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ORIGINAL PAPER

Simultaneous Optimization of Alkaline and Acid–Alkaline Pretreatments Applied to Rice Straw to Produce Glucose Correlated with Chemical and Morphological Effects

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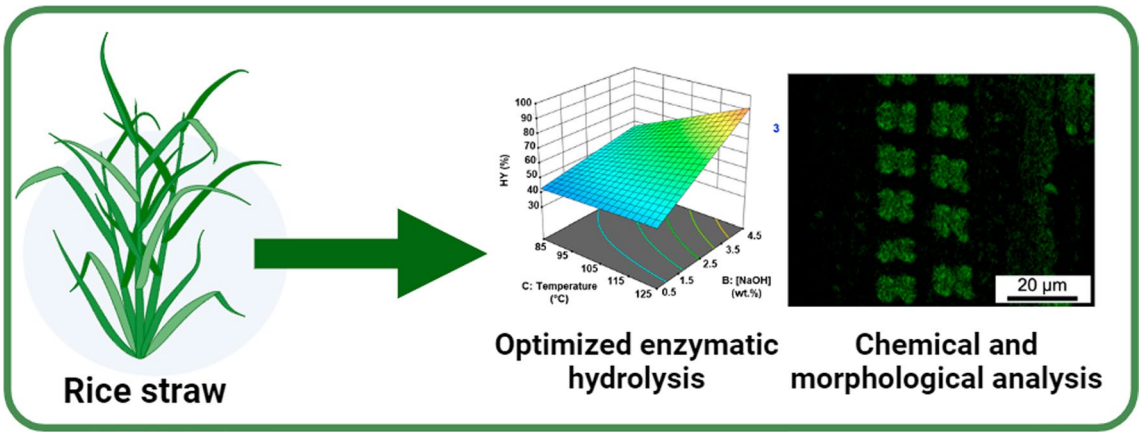
Abstract

Rice straw is a relevant and potential feedstock for bioethanol production due to its abundance and availability around the globe. In this study, a fractional factorial design (FFD 2^{5-1}) was applied to simultaneously evaluate the influence of only alkaline and acid-alkaline pretreatment conditions in glucose release, considering the glucose release after 12 and 24 h of enzymatic hydrolysis as responses and predicting alternatives for the fractionation of rice straw components. Hydrolysis yields (HY) higher than 90% were achieved using low enzyme loads (8 FPU/g substrate) after only 24 h of hydrolysis under optimized pretreatment conditions. Simultaneous DOE optimization showed that the acid step is optional to achieve higher HY but can contribute to a more holistic use of the hemicellulose fraction. Also, it significantly increased the hydrolysis efficiency compared to untreated rice straw (HY = 18%). Enzymatic hydrolysis with a different type of enzymatic cocktail in the optimized conditions using higher solid contents resulted in high cellulose conversion (up to 85%), showing the robustness of the DOE optimization and its applicability as a starting point for optimizations using other types of enzymes. Chemical and morphological analyses were also carried out to understand the effect of the treatments, aiming to achieve pretreatment and hydrolysis processes more effective for lignocellulosic biorefineries.

Graphical Abstract



Keywords Experimental design · Acid pretreatment · Alkali pretreatment · Rice straw · Biorefinery · Silica

Extended author information available on the last page of the article

Statement of novelty

This study used a Factorial Fractional Design of Experiments to optimize alkaline and acid-alkaline pretreatments applied to rice straw, simultaneously in the same experimental chart, allowing to evaluate the interactions between acid pretreatment (step 1) and the variables in alkaline pretreatment (step 2). Results were discussed using different enzymatic hydrolysis conditions and correlated to chemical and morphological changes.

Introduction

Rice is a widely available food source in several regions around the globe and ranks as the third most-produced agricultural commodity, having yielded an estimated 518.14 million metric tons in 2023, prominently in Asia, South America, and Africa [1, 2]. The production of rice results in a significant amount of straw (approximately 1.35–1.5 kg per 1 kg of rice), offering substantial biotechnological potential [3]. However, nearly 50% of rice straw is currently disposed of through burning, landfilling, or used as fodder, increasing thus environmental issues [3].

The potential of rice straw extends to chemical and biotechnological pathways for biofuel and chemical production, showcasing its versatility. The high cellulose content (23–47% wt) in rice straw [4, 5] makes it an attractive source for second-generation (2G) ethanol, a promising alternative to oil-based fuels. 2G ethanol can supplement the production of first-generation (1G) ethanol, produced by direct fermentation of the sucrose present in sugarcane juice or corn starch, and is widely used in some countries such as Brazil. This strategy is a clever alternative to enhance bioethanol production and respond to the increasing demand for more sustainable fuels. Also, it is environmentally beneficial because it allows the production of higher quantities of fuel without the need to increase the cultivated land area, which reduces competition with food production [3].

2G ethanol production involves pretreating lignocellulosic biomass to enhance cellulose accessibility for enzymatic conversion into fermentable sugars like glucose. Pretreatments are a mandatory step in 2G ethanol production due to the intrinsic association of cellulose with hemicellulose (19–27% wt) and lignin (5–24% wt) in the plant cell wall [6], which hinders the polysaccharide conversion into valuable bioproducts [7].

Effective pretreatment is crucial for optimizing 2G ethanol production, especially considering the elevated costs of these processes [8]. Chemical pretreatments, such as those using diluted acids and alkalis, efficiently extract lignin and hemicellulose and increase the substrate surface area and porosity [9]. They are particularly important for rice straw due to its high silica content (up to 20%) [4], considering the detrimental effect of silica on enzymatic action.

Diluted acid and alkaline pretreatments stand out for their ability to remove hemicellulose, lignin, and silica from rice straw, allowing their recovery as byproducts, which is in accordance with the biorefinery concept [10, 11]. Acid treatments extract mainly hemicellulose by hydrolyzing the polysaccharide glycosidic bonds and producing oligomers and monomers, which are solubilized into the liquid fraction [12]. Conversely, alkaline treatments effectively hydrolyze ester and ether linkages between cellulose, hemicellulose, and lignin, solubilizing the aromatic molecule into the alkaline liquid fraction [13]. In sequence, hydroxide ions can efficiently cleave the internal β-*O*-4 linkages of lignin macromolecules, contributing to its solubilization. In rice residues, alkaline pretreatments have a fundamental role in silica extraction and solubilization, which is not efficiently achieved by simple hydrothermal treatments based on hot water [14, 15].

