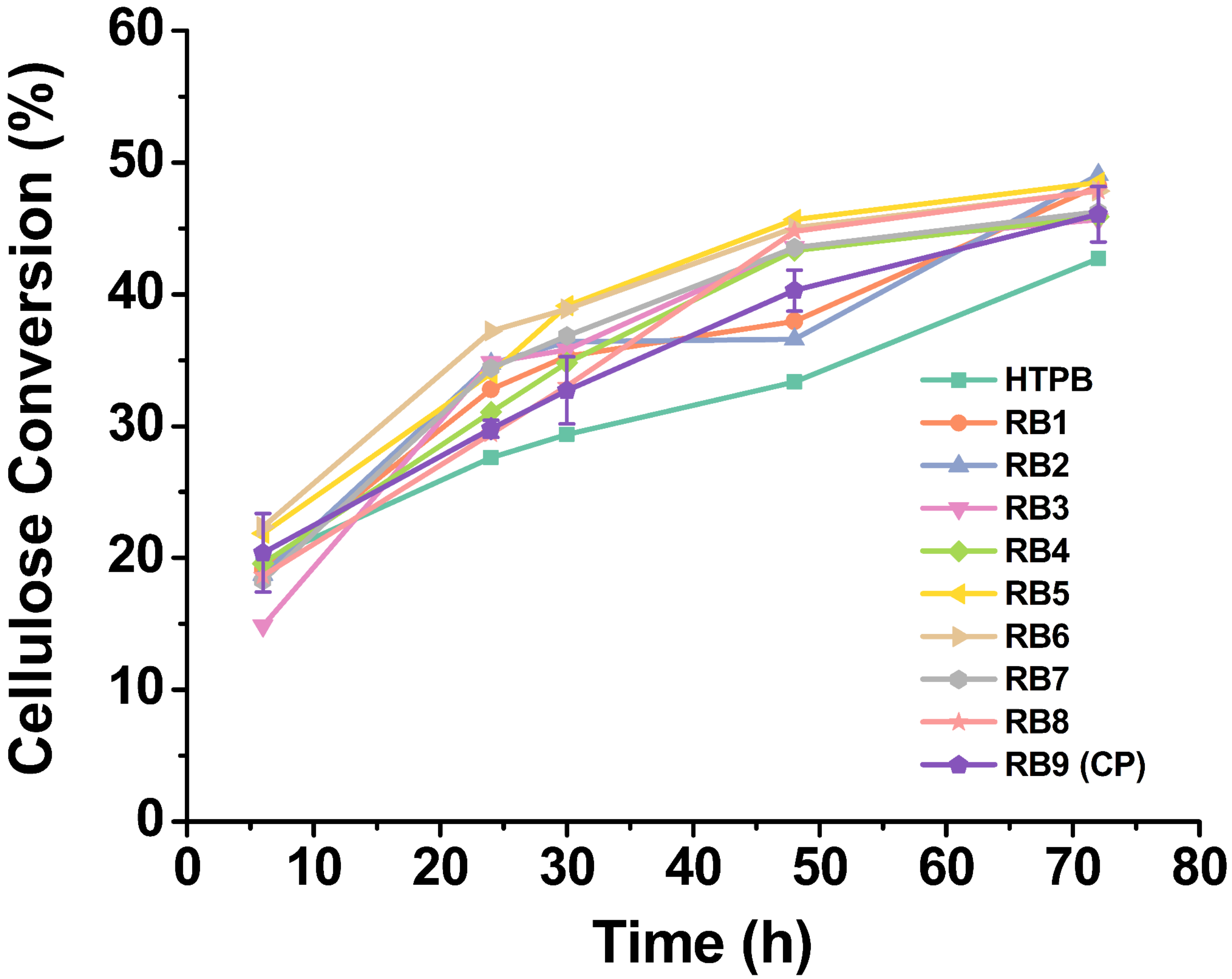

Fig. 2. Enzymatic saccharification kinetics for CCRD performed for mechanical refining (RBs) and pretreated bagasse HPTB. Saccharification assays were conducted with 10% (w/w) solid content and an enzyme cocktail load of 10 FPU.gPTB^{-1}. RB conditions are described in Table 3.

