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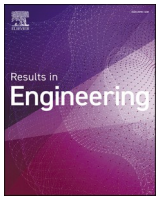

Research paper

Enhanced upstream processing of rice straw by an integrated alkaline pretreatment and enzymatic saccharification

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ABSTRACT

The efficient conversion of lignocellulosic biomass into platform chemicals through enzymatic hydrolysis is often hindered by biomass recalcitrance and limited enzyme accessibility to cellulose. While several studies have addressed individual aspects of rice straw valorization, such as pretreatment, enzymatic hydrolysis, or hydrolysate fermentation, there remains a need for comprehensive research that systematically links pretreatment efficiency, enzyme economy, rapid process kinetics, and mechanistic understanding. Here, we present a systematic, integrated study combining mild NaOH pretreatment of rice straw (1 % NaOH, 73 °C, 4.5 h) with a deliberate pursuing of minimal enzyme dosage, achieving rapid and highly efficient saccharification. Key process variables, pretreatment conditions, enzyme dosage, and processing time, were thoroughly investigated to identify a suitable operation window that balances mild pretreatment with low enzyme input (as low as 6.5 mg protein/g solid, ~4.4 FPU/g), enabling up to 90 % glucose yield within just 8 h—using 50–80 % less enzyme and a much shorter processing time than those commonly reported. Importantly, the study also addresses black liquor reuse, demonstrating pretreatment scale-up and translation to increased solid concentrations (7.5 % w/w DS –dry solid-). Comprehensive kinetic, compositional, and structural analyses—including XRD, SEM, confocal fluorescence microscopy, and enzyme adsorption assays—were integrated to provide new insights into the relationship between substrate modification, enzyme accessibility, and saccharification efficiency.

1. Introduction

Lignocellulosic biomass is an ideal feedstock for biorefineries because it is renewable, abundant, easily accessible, cost-effective and diverse in origin, structure and composition [1–3]. Among these feedstocks, rice straw has an enormous potential for the production of biofuels and biomass-based chemicals due to its high abundance and its composition [4], considering its annual production (800–1000 Mtons). However, its traditional disposal through burning contributes to greenhouse gas emissions. In contrast, optimized upstream processing in biorefineries can convert rice straw into simple sugars and platform chemicals, supporting a broad range of catalytic and fermentative transformations while minimizing environmental impacts.

Rice straw is primarily composed of cellulose (approximately 40 %), hemicellulose (about 20 %), lignin (about 20 %) and ashes (10–15 %, being silica 75 % of this mass) [5]. The complex structure of lignin, hemicellulose and silica contributes to the recalcitrance of rice straw by protecting the cellulose microfibrils, which hinders enzymatic access [6,7]. Thus, an effective pretreatment is crucial to disrupt this structure and enhance the enzymatic saccharification of cellulose by removing ash, lignin and hemicelluloses [8,9]. When selecting a pretreatment method, several key factors affect capital and operating costs including the chemical and energy inputs, waste generation, and the formation of inhibitory compounds. A broad range of pretreatment strategies,

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including physical, chemical, and biological methods, have been explored to improve cellulose accessibility [7,10,11]. High-temperature methods (e.g., autohydrolysis, steam explosion, AFEX) are effective but often require substantial energy and may generate inhibitors that difficult further processing. In contrast, alkaline pretreatment is particularly effective, as it disrupts the lignocellulosic structure by cleaving ester bonds between lignin and hemicellulose, breaking down the crystalline structure of cellulose, and removing lignin and ash, and minimizing the formation of fermentation inhibitors [12–18]. Sodium hydroxide (NaOH) is also favoured due its low cost (US$0.36/Kg as of October 2024 in Europe) and high efficacy [5,19–26].

Recent studies have studied alkaline pretreatment conditions to maximize cellulose conversion, typically using moderate-to-high NaOH concentrations (0.5–12 %), elevated temperatures (80–140 °C), and extended hydrolysis times (24–96 h), often with relatively high enzyme loadings of cellulase cocktails (10–35 FPU/g solid) [23,25,27–31], sometimes supplemented with other β-glucosidase cocktails (30 Uβ/g solid) [5,24,32,33]. These efforts have resulted in considerable improvements in glucose yield (up to 85–90 %), but generally require significant chemical and enzyme inputs, as well as long processing times. Furthermore, many of these studies focus on a single aspect, such as pretreatment optimization or enzyme formulation, without integrating mechanistic or structural analysis, hydrolysis kinetics, process sustainability (e.g., black liquor reuse), or translation to higher solid loadings and process scale-up. For instance, [31] reported 64 % glucose yield from rice straw pretreated with 2 % NaOH (80 °C, 3 h) and hydrolyzed for 72 h at 15 FPU/g, emphasizing lignin valorization but relying on higher enzyme dosages and longer times. Moreira et al. (2024) [28] similarly achieved high sugar yields but at 10–20 FPU/g and with process times of 48–72 h, while rarely evaluating alkali recycling, enzyme adsorption, or rapid kinetics. Prajapati & Kango (2022) [34] evaluated liquor reuse after 2 % NaOH pretreatment (121 °C, 30 min), obtaining 60–85 % conversions that declined upon recycling, but without monitoring hydrolysis progress or exploring time reduction, using only endpoint yields at 24 h with 10 FPU/g. In a more severe condition, Sophonputtanaphoca et al., (2017) [29] employed 5 % NaOH at 70 °C for 3 h, achieving 82.7 % glucose yield after 24 h hydrolysis with 20 FPU/g and 5 % solids loading.

The recent literature also indicates that studies with lower enzyme dosages or milder pretreatment conditions often report much lower glucose yields but hydrolysis kinetics are not analyzed in detail. Thus, recent literature demonstrates significant progress in improving cellulose accessibility through NaOH-based pretreatment, but several process and sustainability challenges persist. One aspect is to find the correct severity of the pretreatment. In this study, “mild pretreatment” is defined quantitatively as the use of NaOH concentrations below 2 % (w/v), temperatures below 80 °C, and pretreatment durations <6 h. These parameters were chosen to minimize energy and chemical input while maximizing cellulose accessibility and maintaining process sustainability.

Although the potential of NaOH pretreatment to enhance enzymatic digestibility has been demonstrate, enzyme economy is typically overlooked in practical applications where compositional, structural modifications face efficient enzymatic hydrolysis [35]. Thus, beyond compositional changes, effective enzymatic depolymerization of lignocellulosic biomass relies on additional factors like degree of polymerization, crystallinity index (CrI), substrate surface area, and porosity [36]. For example, NaOH-pretreated rice straw often exhibits a higher CrI due to the removal of amorphous lignin, hemicellulose, and non-crystalline cellulose, which influences enzyme accessibility [25]. While increased surface area can enhance initial hydrolysis rates, it alone cannot fully address time-dependent delays in hydrolysis [37,38]. Moreover, enzyme costs and limited recyclability pose significant challenges, as biomass heterogeneity and specific endoglucanase and exoglucanase interactions complicate enzyme recovery [39,40]. Moreover, few comparative studies have included mechanistic evaluations such as enzyme adsorption, structural transformation, or compositional analysis linked directly to process performance. To improve economic feasibility, it is crucial to reduce enzyme loading while retaining efficient hydrolysis, necessitating an integrated approach that study compositional and structural changes and considers both pretreatment conditions (NaOH concentration, temperature, and pretreatment duration) and enzyme dosage [41].

Addressing these gaps, our study presents a comprehensive, integrated evaluation of rice straw saccharification under mild alkaline pretreatment conditions combined with deliberate enzyme economy achieving rapid and highly efficient glucose yields. We systematically examine key process variables (NaOH concentration, temperature, pretreatment time, enzyme dosage), aim at low chemical and enzyme input, and thoroughly characterize substrate composition and structure (XRD, SEM, FTIR, enzyme adsorption). We also evaluate black liquor reuse and scaling up to higher solid concentrations as a proof of concept. By integrating compositional, structural, kinetic, and sustainability analyses, our work offers novel insight and a practical framework for intensified, cost-effective biorefinery operations using rice straw as a model substrate.

2. Materials and methods

2.1. Materials

Rice straw was kindly donated by a local agricultural company “Cooperativas Agro-alimentarias de Andalucía” (Seville, Spain). The material was air-dried for 24 h and ground to particle size of approximately 1 mm. Two commercial enzyme cocktails (Biogazyme 2X and β-glucosidase 1000) kindly donated by ASA Spezialenzyme were used in the enzymatic hydrolysis.

A chimera with four substrate bindings domains (CBD) labelled with green fluorescent protein (GFP) was used in the adsorption study. Its molecular weight is 146.25 kDa and it was provided by the research group "Microbial Diversity and Microbiology of Extreme Environments" (IRNAS-CSIC, Seville) as a protein solution with 1.8 mg protein/mL. NaOH, H_2SO_4 and HCl were purchased from Scharlau. Sodium citrate, 3,5-dinitrosalycilic acid (DNSA), d-(+)-galactose, l-rhamnose, l-(+)-arabinose, Avicel pH-101, phenol, Bovine Serum Albumin (BSA) and d-(+)-galacturonic acid monohydrate were supplied by Sigma-Aldrich. d-(+)-glucose and 4-nitrophenyl-β-d-glucopyranoside (pNPG) were from Acros Organics. d-(+)-cellobiose and carboxymethylcellulose sodium salt were from Fluka. d-(+)-xylose, sodium potassium tartrate were from Panreac. Bio-Rad Protein Assay Dye Reagent concentrate solution was purchased to Bio-Rad.