Design of experiments (DOE) is a valuable tool for optimizing pretreatment conditions, allowing efficient screening of an extensive experimental range by simultaneously changing the variables using fewer experiments [16–18]. In other words, DOE allows the detection of interactions between the experimental variables, which is not possible using experiments that vary only one condition at a time [19, 20].

DOE is a strategy already adopted for optimizing pretreatments applied to lignocellulosic biomasses, including rice straw. However, while previous studies have separately optimized only acids [16, 21, 22], only alkalis [17, 23, 24], or sequential acid-alkaline treatments [25, 26], ours aims to simultaneously optimize alkaline and acid-alkaline pretreatments in the same DOE set of experiments. Simultaneous alkaline and acid-alkaline optimization takes advantage of the DOE ability in predicting interactions between acid treatment and the alkaline variables. This approach allows for a comprehensive exploration of diverse scenarios for biomass fractionation, considering the enzymatic hydrolysis outcomes.

The simultaneous optimization emerged as an interesting way to evaluate biomass processing, based on the fact that acid step was considered not strictly necessary in acid-alkaline treatments applied to some biomasses if glucose production is the focus of the process [15, 20]. In these cases, the biomass could be directly forwarded to a delignification step using alkaline methods, avoiding acid step, which is beneficial from an economic point-of-view for a single-product approach (in this case, in glucose). However, the use of an acid step before alkaline treatments is more suitable for an improved fractionation of the biomass components and a preferable alternative to valorize both hemicellulose and lignin fractions [11, 12]. Indeed, alkaline treatments applied

directly to the in natura substrates hinder component fractionation because hemicellulose and lignin are extracted in the same liquid stream. To the best of our knowledge, no studies have simultaneously optimized alkaline and acid-alkaline pretreatments applied to rice straws.

In this study, we obtained optimized conditions for glucose production from acid-alkaline and alkaline pretreated rice straw. First, we used a 2^{5-1} fractional factorial design (FFD) to simultaneously optimize only alkaline and acid-alkaline treatments. This includes assessing the significance of the acid step and four alkaline treatment variables: NaOH concentration, time, temperature, and solid:liquid ratio. We specifically focused on enzymatic hydrolysis outcomes after 12 and 24 h of reaction using a mixture of enzymatic cocktails (Celluclast 1.5 L and Novozyme 188 at 8 FPU/g substrate).

Selected samples from the initial evaluation also underwent enzymatic hydrolysis using the commercial enzymatic cocktail Cellic CTec2, increasing the solid/liquid ratios (up to 5%) and using two different enzyme dosages (8 and 20 FPU/g) to achieve sugar concentrations closer to those industrially used for 2G-ethanol production. Chemical and morphological characterization provided insights into pretreatment conditions, composition, morphology, and hydrolysis yields. This comprehensive understanding enables the prediction of optimal fractionation scenarios applicable in a rice straw biorefinery.

Experimental

Biomass and Materials

Rice straw (variety EPAGRI 121 CL) was kindly donated by Coordenadoria de Assistência Técnica Integral (CATI) (Guaratinguetá, São Paulo, Brazil). Biomass was dried in a convection oven (Tecnal TE-394/3) at 60 °C for 24 h and then grounded in a knife mill (SOLAB–SL 31) until passing through a 2 mm sieve and later stored in packages with airtight closure. NaOH (P.A.) was purchased from Synth®, and H_2SO_4 (98% purity) was acquired from LSChemicals. All reactants were used as received.

Table 1 Factors and levels evaluated in the 2^{5-1} FFD

Factors	Low level (−)	High level (+)	Central point (0)
A-[H_2SO_4] (wt%)	0[a]	3.6	1.8
B-[NaOH] (wt%)	0.5	4.5	2.5
C-Temperature (°C)	85	125	105
D-Time (min)	20	100	60
E-S/L (wt%)	5	12.5	8.75

[a]0 indicates that the acid step was not carried out and the in natura substrate was straightly treated with alkaline solutions

Biomass Pretreatments

In natura rice straw was treated using H_2SO_4 solutions similarly to previously reported procedures [27], following the concentrations indicated by the DOE (Tables 1, 2). These concentrations varied from 0 (when the acid step was not performed and in natura samples were directly forwarded to alkaline treatments) to 3.6 wt%. All acid treatments were conducted in an autoclave (Phoenix AV-75) at 120 °C for 40 min using a solid:liquid ratio of 1:10 (g:mL). At the end of the pretreatment time, the system was cooled to room temperature, and the solid was separated from the liquid fraction by filtration and rinsed until neutral pH was obtained.

In the alkaline step, in natura or acid-treated substrates underwent an alkaline treatment with NaOH solutions following the conditions indicated in Table 1. The range of acid and alkali concentrations was determined based on previous studies for other biomasses, such as rice husks and elephant grass [15, 20]. Experiments above 100 °C were performed in an autoclave (Phoenix AV-75), similarly to the described for acid treatments, while pretreatments below 100 °C were performed in a water bath (Fisatom, model 550), ensuring similar temperature ramp conditions [15]. The solids obtained after each pretreatment were filtered, rinsed until neutral pH and dried.

Experiments were carried out following a 2^{5-1} Fractional Design, in which 5 variables were evaluated at two levels (Table 1), and 5 replicates were used in the central points. The experimental factors considered were: (1) H_2SO_4 concentration in the acid step ([H_2SO_4]; (2) NaOH concentration [NaOH]; (3) temperature; (4) time; and (5) solid-to-liquid ratio (S/L) in the alkaline step. Hydrolysis yields (HY) at 12 and 24 h (calculated following Eq. 1) were evaluated as responses. The range of each factor was defined based on the previous evaluation carried out in rice husks [15].