Time (h)	Mathematical models	R^2	F_{cal}	F_{tab}
6	$Y_1 = 20.40 + 0.63\ x_1 + 0.30\ x_1^2 - 0.37\ x_2 - 1.52\ x_2^2 + 1.27\ x_1\ x_2$	41.26	0.80	5.05
24	$Y_2 = 29.79 + 0.34\ x_1 + 2.81\ x_1^2 - 1.08\ x_2 + 0.96\ x_2^2 - 1.43\ x_1\ x_2$	87.15	8.10	5.05
30	$Y_3 = 32.72 - 0.04\ x_1 + 2.79\ x_1^2 - 0.81\ x_2 + 0.77\ x_2^2 - 0.53\ x_1\ x_2$	68.82	2.60	5.05
48	$Y_4 = 40.29 - 0.31\ x_1 + 1.44\ x_1^2 + 1.77\ x_2 + 0.84\ x_2^2 + 0.28\ x_1\ x_2$	40.63	0.80	5.05
72	$Y_5 = 46.07 + 0.02\ x_1 + 0.95\ x_1^2 - 0.43\ x_2 + 0.40\ x_2^2 - 0.15\ x_1\ x_2$	25.69	0.40	5.05

Table 2. Mathematical models of mechanical refining CRRD for cellulose conversion response as a function of time.

Conclusion

Aiming at reducing biomass recalcitrance, mechanical refining in high biomass consistency was studied. Improvements in enzymatic digestibility of up to 20% were obtained for SETB compared to HPTB, suggesting that steam explosion pretreatment, associated with mechanical refining, may be an interesting alternative for lignocellulosic biorefineries. SEM micrographs showed that mechanical refining increases fiber delamination and exposure of cell wall fibrils, being the fundamental basis for the increase in enzymatic saccharification. In conclusion, this study advances knowledge about using high-consistency mechanical refining in sugarcane biorefineries, complementing the current data available for hardwoods and corn stover.