2.2. Alkaline pretreatment

Five grams of rice straw (dry weight) were mixed with 200 mL NaOH solution in a 250 mL round-bottom flask, resulting in a 2.5 % w/v concentration of dry solids (hereafter referred to as DS concentration). The flask was placed in a heating mantle with integrated magnetic stirring (350 rpm) and equipped with a reflux condenser. Different concentrations of NaOH (0.025–10 %), pretreatment time (1–6 h) and temperature (50–95 °C) were used to study the effect of these variables on solid chemical composition and enzymatic hydrolysis. After pretreatment, the suspension was centrifuged (4–16 K Centrifuge, Sigma) at 9000 rpm for 20 min. The liquid (black liquor) was stored until further use and the solid phase was washed several times with distilled water. This pretreated solid was dried at 60 °C in an oven for at least 12 h, stored in a desiccator at room temperature until further use. All pretreatment experiments were performed in duplicate.

For the liquor reuse, the black liquor resulting from the centrifugation of each pretreatment mixture was used for a new pretreatment of the fresh rice straw, without any additional treatment: no fresh sodium hydroxide was added to recover the original pH. In case of liquid losses

due to centrifugation or retention in the pretreated adsorbed solid before washing, the amount of fresh rice straw was corrected in the next pretreatment, so that it was operated at 25 g DS/L. This was done for the required reuse cycles. For scale-up experiments, the pretreatment scale up was carried out in 1 and 2 L bioreactors with mechanical agitation (IKA RW 20) at 350 rpm and a heating jacket. Reflux condensers minimized evaporation during pretreatment, using as working volume 80 % of the total reactor volume. The final suspension was filtered through a 0.2 mm mesh, being the solid washed with distilled water and dried as performed in the smaller scale process.

2.3. Enzyme activity assays

The activity of total cellulase was measured using the filter paper assay (FPA) according to the NREL standard method [42]. The protein content was measured by the Bradford method [43]. β-glucosidase activity was measured using p-NPG as substrate [44]. Endoglucanase activity was measured using CMCase assay [45].

2.4. Enzymatic hydrolysis

Enzymatic hydrolysis was performed in a 50 mL Falcon tubes, with a working volume of 30 mL and a solid loading of 2.4 % w/v (dry weight). Enzyme dosage was varied in a low enzyme range (6.5–21.9 mg/g solid; 4.4–19.4 FPU/g solid) far below from typical ranges reported in literature (10–60 mg/g solid). The reaction was carried out at several enzyme loadings in a 50 mM sodium citrate buffer pH 5.0, 50 °C and 60 rpm in a roller shaker (Movil-Rod, Selecta) placed in an incubator for temperature control. Samples of 0.5 mL were taken at different times and boiled for 20 min to stop the reaction. These samples were centrifuged and the resulting liquid was diluted with water and centrifuged again to remove impurities and filtered through 0.22 μm for subsequent HPLC analysis. All runs were performed in duplicate with its substrate and enzyme control in the reaction conditions. Cellulose conversion (X), which is, in this case, identical to glucose yield (Y_G), was defined as:

$$X = Y_G = \frac{C_C\left(\frac{g}{L}\right)}{C_{C0}\left(\frac{g}{L}\right)} = \frac{C_G\left(\frac{g_{glucose}}{L}\right)\cdot 0.9\left(\frac{g_{cellulose}}{g_{glucose}}\right)}{Cellulose\,composition\left(\frac{g_{cellulose}}{g_{DS}}\right)\cdot DS\,concentration\left(\frac{g_{DS}}{L}\right)} \quad (1)$$

Where C_C is the concentration of cellulose consumed, C_{C0} the initial concentration of cellulose, C_G is the concentration of glucose produced, 0.9 is the conversion factor, and *Cellulose composition* is the amount of cellulose content in the pretreated or untreated solid. Cellulose conversion (X) was defined as the maximum amount of glucose obtained, since the enzyme cocktails used do not accumulate reaction intermediates such as cellobiose. Therefore, the cellulose conversion coincides with the glucose yield (Y_G).

2.5. Adsorption study

A small solid mass (24 mg dry weight) was suspended in 1 mL of GFP solution (0.18 mg protein/mL) in a 50 mM sodium citrate buffer pH 5.0. The solids were incubated at room temperature for 24 h under constant gentle stirring (20 rpm) in an end-over-end rotator and subsequently centrifuged. The supernatant was extracted and residual protein was quantified by measuring the fluorescence using a spectrofluorometer FP-6500 Jasco, with an excitation wavelength of 490 nm and an emission wavelength of 510 nm. The solids were analysed by confocal fluorescence microscopy.

2.6. Analytical methods and data analysis

The moisture content of the solids was determined by protocol NREL/TP-510–42621 employed a thermogravimetric balance. The chemical composition of raw and pretreated rice straw was determined according to the technical report NREL/TP-510–42619 by the National Renewable Energy Laboratory [46–49]. Sugar concentrations (cellobiose, glucose, xylose, arabinose, galactose, rhamnose and galacturonic acid) were measured with a JASCO (LC-2000 series) HPLC containing a column Benson BP-800 H (300 mm × 7.88 mm), a refraction index detector (RID) RI-2031, a pump PU-2089, an autosampler AS-2059, and a column oven CO-2060. The column was kept at 60 °C, RID detector at 40 °C, and the mobile phase was aqueous H_2SO_4 5 mM flowing at a 0.5 mL /min rate.

Solid samples of pretreated and untreated rice straw as control were analysed by FTIR-ATR (Fourier-Transform Infrared- Attenuated Total Reflection spectroscopy). PerkinElmer Spectrum 100 FT-IR Spectrometer (Perkin Elmer, Massachusetts, United States) equipped with a Universal ATR Sampling accessory was used. The scanning region ranges from 800 cm^{-1} to 4000 cm^{-1} with a total of 4 scans per sample and a resolution of 4 cm^{-1}.

X-ray diffraction (XRD) analysis allowed to obtain the crystallinity index of cellulose (CrI). Samples were analysed by PANanalytical powder diffractometer model X'Pert MPD equipment (Malvern PANanalytical, Malvern, United Kingdom). The analysis was carried out with Cu/kα radiation with $l = 1.54$ Å with a scan range from 4.99 2θ to 40.00 2θ.

The crystallinity index (CrI) was calculated (Eq. (2)) according to the peak height method [50]. The peak I_{002} ($2\theta = 22$) corresponds to the signal of cellulose crystals whereas peak I_{AM} ($2\theta = 18$) corresponds to amorphous cellulose [51].

$$CrI\ (\%) = \frac{I_{002} - I_{AM}}{I_{002}}\cdot 100 \quad (2)$$

It was also calculated by the difference of areas using the method of subtraction of the amorphous crystalline part [50].

Initial enzyme reaction rates were calculated from the initial concentration-time data using finite increments of reaction time/substrate concentration. The morphological alterations caused by alkaline pretreatment were observed by Scanning ElectronMicroscopy with Energy Dispersion Spectroscopy (SEM-EDS). The equipment used was JEOL JSM 6335F microscope (JEOL Ltd., Tokyo, Japan). Several high-resolution images were obtained in the range 20–2000x.

Confocal fluorescence microscopy was performed on a Leica TCS SP8 laser scanning microscope (Leica Microsystems) equipped with 4 fluorescence detectors, two of them high sensitivity HyD detectors, and 4 lasers (405, 488, 552, 638). Confocal images were processed with LAS X Office software (Leica). All images were modified from an intensity of 255 to 100 (on the green scale) so that they could be viewed clearly and compared.

Data analysis of rice straw samples (XRD, FTIR-ATR, CrI and initial rate) were prepared by OriginLab2024 software.

3. Results and discussion

3.1. Influence of the pretreatment in hydrolysis

The first step involved selecting and characterizing suitable enzyme cocktails, combining them to achieve a balanced mixture of depolymerizing enzymes acting on cellulose and residual hemicellulose—such as glucanases and xylanases—alongside accessory enzymes like β-glucosidase and xylosidase, which convert oligomeric intermediates into final monosaccharide products. This strategy prevents the accumulation of oligomers and enhances the final glucose yield [52]. These cocktails were analysed, showing a protein content of 72 mg protein/g preparation for Biogazyme 2X and 84.6 mg protein/mL preparation for β-glucosidase 1000. The quantification of cellulase activities (Table 1) revealed that Biogazyme 2X was 33 % more effective in total cellulose

Table 1
Different cellulase activities of enzyme cocktails.

Cellulase activity	Biogazyme 2X	β-glucosidase 1000
Total cellulase (FPU/$mg_{protein}$)	0.97	0.62
β−1,4-glucosidase (U/$mg_{protein}$)	1.99	15.20
Endoglucanase (IU/$mg_{protein}$)	65.34	48.86

degradation compared to β-glucosidase 1000, showing a total cellulase activity of 0.97 FPU/mg protein versus 0.62 FPU/mg protein, respectively. According to supplier specifications, β-glucosidase 1000 exhibited 7.6 times higher β-glucosidase activity (15.20 U/mg protein) compared to Biogazyme 2X (1.99 U/mg protein), while Biogazyme 2X demonstrated 25 % higher endoglucanase activity, with 65.34 IU/mg protein compared to 48.86 IU/mg protein in β-glucosidase 1000, as expected for enzyme preparations from *Aspergillus fumigatus* [53] and *Trichoderma reesei* [54]. With these enzyme cocktails, we aimed to address the recalcitrance of rice straw to enzymatic saccharification [55].

As a reference, the enzymatic hydrolysis of untreated rice straw was performed using an enzyme loading of approximately 20 FPU/g dry solid –FPU/g DS (15.96 FPU/g DS Biogazyme 2X plus 3.36 FPU/g DS β-glucosidase 1000) and a reaction time of 72 h, conditions commonly reported in the literature for biomass hydrolysis [22,26,27,32,56–58]. Fig. 1.A shows that a 23 % maximum glucose yield was achieved at about 16 h, time at which the enzymatic hydrolysis stopped. This limitation is likely due to low enzyme accessibility [59].