Enzymatic Hydrolysis

Enzymatic hydrolysis was carried out using two types of enzymatic cocktails. Firstly, all samples from DOE were hydrolyzed using a mixture of Celluclast 1.5 L and Novozyme 188 (Novozymes) (ratio 4:1) in a shaking incubator with a minimum of 4 replicates in each experimental condition. Following previous studies, the reactions were conducted with a biomass weight of 4.5 mg and a total volume of 850 µL at 50 °C, pH 4.5 (25 mM sodium acetate buffer) with an enzyme loading of 8 FPU/g biomass [18, 28]. Hydrolysis residence times of 12 and 24 h were evaluated as DOE responses. Additional hydrolysis times (48 and 72 h) were carried out in a kinetic assay using samples with

Table 2 2^{5-1} FFD experiments and the responses of hydrolysis yields (HY) after 12 and 24 h of enzymatic hydrolysis

Sample	Experimental conditions					Responses	
	[H_2SO_4] Step 1 (wt%)	[NaOH] Step 2 (wt%)	Temp Step 2 (°C)	Time Step 2 (min)	S/L Step 2 (wt%)	HY (12 h) (%)[a]	HY (24 h) (%)[a]
SIN[b]	–	–	–	–	–	10.7	18.1
SH1[b]	1.8	–	–	–	–	27.0	32.4
SH2[b]	3.6	–	–	–	–	23.1	33.2
S1	0	0.5	85	20	12.5	33.3	43.0
S2	3.6	0.5	85	20	5.0	32.4	38.1
S3	0	4.5	85	20	5.0	43.3	59.7
S4	3.6	4.5	85	20	12.5	34.0	47.2
S5	0	0.5	125	20	5.0	35.3	57.9
S6	3.6	0.5	125	20	12.5	24.8	36.6
S7	0	4.5	125	20	12.5	43.9	82.4
S8	3.6	4.5	125	20	5.0	45.8	97.2
S9	0	0.5	85	100	5.0	43.1	47.9
S10	3.6	0.5	85	100	12.5	32.6	42.8
S11	0	4.5	85	100	12.5	42.1	72.4
S12	3.6	4.5	85	100	5.0	40.7	49.0
S13	0	0.5	125	100	12.5	33.0	36.3
S14	3.6	0.5	125	100	5.0	43.5	55.6
S15	0	4.5	125	100	5.0	57.6	93.8
S16	3.6	4.5	125	100	12.5	46.7	94.5
S17	1.8	2.5	105	60	8.75	39.4	51.0
S18	1.8	2.5	105	60	8.75	42.1	50.2
S19	1.8	2.5	105	60	8.75	39.5	50.0
S20	1.8	2.5	105	60	8,75	34.9	50.5
S21	1.8	2.5	105	60	8.75	39.7	51.6

[a]Reported values are an average of 5 replicates of enzymatic hydrolysis. The standard deviation calculated for the 5 replicates at the central point is ± 2.6% (12 h) and ± 0.6% (24 h)

[b]Samples SIN, SH1, and SH2 are not part of the DOE chart and are represented only as control samples for the in natura, acid treated using 1.8 wt% and 3.6 wt% of H_2SO_4, respectively

lower (S1) and higher (S8, S15, and S16) cellulose conversion yields at 12 and 24 h of enzymatic hydrolysis (Supplementary Information). This assay was carried out to ensure that 12 and 24 h were the most indicated hydrolysis times to be considered in DOE.

Next, samples S8, S15, and S16, which presented the best results in the first enzymatic evaluation, were also hydrolyzed using the commercial cocktail Cellic CTec2 (Novozymes). Enzymatic hydrolysis was carried out at the same conditions as those using the mixture of Celluclast 1.5L and Novozyme 188 (8 FPU, solid/liquid ratio: 0.47%) for 24 h at 50 °C using a citrate buffer (pH 5) to compare the two enzyme sets. In sequence, samples were also hydrolyzed using higher solid/liquid ratios (2.5 and 5%) with 8 or 20 FPU/g for 24 h and 1 g substrate in an incubator (Marconi MA 832).

Before all enzymatic hydrolysis experiments, substrates underwent a hydration step for 2 h at room temperature. The glucose quantification was performed using High-Performance Liquid Chromatography (HPLC) equipment (Agilent 1200) [11]. Hydrolysis yields (HY) were determined according to Eq. 1, considering the total glucose released on hydrolysis (GL in mg/g substrate), the cellulose content (C, mg/g) in the hydrolyzed substrate, and a correction factor (1.1) due to polysaccharide hydrolysis [29].

$$HY(\%) = \frac{GL(\mathrm{mg/g})}{C(\mathrm{mg/g}) \times 1.1} \times 100 \quad (1)$$

Statistical Evaluation

Analysis of the DOE data was performed in the Design Expert® software. Effect graphs were used to select the significant factors and interactions influencing hydrolysis yields. Analysis of variance (ANOVA) was used to test the regression significance and the lack of fit using F-test. Finally, response surfaces were used to describe the behavior

of the response in the experimental domain and allowed the selection of conditions that lead to the maximization of the evaluated responses.

Chemical Composition

Cellulose, hemicellulose, lignin, ash, and extractive contents were quantified according to the National Renewable Energy Laboratory (NREL) procedure [30]. Briefly, 0.3 g of substrate were hydrolyzed with H_2SO_4 72 wt% (3 mL) for 1 h at 30 °C. Next, 84 mL of deionized water were added to dilute H_2SO_4 to 4 wt% and the system was allocated in autoclave at 121 °C for 1 h. Liquid fraction was separated from solids by filtration using a porous-bottom crucible. Carbohydrates and their degradation products were quantified in liquid fraction by high-performance liquid chromatography (HPLC) (Agilent 1200) using a BIORAD HPX87H column (45 °C, H_2SO_4 5 mmol/L as mobile phase). Acid-soluble lignin present in liquid fraction was quantified by UV–Vis spectroscopy (Agilent, Cary 5000). The solid fraction present in the crucible was dried until constant weight (105 °C) and then calcinated to quantify acid-insoluble lignin. Ashes were quantified by calcinating the substrate (800 °C, 2 h). Soxhlet extraction (ethanol:cyclohexane, 8 h and water, 24 h) was carried out only in *in natura* samples to determine the amount of extractives.