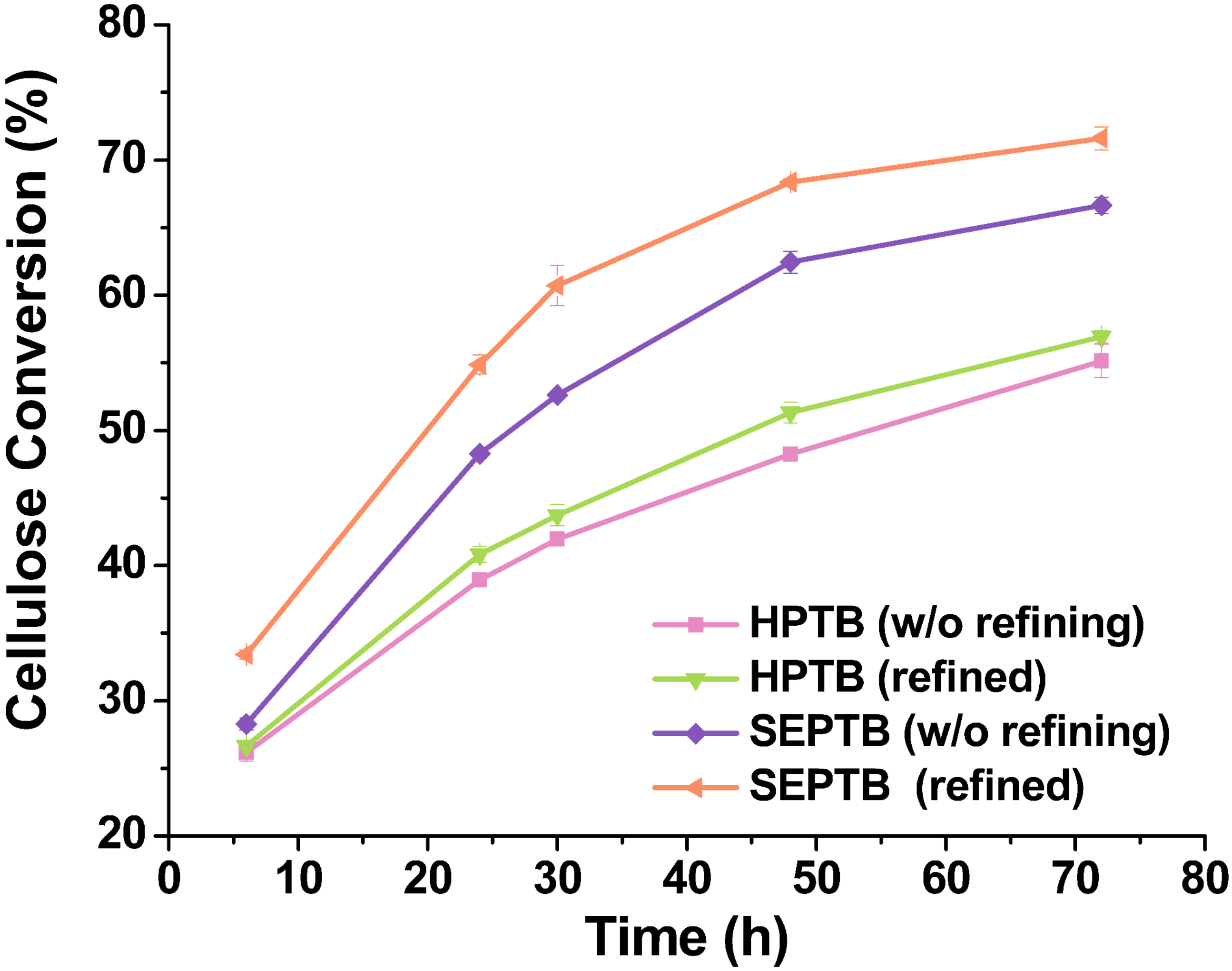

Fig. 3. Enzymatic saccharification kinetics for mechanical refining and pretreated bagasse, HPTB or SEPTB. Saccharification assays were performed with 10% (w/w) solid content and an enzyme cocktail load of 10 FPU. $gPTB^{-1}$.

Experimental procedures

Pretreatment at pilot scale

Two pretreatment conditions were carried out with sugarcane bagasse (SCB) at the Pilot Process Development Plant (PPDP) from Brazilian Biorenewables National Laboratory (LNBR), hydrothermal or steam explosion. Before the pretreatment, SCB was processed in the Dry-Cleaning System from PPDP, where the air flows against the material, promoting its separation from the non-structural inorganics (stones, pieces of iron and steel, and sand). Hydrothermal pretreatment was performed in a 350 L Hastelloy steel 276 batch reactor (Pope Scientific®), stirred by a pitched-blade turbine, using 20 kg (dry basis) of SCB at a solid: liquid ratio of 1:10. The material was added to the reactor when the water temperature was close to 100 °C, taking 15 min to reach 180 °C, with a plateau time of 15 min. After the pretreatment, the liquid fraction was separated using a 100 L Hastelloy steel 276 Nutsche Filter from Pope Scientific®, and the remaining solid fraction (pretreated bagasse) from the eight batches was homogenized and stored in a cold room. Steam explosion pretreatment was performed at a feed rate of 10 $kg.h^{-1}$ and a residence time of 15 min at 190 °C in a stainless-steel continuous reactor from AdvancedBio®, and the resulting pretreated bagasse was homogenized and stored in a cold room. Both pretreated bagasse had their chemical characterization and mass balance of the process determined accordingly to Ref.[24], and the severity parameter (R_0) was calculated using the following Eq. (1)[6]:

$$R_0 = t * exp\frac{(T - 100)}{14.75} \tag{1}$$

Equation 1. Calculation of severity factor. **t** is the pretreatment reaction time (min), and **T** is the reaction temperature (°C).