Next, an intensive pretreatment was applied to the rice straw, followed by enzymatic hydrolysis, to ensure that enzymes act on a highly reactive solid substrate. The high-temperature, high-NaOH concentration process allowed to reach nearly 100 % glucose yield, while a progressive reduction in enzyme concentration, ranging from 8.4 to 1.05 FPU/g DS, was applied to consider economic feasibility issues due to the difficult enzyme recovery (Fig. 1.A) [60,61]. Sampling times were reduced between 24 and 6 h to identify a sensitive reaction zone, achieving approximately 75 % glucose yield in 6 h and 99 % in 16 h. Since the glucose yield difference between 4.2 and 1.05 FPU/g DS was not significant, 1.05 FPU/g DS was chosen, resulting in a significant initial reaction rate boost compared to untreated rice straw. Furthermore, saccharification runs at several rotating speeds were performed to ensure the absence of external mass transfer limitations (see Figure S1). Once a sufficient mixing was provided, further runs not affected by external mass transfer provided unbiased insights into the pretreatment efficiency and the subsequent enzymatic reaction

As the next step, different pretreatment parameters were systematically evaluated with low-enzyme saccharification tests to determine the most suitable and mildest conditions that enhance enzymatic hydrolysis while minimizing enzyme usage [62,63]. With that aim, we first employed a mild temperature (50 °C) and a low NaOH concentration range (0.025–1 % w/v) for 1 hour pretreatment time (Fig. 1.B). NaOH concentrations of only 0.1 % w/v resulted in similar performance to untreated solid, with a slight increase in glucose yield from 0.23 to 0.29. However, concentrations above 0.5 % NaOH significantly improved reactivity, with glucose yields about 72 % after 9 h (Fig. 1.B).

These results indicate that there is a minimum NaOH concentration (0.5 % at 50 °C) that is necessary to achieve effective reactivity in rice straw. Compositional and structural analysis (shown below) will provide experimental evidence about the root of this effect. For higher reactivity, a NaOH concentration of 10 % at 95 °C yields a solid with significantly improved reactivity (Table 2, S13). With a low enzyme loading of 1.05 FPU/g DS of Biogazyme 2X and 3.38 FPU/g DS of β-glucosidase 1000, it is feasible to compare the different pretreatments. Notably, our findings provide a practical threshold for the minimal pretreatment severity and enzyme dosage required for efficient saccharification—offering a new benchmark for process efficiency. In contrast to most previous studies, which have relied on harsher pretreatment conditions and higher enzyme dosages to reach comparable yields, our results demonstrate that high glucose yields can be achieved more sustainably and economically and it the basis of further refinement.

3.2. *Fine-tuning of the pretreatment conditions: impact on enzymatic hydrolysis and chemical composition*

We further examined how various pretreatment conditions affect rice straw proximal composition and their relationship with the enzymatic hydrolysis performance [21]. To better understand and improve the pretreatment process, we fine-tuned key variables, including NaOH concentration, temperature, and process time, with the goal of maximizing enzymatic hydrolysis efficiency [64]. Table 2 and Fig. 2 illustrate the impact of various pretreatment conditions on the rice straw composition and hydrolysis rates, Table S1 shows the relative removal percentage of every component.

The proximal composition of untreated rice straw (URS) included 28.56 ± 0.4 % cellulose, 17.28 ± 0.45 % hemicellulose, 19.81 ± 0.46 % lignin, 21.78 ± 0.63 % water extractives, 2.96 ± 0.35 % ethanol extractives, and 10.5 ± 0.5 % ash. When extractives were excluded (Table 2), the composition was 38.94 ± 0.53 % cellulose, 23.56 ± 0.6 % hemicellulose, 27 ± 0.61 % lignin, and 10.5 ± 0.5 % ash, which aligns with prior studies [62,65,66]. The intensive pretreatment at 95 °C and

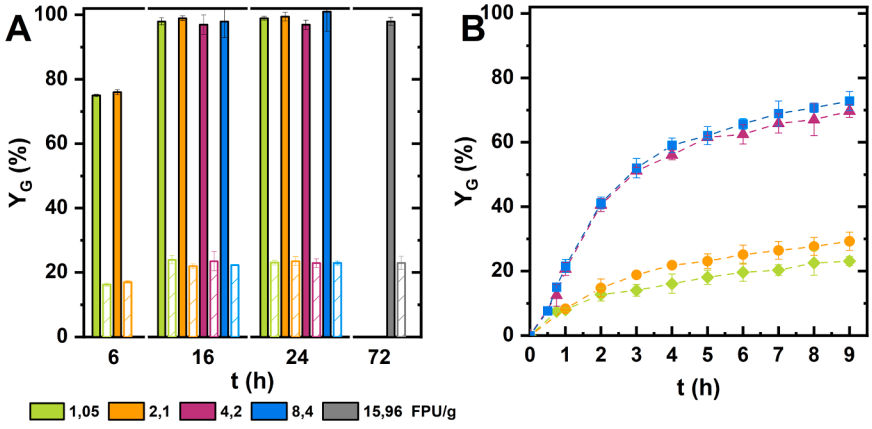

Fig. 1. Evolution of glucose yield (%). A) Effect of enzyme concentration on enzymatic hydrolysis of untreated rice straw ( ) and pretreated rice straw (S13,) using a β-glucosidase 1000 enzyme load of 3.38 FPU/g DS and different Biogazyme 2X loadings (1.05–15.96 FPU/g DS). B) Effect of minimum pretreatment conditions (50 °C and 1 h) on enzymatic hydrolysis for 0.025 % (w/v) NaOH (S1,), 0.1 % (w/v) NaOH (S2,), 0.5 % (w/v) NaOH (S3,) and 1 (w/v) % NaOH (S4,) using a 1.05 FPU/g DS of Biogazyme 2X and 3.38 FPU/g DS of β-glucosidase 1000.

Table 2
Chemical composition the untreated rice straw (free extractives) and rice straw pretreated under different conditions.

Solid	Pretreatment conditions			Chemical composition (NREL) (%)			
	T (°C)	C_{NaOH} (%w/v)	t (h)	Cellulose	Hemicellulose	Lignin	Ash
URS	–	–	–	38.94 ± 0.53	23.56 ± 0.60	27.00 ± 0.61	10.50 ± 0.50
S1	50	0.025	1	39.25 ± 0.11	25.15 ± 1.45	26.60 ± 1.76	9.00 ± 0.40
S2	50	0.1	1	42.21 ± 0.56	26.68 ± 1.55	27.00 ± 0.34	7.37 ± 0.03
S3	50	0.5	1	46.41 ± 1.06	27.61 ± 0.88	20.97 ± 3.12	5.00 ± 0.30
S4	50	1	1	50.01 ± 0.4	24.96 ± 2.96	20.23 ± 1.13	4.80 ± 0.05
S5	50	10	1	67.24 ± 0.1	15.14 ± 0.02	12.82 ± 1.94	4.80 ± 0.55
S6	73	1	1	59.65 ± 0.26	24.55 ± 0.11	11.4 ± 0.02	4.40 ± 0.19
S7	73	1	3	59.21 ± 0.26	24.63 ± 0.12	12.14 ± 0.31	4.02 ± 0.28
S8	73	1	4.5	60.67 ± 0.77	23.44 ± 1.34	11.89 ± 0.46	4.00 ± 0.28
S9	73	1	6	62.89 ± 0.28	23.81 ± 0.11	9.50 ± 0.70	3.80 ± 0.38
S10	73	3	1	68.61 ± 0.62	14.69 ± 1.04	11.90 ± 1.67	4.80 ± 0.40
S11	73	5.5	1	69.79 ± 0.10	16.01 ± 0.02	9.60 ± 0.78	4.60 ± 0.64
S12	73	10	1	74.03 ± 0.14	10.27 ± 0.20	10.90 ± 0.55	4.80 ± 0.01
S13	95	10	1	80.85 ± 0.24	5.13 ± 0.84	9.42 ± 0.59	4.60 ± 0.37

10 % NaOH led to significant changes cellulose content increased to 80.85 %, while hemicellulose and lignin decreased to 5.13 % and 9.42 %, respectively (Table 2, S2 and S13; Table S1). In contrast, mild pretreatment (0.025 or 0.1 % NaOH) resulted in minimal compositional changes for example at 0.1 % NaOH (Table 2, S1 and S2) cellulose increased by only 3 % though ash content dropped by 14 % and 30 %. Glucose production yields were very similar to those of the untreated waste (Fig. 1.A).

In contrast, at 0.5 % - 1 % NaOH (Table 2, S3 and S4), lignin and ash content significantly decreased by about 22 % and 50 %, respectively, while cellulose content increased by 19 % and 28.5 %. These changes resulted in an accelerated hydrolysis rate and increased glucose yield (Fig. 1.B). These results highlight the importance of achieving an optimal balance between pretreatment severity and low enzyme use. At higher NaOH concentrations, lignin and hemicellulose are effectively removed, thereby improving substrate accessibility for enzymatic hydrolysis by reducing lignin and hemicellulose, key to overcoming biomass recalcitrance. Lignin reduction decreases structural rigidity, and hemicellulose removal enhances substrate reactivity, both contributing to more efficient saccharification, while more severe conditions (e.g., 10 % NaOH at 95 °C) are highly effective at achieving near-complete delignification.

Achieving a suitable balance between pretreatment severity and efficient glucose production, and understanding its relationship with compositional changes, requires a more detailed analysis. Ash solubilization showed minimal variation—ranging between 4.4 % and 5 %—regardless of changes in temperature and NaOH concentration, although it was reduced by up to 50 % under the mildest pretreatment conditions (S3). Increasing pretreatment time had only a minor impact, with a maximum variation of 14 %. In contrast, lignin and hemicellulose removal, as well as their influence on enzymatic hydrolysis, exhibited more complex and varied behaviors. Lignin content remained relatively stable with varying NaOH concentrations at mild temperature. Elevated temperatures (73 °C and 95 °C) in combination with higher NaOH concentrations (10 %) led to substantial lignin removal (~55–65 %), while hemicellulose content was significantly reduced, especially at 73 °C and higher NaOH concentrations (Table 2, S10). Higher NaOH concentrations (3 % or more) resulted in improved cellulose content (~68 %) and a significant reduction in hemicellulose (~15 %).