Morphological Analysis

Sample morphology was analyzed in a field-emission scanning electron microscope (FESEM) (Quanta 250, FEI), operating at 5 kV. Prior to the analysis, all samples were coated with an Iridium film (*ca.* 5 nm) using a BALTEC MED 020 sputter coater, operating at 11.3 mA for 90 s. At least 20 images were obtained from each sample to ensure the reproducibility of the results. Elemental analyses were carried out in the same microscope, using an Oxford X-max N 50 dispersive energy spectroscopy analyzer (EDS) (Oxford Instruments) with 10 kV of acceleration voltage.

Results and Discussion

Fractional Factorial Design (FFD) Analysis

FFD was selected as the DOE tool in this study since it reduces the number of runs compared to a full factorial design. In the case of a DOE using five variables, the 2^{5-1} FFD presents resolution V, indicating that the main effects are aliased with fourth-order interactions (which are unlikely to be significant), and second-order interaction effects are aliased with the third-order interactions (also unlikely to be significant). Therefore, primary and second-order interactions can be estimated in 16 runs (central points not considered) against 32 runs needed in a full factorial design [19, 20].

Table 2 describes the experiments conducted according to the 2^{5-1} FFD and the two evaluated responses (HY after 12 or 24 h of enzymatic hydrolysis using a mixture of Celluclast 1.5 L and Novozyme 188 enzymatic cocktails). The results showed HY up to sixfold higher than in natura straw (SIN), even after only 12 h of hydrolysis, indicating the efficiency of the pretreatment approach. At this hydrolysis time, the best performance was achieved under condition S15 (HY = 57.6%), using no acid step and NaOH 4.5 wt% at 125 °C for 100 min with S/L of 5 wt% in the alkali step. Considering 24 h of enzymatic hydrolysis, the efficiency was even higher, achieving values higher than 90% for several experimental conditions (S8, S15, and S16).

Previous studies using acid or alkaline diluted solutions to pretreat rice straw typically used enzymatic hydrolysis times longer than 24 h and higher enzyme load to achieve similar results. The yields of enzymatic hydrolysis of rice straw treated using sequential acid-alkaline treatments resulted in 70–90% cellulose conversion to glucose after 72 h of enzymatic hydrolysis [25, 26]. Nevertheless, alkaline treatments used directly in rice straw also resulted in high cellulose conversion rates, closer to 60% [24]. For example, sequential treatment with 0.5% H_2SO_4 (130 °C for 2 h) and 1.5% NaOH (80 °C for 3 h) resulted in HY of 92.7% using an enzymatic load of 17 FPU/g substrate after 72 h of enzymatic hydrolysis [25]. Considering other pretreatment approaches, such as micro-emulsions of eutectic solvents [31] and ionic liquid systems [32, 33], HY range between 61 to 88% using an enzymatic content of around 20 FPU/g cellulose for 72 h.

HY should be compared with caution with other studies since hydrolysis conditions depend on the type and combinations of enzymes and on the experimental conditions used in hydrolysis, which vary significantly from one study to another. Nonetheless, the HY achieved here are closer to the maximum conversion of cellulose into glucose, thus showing the efficiency of the pretreatments.

Higher HY were achieved here using hydrolysis conditions typically milder than those reported in the literature (15–20 FPU, 48–72 h) [11, 34, 35]. Two main causes that can be associated with our high HY are the high cellulose content in the substrates (which will be discussed in section "Chemical Compositions and Their Relations with Glucose Production") and the use of a reduced solid concentration (0.47%), which will be assessed in section "Considerations of Enzymatic Hydrolysis with Different Enzymes". Morphology of the substrates, will be assessed in section "Morphological Characterization", respectively.

The Half-Normal plots of the effects are shown in Fig. 1, where the most important effects are those that deviate more from the straight line (centered in zero). In this case, NaOH