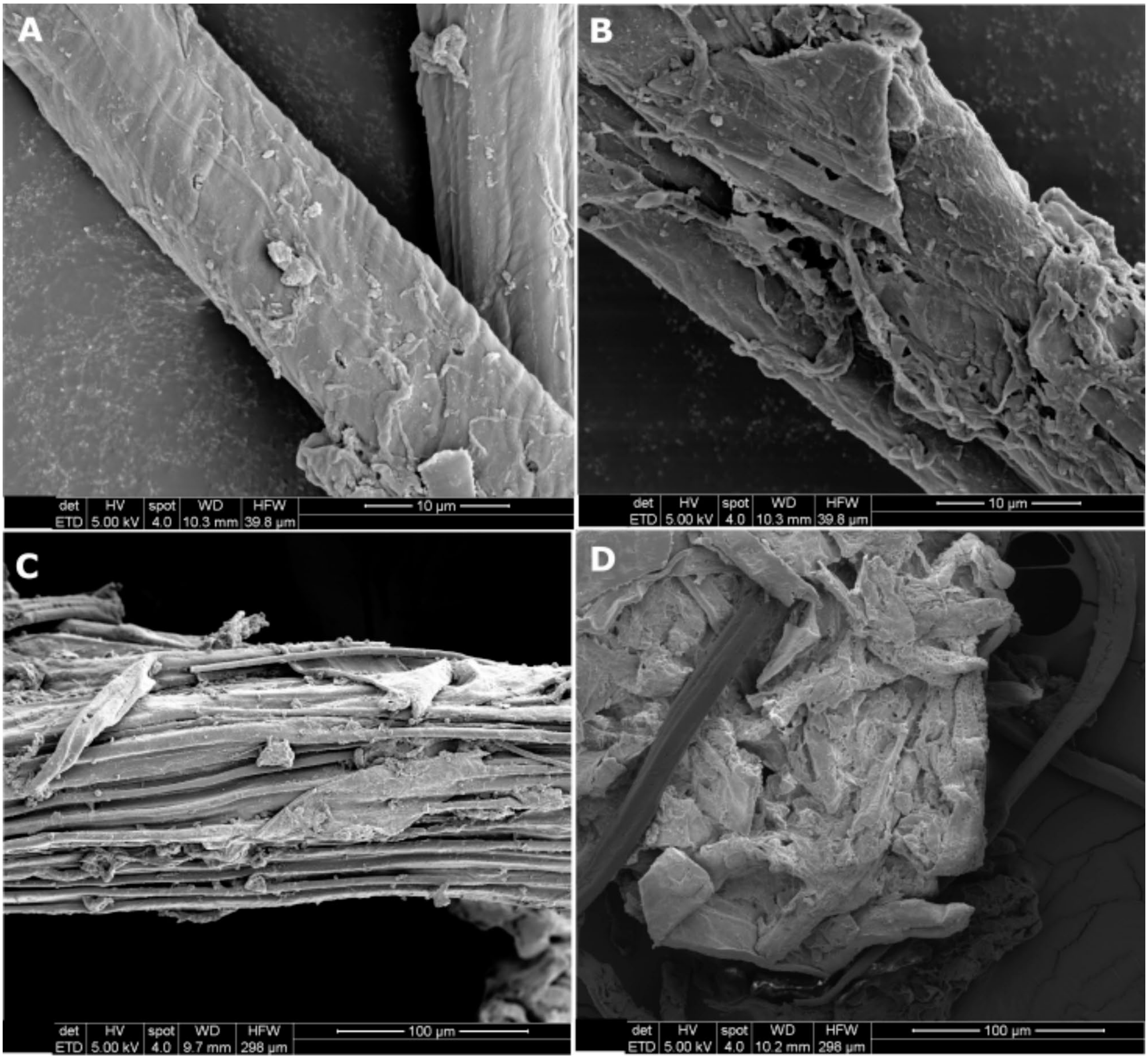

Fig. 4. Scanning electron microscopy of unrefined and refined pretreated sugarcane bagasse. (**A**) HPTB unrefined; (**B**) HPTB refined (1 mm x 3000 RPM); (**C**) SEPTB unrefined; (**D**) SEPTB refined (1 mm x 3000 RPM).

High-consistency mechanical refining at pilot scale

High-consistency mechanical refining was applied on the pretreated bagasse using a 12-in continuous disc refiner model 12–1 C from Andritz®. The disc refiner comprises two vertical disks with a three-zone fine, one rotates while the other remains stationary. The refining effect was evaluated by a Central Composite Rotatable Design – CCRD (2^2 factorial design with 4 trials under the axial conditions and 3 repetitions at the central point) totaling 12 trials (Table 3)[25], and the variables investigated were the gap width (GW) and disc rotation (DR). In the terminology of the tests, RB means "refined bagasse", and the number represents the experiment that was performed as part of the design. The RB9 investigation represents the center point (CP) repeated in four independent assays. For each experimental condition, the GW (from 1 to 2.5 mm) and DR (from 1500 to 3000 RPM) used were adjusted, and 5 kg of pretreated bagasse with a moisturizing average of 65% was fed by a screw at a speed of 20 rpm, and a dilution water flow of 400 mL/min resulted in a high-consistency refined pulp with 15% of solids. The material was collected at the bottom of the refiner. The resulting refined pretreated sugarcane bagasse was homogenized and stored at -10 °C. The chemical composition of the refined samples was assumed to be the same as the pretreated biomass presented in the above section.