To isolate temperature effects, we used a NaOH concentration of 10 % (S5, S12, and S13) in the range of (50–95 °C). The cellulose content varied between 67 % and 80 %, and hemicellulose content ranged from 15 % to 5 %. Although changes in pretreatment temperature affected the cellulose composition of the solids, the impact on enzymatic hydrolysis was less pronounced. At 50 °C, 73 °C, and 95 °C, glucose yields reached 72 % after 6 h. However, at 95 °C, the glucose yield increased to 93 % within 12 h, while at lower temperatures (50 °C and 73 °C) glucose yields plateaued at around 83 %. This enhanced glucose yield at higher temperatures likely results from reduced hemicellulose content and a higher proportion of amorphous cellulose, which improves substrate

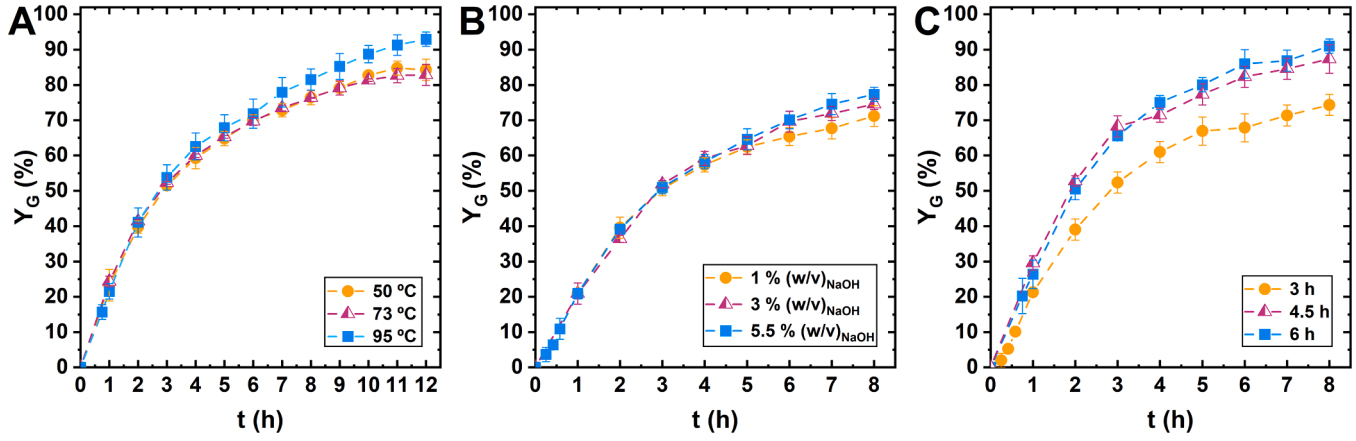

Fig. 2. Evolution of glucose yield (2.4 % solid loading, 1.05 FPU/g DS of Biogazyme 2X and 3.38 FPU/g DS of β-glucosidase 1000) with the effect of different pretreatment conditions. A) Effect of temperature (10 % (w/v) NaOH and 1 hour): 50 °C (S5, ●), 73 °C (S12, ▲), 95 °C (S13, ■). B) Effect of NaOH concentration (73 °C and 1 h): 1 % (w/v) NaOH (S6, ●), 3 % (w/v) NaOH (S10, ▲), 5.5 % (w/v) NaOH (S11, ■). C) Effect of pretreatment time (73 °C and 1 % (w/v) NaOH): 3 h (S7, ●), 4.5 h (S8, ▲), 6 h (S9, ■).

accessibility for enzymatic saccharification enzymes [22].

Since 73 °C was sufficient for effective treatment under not-limiting conditions of NaOH, we next analysed the hydrolysis rate with varying NaOH concentrations (1–5.5 %) (S6, S10, and S11). Hydrolysis rates were similar for up to 5 h across all treatments, with ~64 % glucose yield (Fig. 2.B). At 1 % NaOH, the reaction slowed afterward, reaching 71 % glucose yield at 8 h—comparable to results at 50 °C (Fig. 1.B) [21,67,68]. In contrast, higher NaOH concentrations (3 % and 5.5 %) yielded reactivity comparable to that at 10 % NaOH, with glucose yields of 78 % in 8 h. Below 3 % NaOH, hemicellulose content remained at 25 %, similar to untreated straw, while cellulose content was 60 %. In contrast, concentrations above 3 % NaOH reduced hemicellulose to about 15 % and increased cellulose to approximately 68 %. Interestingly, pretreatment with 10 % NaOH at 50 °C gave similar results to 3 % NaOH at 73 °C, suggesting that higher temperature compensates for lower NaOH concentration. Given the environmental and economic burden of using 10 % NaOH [2,68], milder conditions at higher temperature are more sustainable.

Since one of our main motivations was to explore conditions for an environmentally friendly pretreatment, we studied whether better results could be obtained with an even lower NaOH concentration (1 %) but increasing the contact time to between 1 and 6 h. Mild pretreatments usually require longer contact times to achieve higher solubility of hemicelluloses, lignin, or ashes [2]. While solid composition remained largely unchanged, longer pretreatment enhanced reactivity, enzymatic hydrolysis results showed that increased pretreatment time made the solid more reactive and accessible, sharply increasing the reaction rate (Fig. 2.C). At 3 h, hydrolysis was still slow, with 74 % glucose yield at 8 h. However, a further increase of pretreatment time to 4.5 h dramatically improved performance: 68 % glucose yield was reached within 3 h, and 88–91 % in 8 h. Minimal differences between 4.5 and 6 h of pretreatment time were observed. This shows that long enough contact times enable high efficiency despite milder chemical conditions and lower cellulose enrichment, making pretreatment time a key variable for improving saccharification. These results exemplify how a comprehensive, integrated approach, combining detailed compositional analysis, systematic variation of pretreatment variables, and direct measurement of enzymatic hydrolysis kinetics, can identify process conditions that simultaneously enhance both substrate reactivity and enzyme economy. Specifically, our workflow enabled the localization of a mild pretreatment regime (1 % NaOH, 73 °C, 4.5 h) where the resulting solid is particularly amenable to low enzyme dosages and rapid saccharification. This stands in contrast to many prior studies, which have generally evaluated pretreatment or hydrolysis optimization in isolation, often leading to unnecessarily high enzyme consumption or severe pretreatment (e.g., Wang et al., 2025; Moreira et al., 2024; Kaur & Kuhad, 2019) [27,28,31].

3.3. Influence of pretreatment on initial hydrolysis kinetic

As we identified mild pretreatment conditions and low enzyme loadings resulting in a suitable balance of significant compositional changes and efficient cellulose saccharification, we subsequently explored the initial apparent substrate reactivity, before the onset of slow-down factors [37]. This approach is particularly valuable for benchmarking different pretreatment strategies, as it highlights how efficiently substrate reactivity is enhanced following various treatments [69,70]. After calculating the apparent initial velocity when using the untreated residue or with a low concentration of NaOH (see material supplementary, Figure S2), this was constant and approximately 0.15 h^{-1}. However, at higher NaOH concentration, the solid starts to become more reactive, doubling the initial rate. Once a minimum NaOH concentration and temperature condition is reached (0.5 % NaOH at 50 °C), hardly any differences are observed among the different solids subjected to a short pretreatment, all being in the range of 0.3 h^{-1}. The most noticeable difference was observed with a higher pretreatment time: at 3 h a high glucose yield (82 % at 8 h) was achieved, but at 4.5 and 6 h, the initial saccharification velocity incremented by 50 %, obtaining velocities of 0.45 h^{-1}, and a better yield at 8 h (95–96 %), with an 82 % glucose yield at 4 h saccharification. This confirms the hypothesis that pretreatment time is a key parameter in the alkaline pretreatment of rice straw to improve cellulose reactivity, even when composition remained largely unchanged, which was initially surprising. By systematically quantifying both composition and initial hydrolysis rates across pretreatment conditions, our work provides mechanistic insight into how even subtle changes in pretreatment duration can yield major improvements in hydrolysis efficiency. Few studies to date have simultaneously addressed this coupling of process variables at mild pretreatment severities and low enzyme dosages [71].

3.4. Structural characterization of pretreated solids: insights into structural changes

To validate our previous findings and provide additional evidence explaining final glucose yield, we conducted a structural characterization to deepen our understanding of the relationship between pretreatment conditions and substrate reactivity.

The IR spectra (see Figure S3) confirm a decrease in lignin content with increasing pretreatment severity. Untreated solid and those treated with lower concentrations (S2 and S3) exhibit a high lignin content. In contrast, more aggressively treated solids show a consistent delignification ranging between 50–60 %. The IR spectra reveal that peaks related to cellulose become more prominent with increased pretreatment severity. Additionally, the broad peak associated with silica diminishes as ash content decreases, suggesting silica removal. The peak around 2916 cm^{-1} changes shape with pretreatment time [72]. This shift, more distinctly observed in solids treated longer (S9), reflects structural alterations in cellulose. Moreover, the strengthening of the peak at 3300 cm^{-1} indicates enhanced cellulose crystallinity resulting from pretreatment.