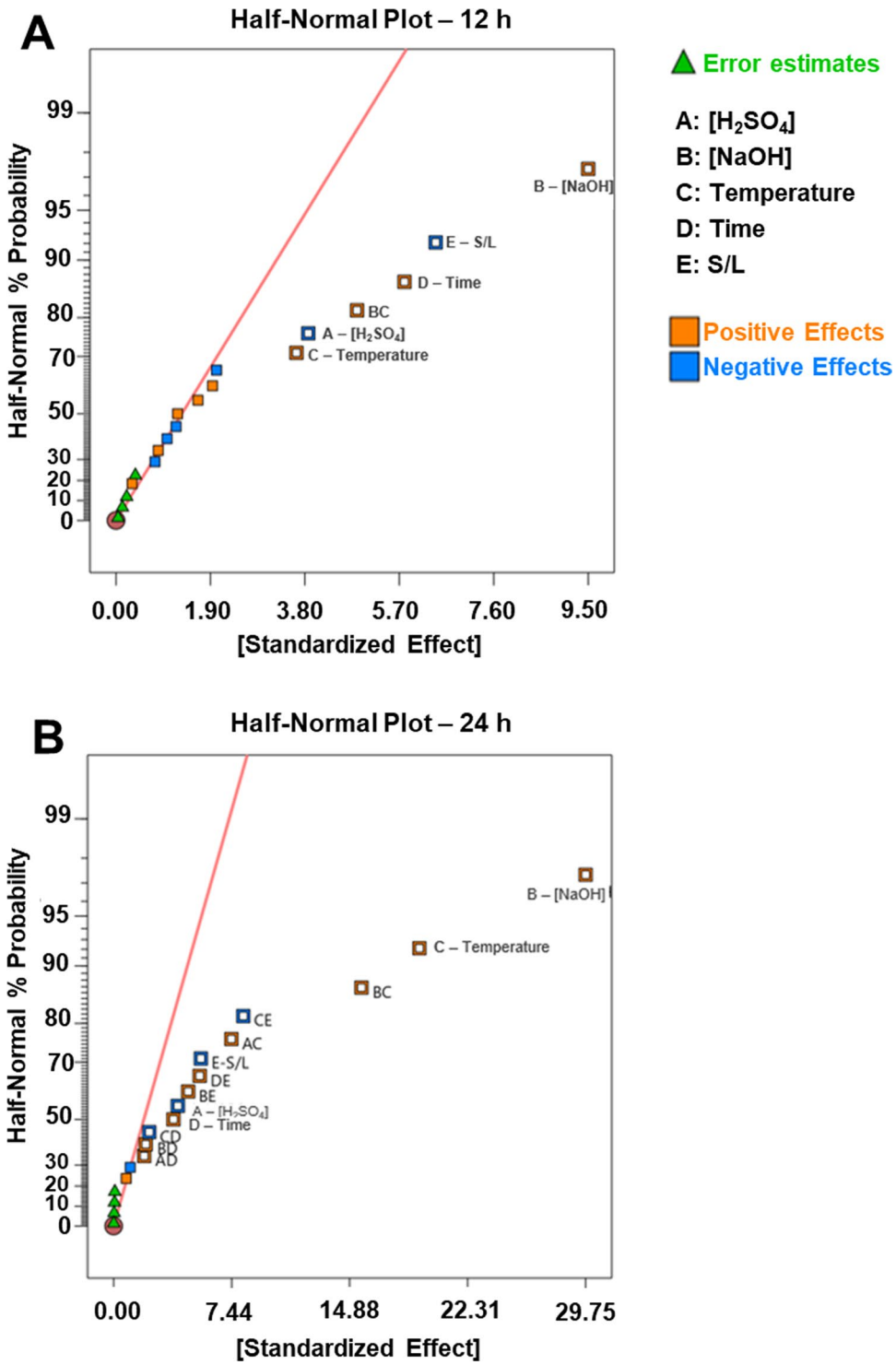


Fig. 1 The Half-normal plot of the standardized effects of the 2^{5-1} fractional factorial design for hydrolysis residence times of **A** 12 h; and **B** 24 h

concentration is the factor that most influences the response since it is far from the straight line, having a positive effect. It indicates that the increase of NaOH concentration is expected to increase the hydrolysis yields both for 12 and 24 h.

It is noticeable that the factors and their degree of influence on HY differ depending on the residence time in enzymatic hydrolysis. For 12 h of enzymatic hydrolysis, the most important factors after NaOH concentration are the S/L (negative effect), Time (positive effect), [H_2SO_4] (negative effect), and Temperature (positive effect). Also, the binary BC ([NaOH]-Temperature) interaction proved significant. On the other hand, if hydrolysis residence time is changed

to 24 h, the main factors influencing HY after [NaOH] are Temperature (positive effect) and the BC interaction.

Comparing the two enzymatic hydrolysis times, Temperature became more relevant as enzymatic hydrolysis times increased. In contrast, the S/L ratio, Time, and [H_2SO_4] concentration, which were relevant for 12 h, were less expressive and virtually unimportant for HY obtained at 24 h. This difference indicates that HY are more sensitive to pretreatment conditions using shorter hydrolysis residence times. This can be explained by considering that at 12 h of enzymatic hydrolysis, enzymes have a more limited time to convert cellulose into glucose. Hence, the accessibility of the substrate becomes more critical (more accessible substrates present higher cellulose conversion). On the other hand, longer hydrolysis times allowed a slower kinetic for cellulose conversion, and the specific limitations of the substrate were thus less crucial (enzymes have time to convert higher amounts of cellulose even in less accessible substrates). Likewise, different pretreatment conditions allowed an almost total conversion of the available cellulose [27].

The Analysis of Variance (ANOVA) using HY after 12 h of enzymatic hydrolysis (Table S1) showed that the regression was significant. This information is based on the F value, calculated by the $MS_{Regression}/MS_{Residual}$. The regression is considered significant if the F value calculated is higher than the F value tabulated. For HY after 12 h, the F value calculated is 20.61, while the F value tabulated is 2.92 (6, 13, 95% confidence level). Additionally, there was no lack of fit in the model since the $MS_{lack\ of\ fit}/MS_{pure\ error} = 1.06$, which is less than the tabulated F value of 6.00 (9, 4, 95% confidence level). It is noteworthy that the "curvature" term in ANOVA (Table S1) refers to the difference between the average experimental center points and the predicted value, considering a linear model that did not include them.

The response surfaces using 12 h of enzymatic hydrolysis as a function of the most critical factors (NaOH concentration and S/L ratio in step 2) are shown in Fig. 2. The surface curvature is not significant (p-value = 0.7804 in Table S1), indicating the linear model adequacy in describing experimental results within the studied domain. Therefore, based on the model, it is possible to predict that an optimal HY (12 h) could be reached at [NaOH] at the high level, keeping S/L at lower values. The highest conversion value (*ca.* 57%) was obtained without the acid step and using the following conditions in the alkaline pretreatment: [NaOH] = 4.5 wt% at 125 °C and 100 min using an S/L = 5 wt%, which coincides with the pretreatment conditions used to obtain S15.

Regarding 24 h of enzymatic hydrolysis, ANOVA (Table S2) showed that the regression was significant, considering that the F value calculated by the $MS_{Regression}/MS_{Residual}$ is 373.41, while the F value tabulated (13, 6, 95% confidence level) is 4.0. Nevertheless, for this response, the linear model presented a lack of fit (Curvature p-value < 0.05). This indicates that the effects can still be calculated and interpreted, but the model cannot be used for prediction within the experimental domain. However, it is important to highlight that the goal of the DOE was to optimize the glucose release and the current experiments present HY already closer to 100%. Therefore, additional experiments are not needed since the goal is not to propose a statistical model. The calculated effects give the directions of response surfaces, which allows the graphical interpretation of the influence of each factor.