The mathematical modeling of the resulting CCRD was carried out based on the function cellulose conversion (%) after the enzymatic saccharification. Data were analyzed via Protimiza Experiment Design Software (http://experimental-design.protimiza.com.br). The effect on the response function of all individual process variables

Experiment	Gap width (mm)	Disc rotation (RPM)
RB1	1.22 (-1)	1718 (-1)
RB2	2.28 (+1)	1718 (-1)
RB3	1.22 (-1)	2782 (+1)
RB4	2.28 (+1)	2782 (+1)
RB5	1 (-1.41)	2250 (0)
RB6	2.5 (+1.41)	2250 (0)
RB7	1.75 (0)	1500 (-1.41)
RB8	1.75 (0)	3000 (+1.41)
RB9 (CP)	1.75 (0)	2250 (0)

Table 3. The central composite rotatable design conditions for investigating the high-consistency mechanical refining.

and their binary interactions was calculated for each selected reaction time during a saccharification time course of 6, 24, 30, 48, and 72 h using the confidence level of 95% ($p = 0.05$).

Scanning electron microscopy

Scanning electron microscopy (SEM) was performed on an INSPECT F50 from a Thermo Fischer Scientific field-emission scanning electron microscope operating at 5 kV. Fiber samples were vacuum-dried before analysis. Samples were analyzed coated with a thin layer of gold (20 nm) in a sputtering BAL-TEC SCD 050.

Enzymatic saccharification of refined pretreated bagasse

Enzymatic saccharification assays were performed with refined pretreated bagasse and the commercially available blend Cellic® CTec2, and cellulase activity (183 FPU/mL) was determined according to Ref.[26]. The tests were performed with 10% of total solids, an enzyme load of 10 FPU.gPTB^{-1}, 50 °C, pH 5 (100 mM sodium citrate buffer solution), and 150 rpm. Over 72 h, aliquots were taken at a pre-determined time to monitor the sugar release. The samples were subjected to glucose analysis using the D-Glucose Assay Kit. Alternatively, the carbohydrates were measured by high-performance liquid chromatography (HPLC). The cellulose conversion was calculated using Eq. (2), and hemicellulose conversion by Eq. (3).

$$\eta = \frac{M_{Glu\cos e} \times f}{M_{Cellulose}} \times 100 \tag{2}$$

Equation (2) Calculation of glucan conversion into monomeric glucose. $\mathbf{M_{Glucose}}$ is the mass of glucose present in the hydrolysate, and $\mathbf{M_{Cellulose}}$ is the initial mass of cellulose present in the pretreated bagasse. *f* is the conversion factor of glucose into cellulose ($f = 162/180 = 0.9$).

$$\eta = \frac{M_{Arabinose+Xylose} \times f}{M_{Hemicellulose}} \times 100 \tag{3}$$

Equation (3) Calculation of xylan conversion into monomeric xylose and arabinose. $\mathbf{M_{Xylose + Arabinose}}$ is the mass of xylose and arabinose present in the hydrolysate, and $\mathbf{M_{Hemicelulose}}$ is the initial mass of hemicellulose present in the pretreated bagasse. *f* is the conversion factor of xylose and arabinose into hemicellulose ($f = 132/150 = 0.88$).

Analysis of carbohydrate, organic acids, and furan concentrations

HPLC was used to quantify glucose, xylose, arabinose, cellobiose, acetic acid, formic acid, succinic acid, and lactic acid present in the enzymatic hydrolysate and chemical composition samples. The chromatographer was the 1260 Infinity from Agilent, equipped with a refractive index detector and an Aminex HPX 87 H column (300 × 7.8 mm) and pre-column (30 × 4.6 mm) at 35ºC with a flow of 0.6 mL.min^{-1} of 5 mM sulfuric acid solution eluted isocratically.

Furfural and hydroxymethylfurfural (HMF) present in the hydrolysates and compositional analysis samples were quantified in the same HPLC equipped with a UV-visible detector at 274 nm, an Acclaim C18 3 µm column (4.6 × 150 mm) at 25 °C with a flow of 0.8 mL.min^{-1} and acetonitrile: water solution (1:8 *v/v*) containing 1% acetic acid.

The samples were homogenized and filtered through a Millex syringe filter with a diameter of 13 mm, 0.22 µm (for enzymatic hydrolysates), and 0.45 µm (for chemical composition samples). Sample quantification was performed by external calibration.

Data availability

The datasets used and/or analyzed during the current study are presented in the source data file and/or are available from the corresponding author upon reasonable request.

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Author contributions

LPA, GJMR, and MTM conceived and designed the study; LPA collected and analyzed the data, and drafted the manuscript; MIR contributed to the data analysis and interpretation. All authors reviewed and approved the final manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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