The crystallinity was also investigated by XRD (see Figure S4). The characteristic peaks of cellulose types (I-IV) were significantly different depending on the sample [50,73–76]. Untreated rice straw and mildly pretreated samples (e.g., S3) show a nearly flat plateau between 14° and 17°, indicating a lack of crystallinity due to the presence of amorphous materials such as lignin and hemicellulose. With harsher pretreatments, the peak around this zone becomes more pronounced (S6 and S9), suggesting an increase in crystal content. However, when aggressive conditions are applied (S13), these peaks begin to separate, distinguishing the two faces when the amorphous part is removed, since this solid is 80 % cellulose. The peak at 22.3°, characteristic of type I cellulose, becomes more pronounced with pretreatment, resulting in a higher purity of cellulose I due to the removal of amorphous components such as hemicellulose and lignin. However, under highly aggressive conditions (e.g., S13), the diffraction pattern begins to show a slight separation of peaks, with a shoulder appearing around 20°, suggesting the formation of cellulose II [76] . This structural transformation is attributed to mercerization, as cellulose II can be formed from cellulose I through treatment with concentrated aqueous sodium hydroxide [77].

The peak around 35° is also much more accentuated in this solid than in the others, which indicates the presence of a purer cellulose I [22,78,79]. Notably, while the solid treated for 6 h exhibits superior hydrolysis compared to the 1 h treatment, the crystallinity remains consistent across these samples, as shown by similar XRD patterns (see Figure S4). The crystallinity index [80] estimated using the Segal and area methods (see Table S2), ranges from 74–80 % and 61.2–67.32 %, respectively, and shows minimal variation among samples. Despite the increase in cellulose content with pretreatment, the percentage of amorphous cellulose also rises, indicating greater reactivity. However, the samples with similar compositions (S6, S8, and S9) showed the same amorphous cellulose content (Table S2), irrespective of the pretreatment time.

The surface features of the pretreated solids were examined using

scanning electron microscopy (SEM). The untreated residue exhibits a parallel macrofibre structure interspersed with layers of parenchyma cells [81]. As the pretreatment severity increases, this organised structure disintegrates, resulting in a network of fine, fragmented fibres. Images show how untreated residue displays a compact, rigid structure with minimal surface cracks and granules, likely silica deposits (Fig. 3, A.1 and A.2). The most significant difference is observed along the increase of the pretreatment time, same trend than in the observation based on hydrolysis, when pretreated for 1 hour at 73 °C with 1 % NaOH, the structure remains relatively ordered but shows increased fissures and emerging granules that form fibres (Fig. 3, B.1 and B.2), showing an improved accessibility compared to the untreated straw. In contrast, pretreatments for 4.5 to 6 h lead to a structure of loose, separated fibres (Fig. 3, C.1, C.2, D.1, and D.2), indicating enhanced enzyme accessibility and potentially improved cellulase adsorption and hydrolysis efficiency. The most aggressive pretreatment conditions reveal a fully fibrous structure with well-defined fibres (Fig. 3, E.1 and E.2). Although this structure is more defibrillated than that of the 6-hour pretreatment, it showed a slightly lower initial reaction rate and less efficient hydrolysis at extended processing times, probably due to a higher accumulation of inhibitory compounds in the pretreated solid [15].

Cellulase access to cellulose was also investigated using a green fluorescent protein (GFP) labelled with carbohydrate-binding modules (CBM), which specifically bind to cellulose. The quantitative analysis of GFP labelling revealed that the supernatant fluorescence of the untreated rice straw was equivalent to that of the initial GFP solution, indicating minimal GFP adsorption on the untreated residue. In contrast, the supernatant from the pretreated solid displayed only 7.7 % of the initial fluorescence, demonstrating a 92.3 % adsorption yield on the pretreated solid. Fluorescence microscopy of the solid corroborated these findings. Solids without GFP showed fluorescence per se (Fig. 4, A,1 and B.1). The treated solid showed lower fluorescence than the untreated residue, as lignin exhibits natural fluorescence. Thus, the decrease in solid fluorescence may result from lignin removal during pretreatment [82,83].

The untreated straw, when treated with GFP, exhibited uniform fluorescence across the surface with no intensity peaks (Fig. 4, B.2 and B.3). In contrast, the pretreated solid showed significant GFP adsorption, with marked fluorescence intensity revealing the structural features of the surface, consistent with SEM observations (Fig. 4, A.2 and A.3). This result highlights improved cellulose accessibility following pretreatment, aligning with the enhanced hydrolysis behaviour observed.

Based on these findings, where pretreatment time emerged as a critical factor, we opted for a pretreatment with a duration of 4.5 h at 73 °C with 1 % NaOH concentration. These structural and compositional insights, when combined with our systematic kinetic and compositional analyses, allowed us to directly link mild pretreatment conditions to increased cellulose accessibility and reactivity—at low enzyme dosages and rapid hydrolysis times. To our knowledge, our study is exemplary in terms of providing a comprehensive, integrated analysis connecting pretreatment variables, substrate modification, enzyme accessibility (via direct GFP adsorption), and overall hydrolysis efficiency.

3.5. *Improvement of pretreatment sustainability: Black liquor reuse and process scale-up*

To further address the applicability of our approach, we evaluated two critical aspects often neglected in comparable studies: the reuse of alkaline black liquor and the scale-up of both the pretreatment and the process to higher solid concentrations.

Once the suitable mild pretreatment conditions were established, we finally aimed at evaluating the potential for reusing the liquid phase, or black liquor, obtained from the pretreatment. The residual alkaline black liquor from the rice straw pretreatment was recycled and employed to treat fresh rice straw under the same conditions used previously. This recycling process was conducted over four consecutive cycles without any additional treatment or pH adjustment of the liquor, monitoring chemical compositions and pH values of the produced black liquors.

The first reuse of the liquor (SR1) led to a solid with a composition similar to that of the solid treated with fresh liquor, showing only minor increases in lignin and ash content (Table 3). In this case, enzymatic saccharification of rice straw pretreated with the recycled black liquor yielded results comparable to those obtained with the freshly prepared pretreatment solution (Fig. 5). However, with subsequent reuses, a noticeable decrease in cellulose content and a significant increase in lignin and ash were observed. Specifically, after the second reuse (SR2), cellulose content dropped to 50 %, while lignin content rose by 44 %, and ash content nearly doubled. By the third and fourth reuses (SR3 and SR4), the composition approached that of the untreated residue, with lignin content reaching 27 %. Enzymatic saccharification mirrored these

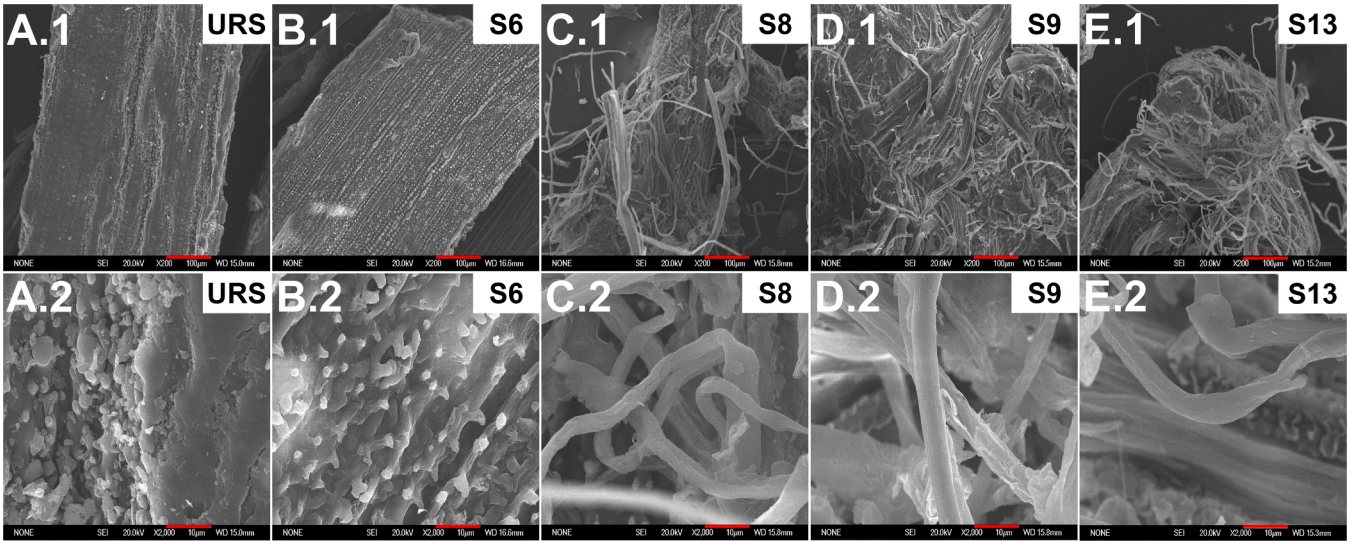

Fig. 3. SEM pictures of the URS (A.1 and A.2) and different samples of pretreated rice straw (pretreatment conditions: T, C_{NaOH}, t): S6, B.1 and B.2 (73, 1, 1); S8, C.1 and C.2 (73, 1, 4.5); S9, D.1 and D.2 (73, 1, 6); S13, E.1 and E.2 (95, 10, 1).The upper figures (A.1 to E.1) correspond to a magnification of 200x (the scale bar corresponds to 100 μm) and figures A.2 to E.2 have a magnification of 2000x (the scale bar indicates 10 μm).