The response surfaces after 24 h of enzymatic hydrolysis as a function of the most important factors ([NaOH] and Temperature) are shown in Fig. 3. The condition for the highest HY was [NaOH] = 4.5% (wt%), Temperature = 125 °C, S/L = 5% (wt%). [H_2SO_4] and Time are practically indifferent and have been shown at their lowest levels. It is noteworthy that between the conditions that led to the highest (Fig. 3B) and the lowest results (Fig. 3C), the maximum value is always when Temperature and [NaOH] are at their highest levels. This observation is also valid for response surfaces evaluating the HY after 12 h of enzymatic hydrolysis when [NaOH] and Temperature are varied (Fig. S1). Still, the maximum values using 12 h of enzymatic hydrolysis reached up to 57.6%.

A kinetic assay was carried out using samples S1, which showed the lowest HY at 24 h, and samples S8, S15, and S16, which presented the highest HY at 24 h (Fig. S2). Residence times of enzymatic hydrolysis higher than 24 h did not increase HY for any samples. In the case of sample S1, HY were practically constant (around 40%) after 12 h. The results suggest that the pretreatment conditions are the key parameters for optimizing hydrolysis. In addition, the hydrolysis time had more influence in shorter residence times, but not further. Therefore, the kinetic assay showed that 24 h of enzymatic hydrolysis is the optimum hydrolysis time for the pretreatment conditions tested within these experimental ranges, as the DOE evaluation and kinetic assay suggested.

Pretreatments carried out under the conditions indicated in the assays S8, S15, and S16 allowed the conversion of almost all the cellulose contained in the samples using 8 FPU of enzymatic load (Celluclast + Novozyme 188) and 24 h of residence time. These conditions were considered along with the chemical composition in the next sections and will be further discussed.

Chemical Compositions and Their Relations with Glucose Production

Concomitant with evaluating the efficiency of the pretreatments in the enzymatic action, it is also essential to assess their effect on the chemical composition of the substrates.

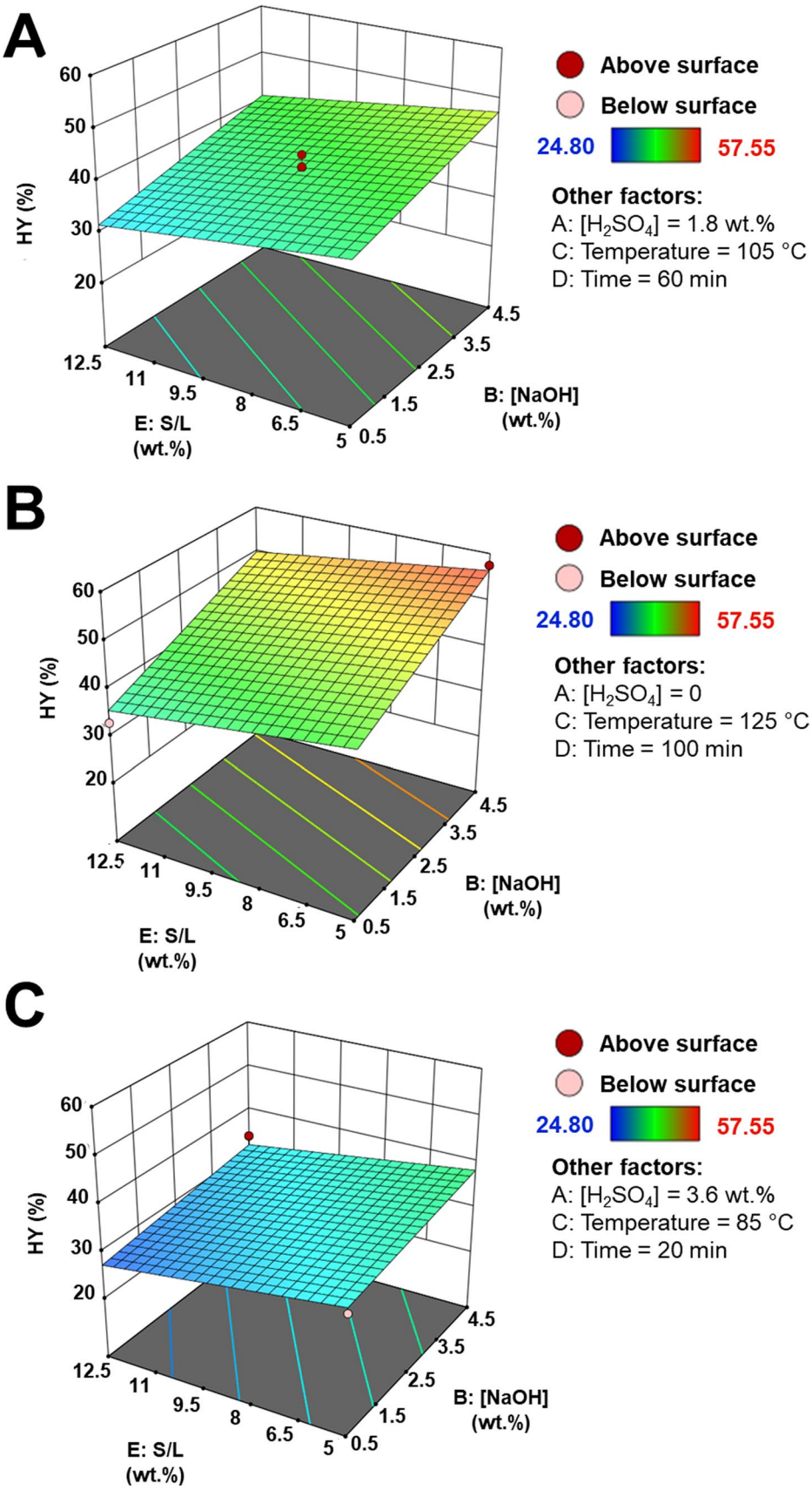


Fig. 2 Response surface (HY after 12 h of enzymatic hydrolysis) of the most relevant factors for HY in rice straw samples ([NaOH] and S/L): **A** with all the other factors kept at their center points ($[H_2SO_4] = 1.8$ wt%, Temperature = 105 °C, Time = 60 min); **B** under the conditions that resulted in the highest conversion (without acid step, Temperature = 125 °C; Time = 100 min); and **C** under the conditions that resulted in lowest conversion ($[H_2SO_4] = 3.6$ wt%, Temperature = 85 °C; Time = 20 min). Surface points above and below are shown to highlight the curvature analysis