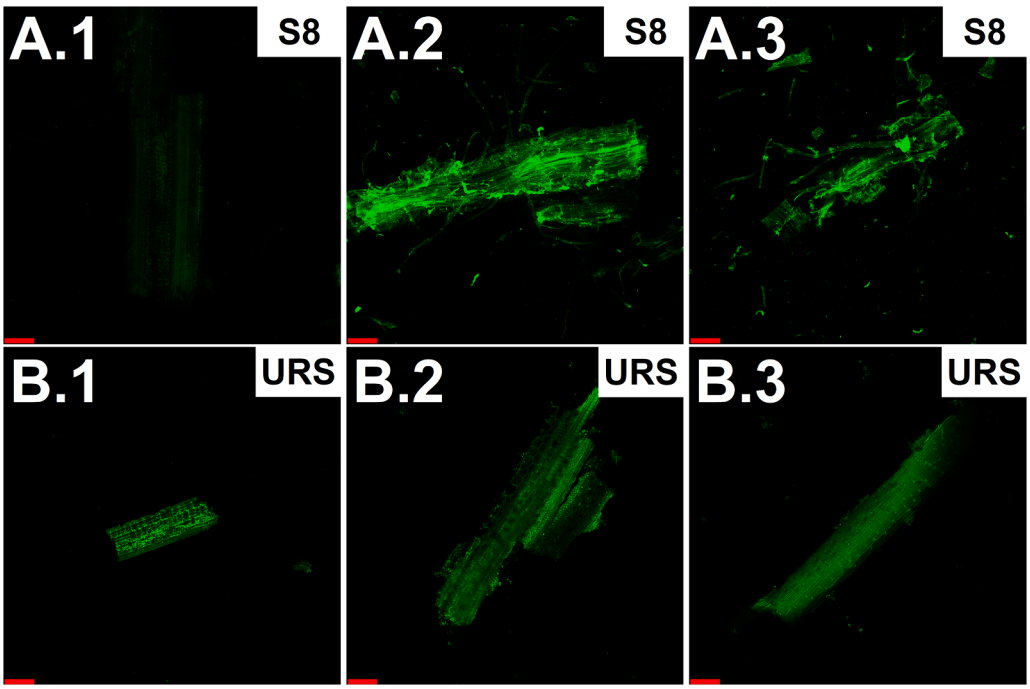

Fig. 4. Confocal microscopy micrographs at 40x magnification. Number 1 represents the control solid without enzyme, while numbers 2 and 3 correspond to the solids after 24 h of incubation. A represents Solid 8 (pretreatment conditions: 73 °C, 1 % (w/v) NaOH, 1 h), and B represents untreated rice straw. The red line indicates 100 μm.

Table 3
Chemical composition of pretreated rice straw with liquor reuse. Value of pH at zero time in the first cycle: 12.95 ± 0.04.

Solid	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Ash (%)	pH
SR1	58.69 ± 1.55	22.86 ± 0.88	13.95 ± 3.04	4.5 ± 0.3	12.77 ± 0.05
SR2	51.26 ± 1.76	24.72 ± 1.25	16.86 ± 2.06	7.16 ± 0	12.30 ± 0.07
SR3	40.32 ± 1.51	21.48 ± 0.67	27.2 ± 2.33	11 ± 1	11.68 ± 0.04
SR4	39.18 ± 1.28	21.32 ± 1.08	28.5 ± 1.46	11 ± 0.9	10.41 ± 0.07

changes: glucose yield was 73 % after the first two reuses but fell to 60 % and 47 % in the third and fourth reuses, respectively. This decline in efficiency is attributed to the progressive increase in lignin and ash content, as well as a lower liquor basicity, with the pH dropping from above 12 to 10.4 after the fourth reuse [34]. Therefore, maintaining a high pH is crucial for optimal pretreated rice straw saccharification efficiency.

To determine whether the observed decline in hydrolysis efficiency was due to with pretreatment pitfalls or to enzyme concentration, solids SR2 and SR4 were subjected to hydrolysis using five times the enzyme concentration employed in previous experiments. For SR2, which was treated under optimal conditions and pH, 95 % glucose yield was achieved within 8 h (Fig. 5.B). In contrast, SR4, pretreated with recycled

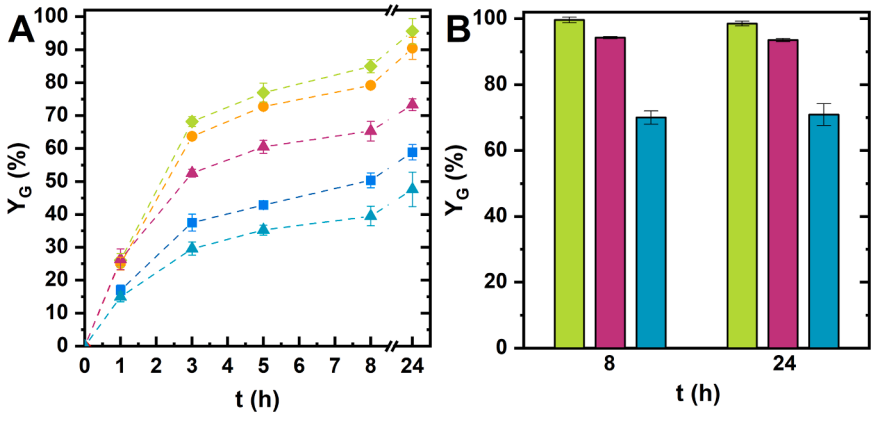

Fig. 5. Enzymatic hydrolysis of pretreated rice straw with black liquors: A) Enzyme concentration of 1.05 FPU/g DS of Biogazyme 2X and 3.38 FPU/g DS of β-glucosidase 1000; solid with fresh liquor (◆), SR1 (first reuse liquor, ●), SR2 (second reuse liquor, ▲), SR3 (third reuse liquor, ■) and SR4 (fourth reuse liquor, ▲); and B) Enzyme concentration of 5.25 FPU/g DS of Biogazyme 2X and 16.9 FPU/g DS of β-glucosidase 1000; solid with fresh liquor (■), SR2 (■) and SR4 (■).

liquor over four cycles, reached a maximum glucose yield of 70 % within 8 h. Despite extending the reaction time to 24 h, the glucose yield did not increase, suggesting that excessive reuse of the liquor without pH adjustment impairs pretreatment effectiveness and much higher enzyme loadings only partially compensate for it. These findings are consistent with previous results (Fig. 1.B), which indicates that a minimum NaOH concentration of 0.5 % at 50 °C is necessary to ensure solid reactivity. While a first reuse cycle was effective, subsequent cycles led to a lower pH liquor, to a less efficient delignification, and, subsequently, to reduced hydrolysis yields, highlighting the need for pH control and management of accumulated inhibitors in recycled streams.

Furthermore, we scaled up the pretreatment process by increasing the solid concentration to 5 % w/w and by working with larger volumes (800 and 1600 mL) in cylindrical bioreactors provided by one/two Rushton 6-blade turbines spinning at 350 rpm to ensure effective liquid-solid mixing and mass transfer. The final solid composition was similar to that obtained from smaller-scale hydrolysis experiments (200 mL in a 250 mL round-bottom flask), as well as the reactivity of the solid fractions (see Table S3).

To demonstrate the practical applicability of our enhanced integral workflow, we performed a proof-of-concept scale-up by increasing the solid concentration during pretreatment and hydrolysis to 7.5 % (w/v), which is in the direction to industrial relevance (Fig. 6), slightly increasing the enzyme load to 9 mg protein per gram dry solid, still a low enzyme concentration not to endanger process economic feasibility, while targeting for high glucose concentrations. The results showed that the compositional changes achieved under optimal conditions (1 % NaOH, 73 °C, 4.5 h) were consistent at this larger, industrially-relevant solid concentration, and the hydrolysis efficiency remained high—achieving ~82 % glucose (40 g/L) yield at only 9 h. In the literature, in a vertical ball mill reactor with 8 % solids and a high enzyme loading (21 FPU/g and 26.5 Uβ/g), a sugar concentration about 50 g/L was reached in just 9 h [84]. In contrast, using sulfuric acid–pretreated rice straw at solid loadings between 2.5 and 10 %, glucose concentrations ranging from 10 to 40 g/L were obtained, but only after 24 h of hydrolysis [85].

These results indicate that our approach is robust and transferable to more industrially relevant substrate concentrations and enzyme loads, operating for short process time to reduce energy input. Thus, process intensification is achieved without efficiency loss.

4. Conclusions

In conclusion, this work exemplary shows that mild alkaline pretreatment conditions, specifically 1 % NaOH at 73 °C, at several scales are highly effective in improving rice straw saccharification, achieving hydrolysis results at low enzyme loadings (approx. 4.4 FPU/g DS at 2.4 % g DS concentration in the reacting slurry under non-limiting external mass transfer conditions) similar to those obtained with harsh alkali pretreatments. Longer pretreatment time (4.5 h) proved crucial for enhancing glucose yield, highlighting the importance of processing time in boosting hydrolysis efficiency.

This study stands out from previous research by presenting an integrated, systematic evaluation of rice straw mild alkaline pretreatment combined with low enzyme fast saccharification, obtaining a deeper understanding of the results based on multiple physicochemical analyses. Our approach achieves up to 90 % glucose yield within 16 h—using 50–80 % less enzyme and significantly shorter process times than most reported studies. Structural analyses (FTIR, XRD, fluorescent microscopy and SEM) showed that mild alkaline pretreatments increase cellulose crystallinity and create fibrous structures with a potentially improved enzyme accessibility, an aspect further confirmed with GFP adsorption tests: pretreated solids exhibited significantly higher enzyme binding compared to untreated straw, thus explaining improved hydrolysis efficiencies. The combined assessment of pretreatment efficiency, enzyme accessibility, black liquor recycling, and scale-up within a single workflow fills key gaps in the literature and provides new perspectives for process intensification and sustainability in lignocellulosic biorefineries. While the majority of experiments in this study were intentionally performed at low solid loading to minimize confounding variables and enable a detailed understanding, we also demonstrated proof-of-concept at higher solid concentrations, maintaining composition of pretreated solids and saccharification efficiency, which supports the industrial applicability of our workflow. These promising results could be applicable to small rice straw particles in a continuous pretreatment-saccharification upstream processing. Future studies will build on this foundation, systematically exploring high solid loadings and high-solid operations, addressing additional challenges, such as limited water availability, mass transfer barriers, and mixing efficiency, that are critical for large-scale industrial implementation.

CRediT authorship contribution statement

Celia Alvarez-Gonzalez: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **José A. Delgado:** Investigation, Methodology, Writing – original draft, Writing – review & editing. **Juan M. Gonzalez:** Conceptualization, Funding acquisition, Project administration, Writing – review & editing. **Mauricio Zurita-Gotor:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing. **Miguel Ladero:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Supervision, Visualization, Writing – original draft, Writing – review & editing. **Juan M. Bolivar:** Conceptualization, Data curation, Formal analysis,

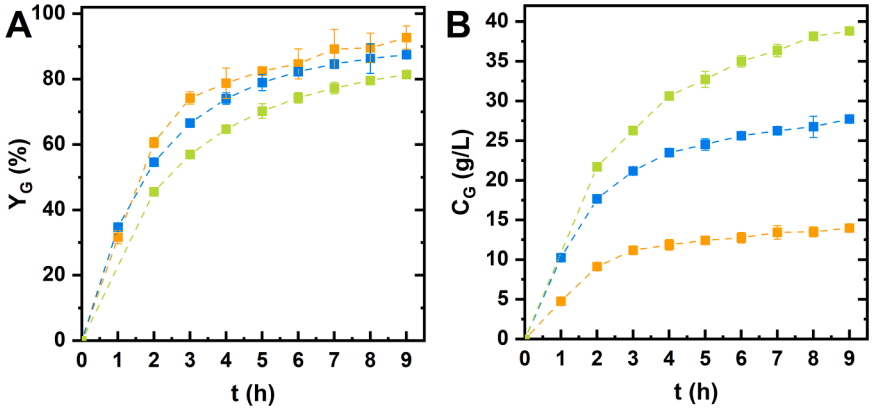

Fig. 6. Enzymatic hydrolysis of pretreated rice straw with different solid loadings: 24 g/L (■), 50 g/L (■) and 75 g/L (■). Enzyme concentration of 4.3 FPU/g DS of Biogazyme 2X and 2.4 FPU/g DS of β-glucosidase 1000. A) Glucose yield and B) Glucose concentration.

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Declaration of competing interest

The authors declare that they have no knowledge of competing financial interests or personal relationships that could have influenced or appeared to influence the work here reported.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.rineng.2025.106573.

Data availability

Data will be made available on request.

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Enhanced saccharification process of rice straw by integrated study of alkaline pretreatment and enzymatic saccharification.

Supplementary material

Supplementary tables

Table S1 Relative changes of the key components upon pretreatments**.**

Solid	Pretreatment conditions			Relative content change (NREL) (%)			
	T (ºC)	C_{NaOH} (%w/v)	t (h)	Cellulose	Hemicellulose	Lignin	Ash
URS	-	-	-				
S1	50	0.025	1	1	7	-1	-14
S2	50	0.1	1	8	13	0	-30
S3	50	0.5	1	19	17	-22	-52
S4	50	1	1	28	6	-25	-54
S5	50	10	1	73	-36	-53	-54
S6	73	1	1	53	4	-58	-58
S7	73	1	3	52	5	-55	-62
S8	73	1	4.5	56	-1	-56	-62
S9	73	1	6	62	1	-65	-64
S10	73	3	1	76	-38	-56	-54
S11	73	5.5	1	79	-32	-64	-56
S12	73	10	1	90	-56	-60	-54
S13	95	10	1	108	-78	-65	-56

Table S2. Crystallinity index of different samples of untreated and pretreated rice straw.

Solid	Segal method			Area method		
	CrI (%)	Crystalline cellulose (%)	Amorphous cellulose (%)	CrI (%)	Crystalline cellulose (%)	Amorphous cellulose (%)
S13	80	64.68	16.17	67.32	54.43	26.42
S9	78.18	49.17	13.72	65.80	41.38	21.51
S6	74.94	44.70	14.95	63.60	37.94	21.71
S8	76.68	46.52	14.15	66.21	40.17	20.50
S3	75.91	35.23	11.18	63.24	29.35	17.06
URS	74.01	21.14	7.42	61.20	17.48	11.08

Table S3. Change of scale of the pretreatment.

Scale (L)	Solid loading (%)	Cellulose (%)	Hemicelullose (%)	Lignin (%)	Ash (%)	X_{8h}
0.8	2.5	60.81 ± 1.66	23.83 ± 0.63	11.56 ± 1.27	3.8 ± 0.8	0.87 ± 0.08
0.8	5	60.14 ± 0.89	24.53 ± 0.48	11.53 ± 3.62	3.8 ± 0.6	0.88 ± 0.05
1.6	5	59.89 ± 1.30	24.27 ± 2.68	11.85 ± 2.18	4 ± 0.6	0.87 ± 0.04

Supplementary figures

Effect of external mass transfer on saccharification kinetics

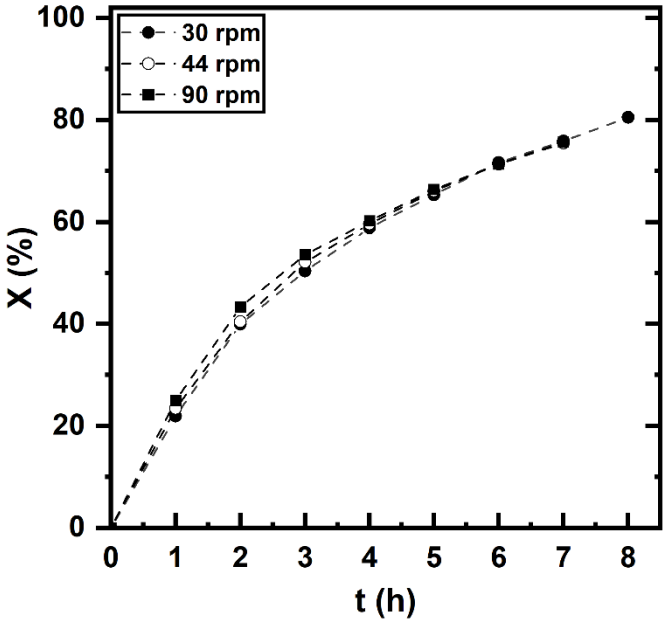

Figure S1. Effect of external matter transfer through the evolution of cellulose conversion with time using different stirring rates.

The effect of external matter transfer was studied by hydrolysis of solid 13 with an enzyme concentration of 1.05 FPU/g dry solid of Biogazyme 2X and 3.38 FPU/g dry solid of β-glucosidase 1000. The stirring speed was studied in the range of 30-90 rpm. We found that the global hydrolysis rate was not limited by this phenomenon in the range studied, being the process controlled by the chemical reaction.

Effect of pretreatment on initial hydrolysis rate

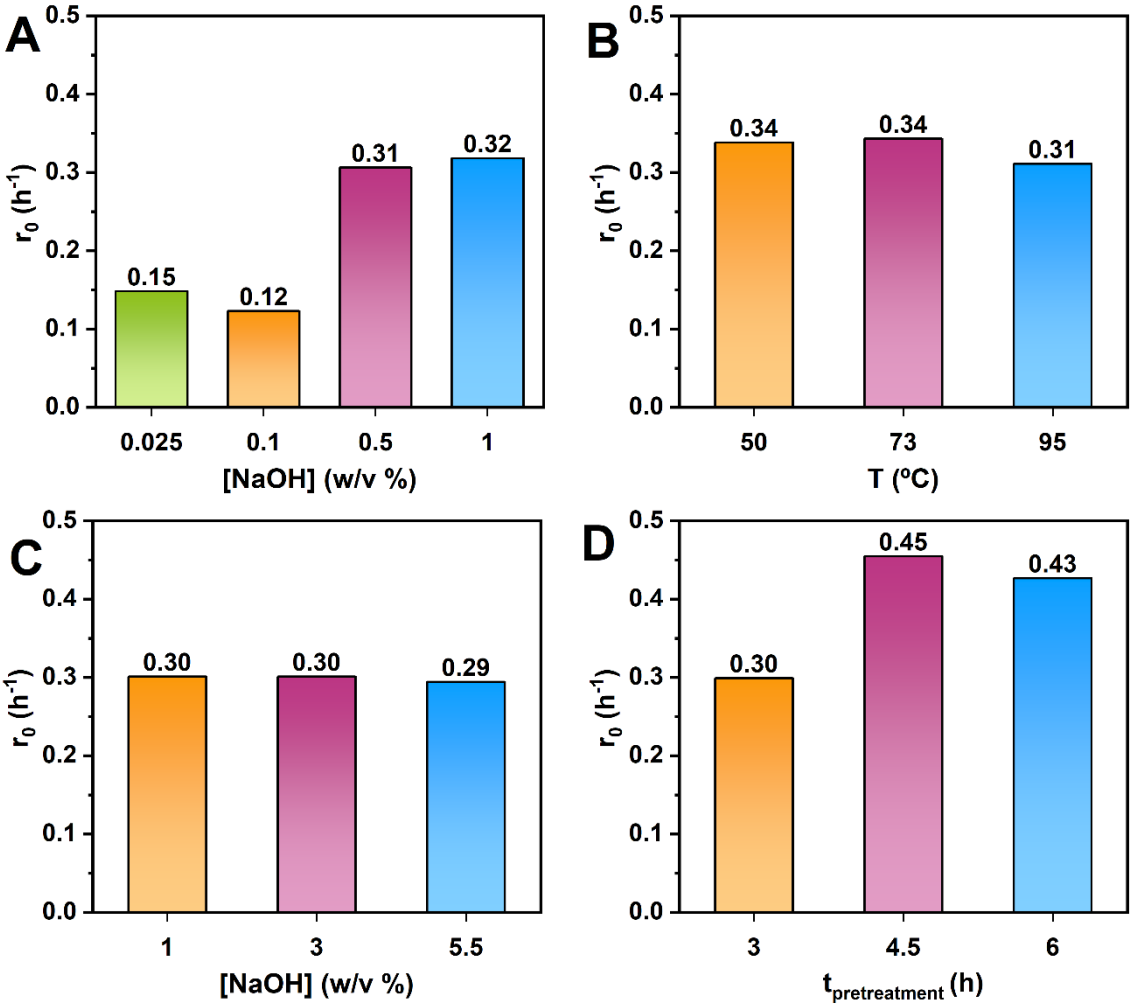

Figure S2. Effect of pretreatment on initial hydrolysis rate using a 1.05 FPU/g dry solid of Biogazyme 2X and 3.38 FPU/g dry solid of β-glucosidase 1000. A) Effect of minimum pretreatment conditions (50ºC and 1 h) B) Effect of temperature (10% (w/v) NaOH and 1 hour). C) Effect of NaOH concentration (73 ºC and 1h). D) Effect of pretreatment time (73 ºC and 1% (w/v) NaOH).