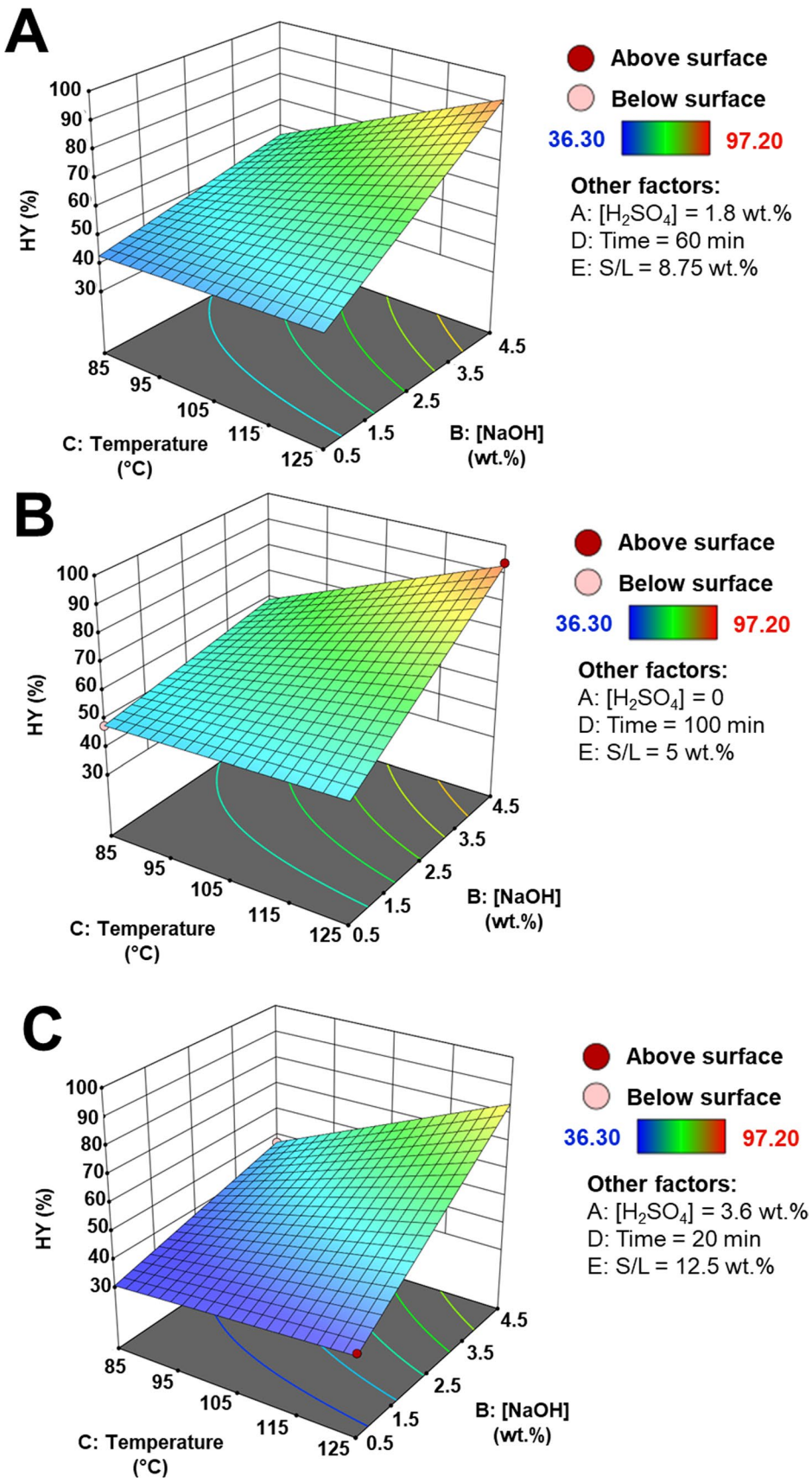


Fig. 3 Response surface (HY after 24 h of enzymatic hydrolysis) of the most relevant factors for HY in rice straw samples ([NaOH] and Temperature): **A** with all the other factors kept at their center points ([H_2SO_4] = 1.8 wt%, Time = 60 min, S/L = 8.75 wt%); **B** under the conditions that resulted in the highest conversion (without acid step, Time = 100 min, S/L = 5 wt%); and **C** under the conditions that resulted in lowest conversion ([H_2SO_4] = 3.6 wt%, Time = 20 min; S/L = 12.5 wt%). Surface points above and below are shown to highlight the curvature analysis

This evaluation is crucial because it explains the enzymatic hydrolysis results and defines the best conditions between similar HY, considering a biorefinery scenario.

The chemical composition and the quantity of glucose released after enzymatic hydrolysis using the mixture of Celluclast 1.5 L and Novozyme 188 in the substrates in natura and after pretreatments indicated by DOE (Table 2) are reported together in Fig. 4 and also in Table S3. The compositional analysis showed that the rice straw in natura (SIN) comprises 36.9% cellulose, 25.0% hemicellulose, 12.9% lignin, 7.7% ash, and 17.4% extractives. The lignin content is typically lower than that reported for other grasses, such as elephant grass [11], sugarcane bagasse [27], and residues of corn [36], but similar to the values reported in the literature for other rice straw samples [1, 37]. As lignin is a major factor hindering enzymatic action, these relatively lower contents highlight the applicability of rice straw for glucose production by enzymatic hydrolysis.