FTIR analysis

Peaks at 896 cm^{-1} (C-H deformation of β-glycosidic linkages), 1030 cm^{-1} (C-O-H), 1100 cm^{-1} (C-O-C), 1160 cm^{-1} (C-O-C), 2916 cm^{-1} (C-H) and 3300 cm^{-1} are peaks related to cellulose. The peak around 2916 cm^{-1}, which is linked to O-H stretching vibrations in lignin and hemicellulose [1]. Peak at 1060 cm^{-1} is related to silica. Lignin has absorption bands around 1514 and 1605 cm-1. Peaks at 1740 cm^{-1} and 1250 cm^{-1}, indicating the destabilisation of ester bonds between hemicellulose and lignin and the breakdown of the C-O-C functional group in hemicellulose [2].

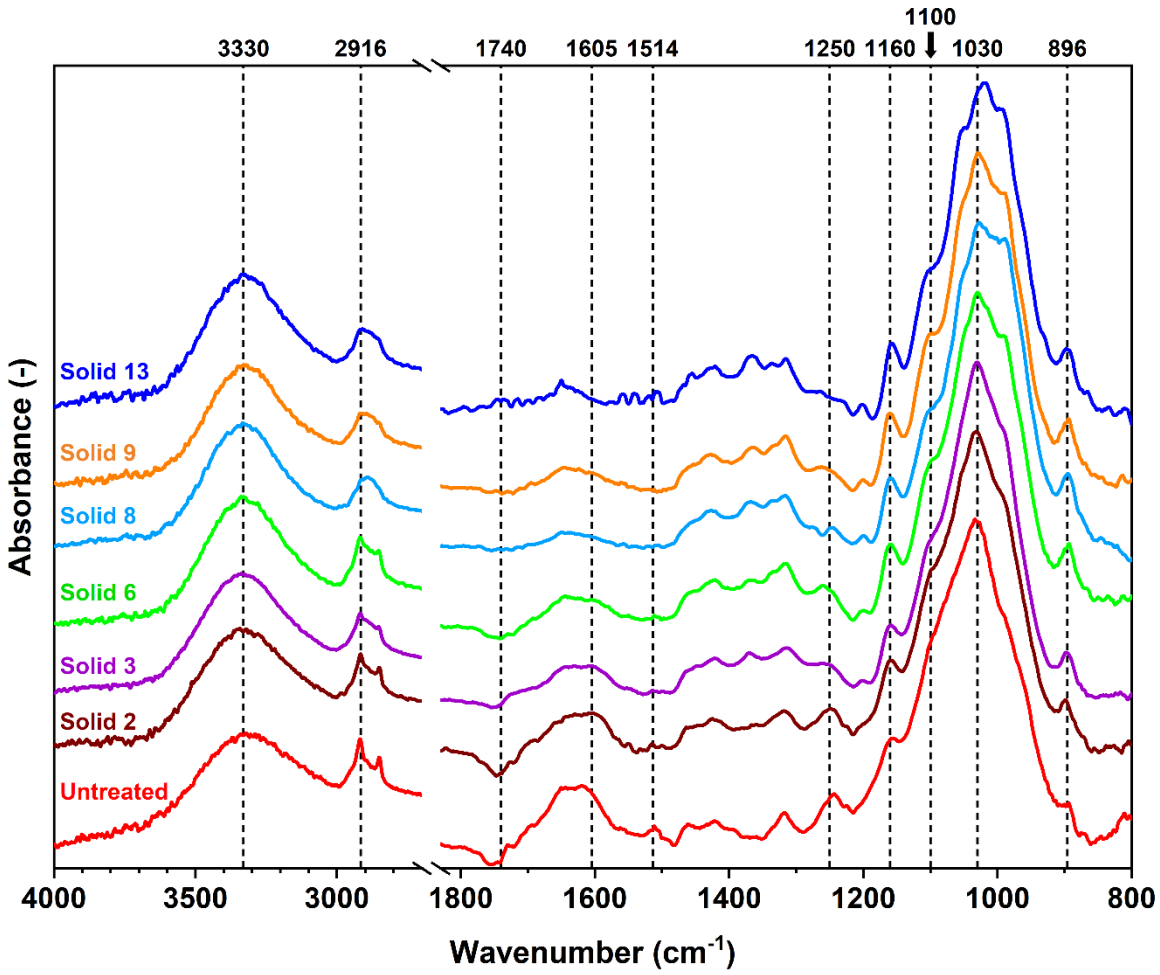

Figure S3. FTIR-ATR spectra of the URS and different samples of pretreated rice straw (Conditions: T,C_{NaOH},t): — untreated rice straw ; — S2 (50, 0.1, 1); — S3 (50, 0.5, 1); — S6 (73, 1, 1); — S8 (73, 1, 4.5); — S9 (73, 1, 6); — S13 (95, 10, 1).

DRX analysis

There are four types of cellulose (I, II, III and IV). Cellulose I is the most abundant in nature and exists in parallel strands without intersheet hydrogen bonding. Cellulose II is thermodynamically more stable, characterized by a more compact and ordered structure than Cellulose I and exists in antiparallel strains with intersheet hydrogen bonding. Cellulose II can be irreversibly obtained from cellulose I by alkaline pretreatment [3]. This conversion alters the crystalline structure, results in a higher degree of crystallinity of cellulose I [4]. The diffraction peaks characteristic of cellulase I are 14.7 (101), 16.5 (10i), 22.5 (002) and 34.5 (040). The diffraction peaks characteristic of cellulase II are around 12.0 ($1^{-}10$), 20.0(110) and 22.0 (020).

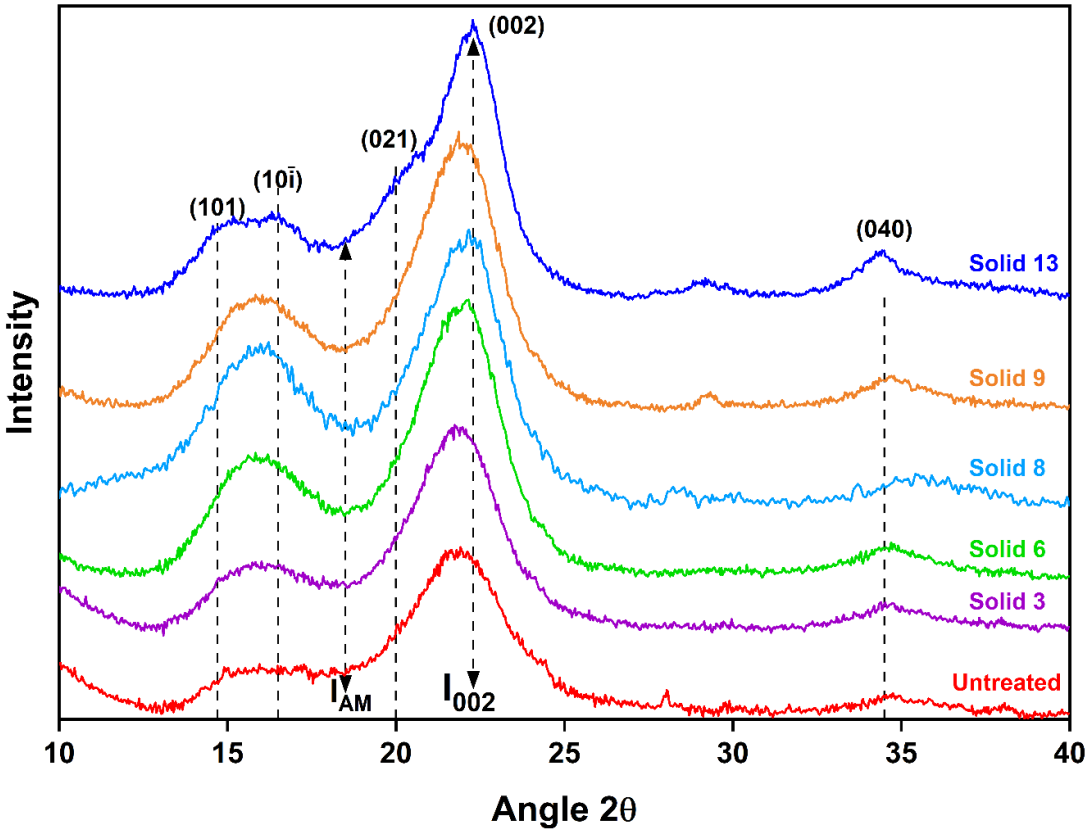

Figure S4. XRD patterns of the URS and different samples of pretreated rice straw (pretreatment conditions: T,C_{NaOH},t): untreated rice straw ; S3 (50, 0.5, 1); S6 (73, 1, 1); S8 (73, 1, 4.5); S9 (73, 1, 6); S13 (95, 10, 1).

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