As expected, acid treatments extracted mainly hemicellulose, reducing its content by 65% and 75% as the in natura substrate is treated using $[H_2SO_4] = 1.8$ wt% (SH1) and 3.6 wt% (SH2). Alkaline treatments applied in acid-treated samples resulted in minimum hemicellulose contents (up to 2.2 wt%). On the other hand, samples treated only with alkaline treatments had a maximum reduction of 43% in the hemicellulose content in the assay using the different conditions in high levels (S11). Indeed, alkaline treatments resulted mainly in lignin extraction (up to 77% in S8 using $[H_2SO_4]$, [NaOH], and Temperature in high levels and Time and S/L in low levels), which follows the known effect of these treatments [38]. Alkaline treatments also effectively reduced the ash content to 0.7 wt% in the most efficient conditions, essential for rice straw processing.

As discussed in the previous section, the quantity of glucose released significantly increased from 74.5 (in natura) to 965.2 mg/g (sample S8) after 24 h of enzymatic hydrolysis. Indeed, the chemical composition analysis showed a linear correlation between the increase in cellulose content and the increase in glucose released, which is most noticeable after 12 h of enzymatic hydrolysis (Pearson's $r = 0.82$) (Fig. S3). In addition, other relations can be noticed, such as the reduction in lignin, hemicellulose, and ash content with the increase in glucose release after 12 h of hydrolysis (Pearson's $r = -0.87, -0.65, -0.62$, respectively) (Fig. S3, SI). All these correlations are expected since increasing cellulose content results in increasing substrate accessibility, which results in higher HY. Likewise, solids are enriched in cellulose due to the removal of hemicellulose, lignin, and ashes by acid and alkaline treatments, justifying the negative values of Pearson's r for these components.

In addition to the changes in chemical composition, the crystallinity of samples also changed because of the pretreatments, according to the determination of the Crystallinity Index (CrI, Table S4) based on the X-Ray diffraction (Fig. S4) [39]. CrI increased from 46 to 52–54% in samples SH2 and S1, which still presented high lignin contents, and to 63–64% in samples with high cellulose content (S8, S15, and S16). Samples S8, S15, and S16 presented the highest cellulose conversion, which indicates that cellulose accessibility caused by hemicellulose and lignin removal played a more significant role than the increase in CrI. Indeed, the increase in CrI could be interpreted as a drawback for enzymatic hydrolysis since enzymes usually present better performance in amorphous substrates, which are less organized and easily converted into monosaccharides [40]. However, it is essential to highlight that the increase in CrI observed here is a consequence of the extraction of the amorphous

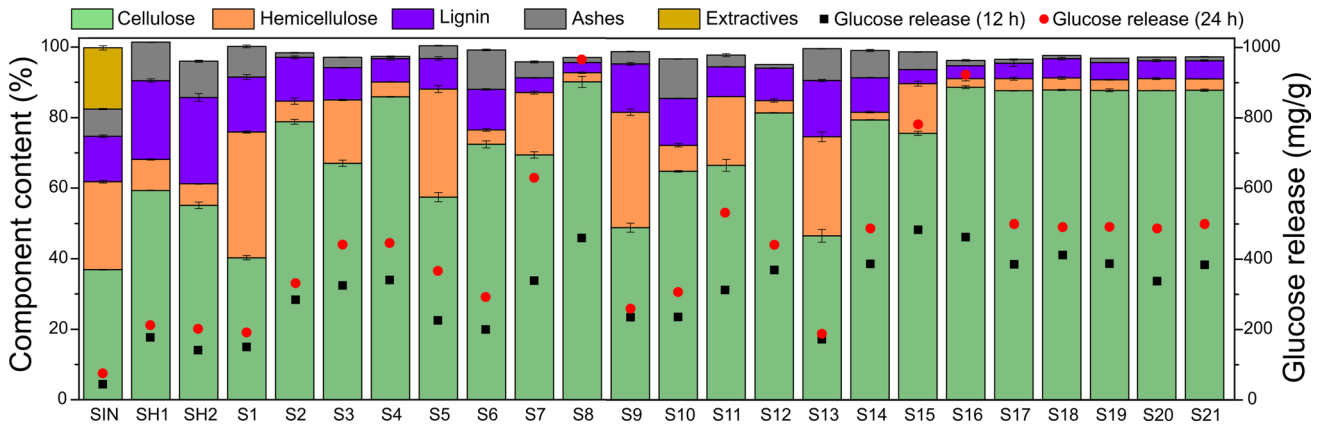


Fig. 4 Chemical composition of solid substrates before and after pretreatments and glucose released (mg/g substrate) after 12 and 24 h of enzymatic hydrolysis. Error bars are standard deviation values of replicates. SIN = raw rice straw; SH1 = sample pretreated only with $[H_2SO_4] = 1.8$ wt%; SH2 = sample pretreated only with $[H_2SO_4] = 3.6$ wt%; S1 to S21 = FFD samples with experimental conditions detailed in Table 1. Detailed values are described in Supplementary Information, in Table S3

components of lignocellulosic biomasses (hemicellulose and lignin) [11, 41]. Since cellulose is a semicrystalline polymer [42], cellulose-rich substrates presented a higher CrI than *in natura* or less modified substrates.

Based on the chemical characterization of the solid substrates and HY, it is possible to suggest some approaches for rice straw processing (Fig. S5). Condition S15 is the most economical because it uses no acid step. However, the acid step plays a significant role in hemicellulose extraction (Fig. 4). Therefore, acid steps can be conveniently applied in a cascade approach to separate hemicellulose and lignin in different streams. Amongst the conditions using acid-alkaline pretreatments, S8 and S16 showed the highest HY. However, S8 is more advantageous because it demands only 20 min of alkaline pretreatment, extracting the same quantity of lignin (87–88%, according to Table S5) and producing the same amount of glucose from each 100 g of in natura substrate (20.6 g, Fig. S6). A more detailed mass balance of the solid fractions and an estimate of component recovery from liquid fractions can be found in Supplementary Information (Fig. S5).

The recovered hemicellulose can be further converted into bio-based chemicals, such as furfural [43, 44] and xylitol [45]. If hemicellulose remains in the substrate, it can enzymatically be converted to pentoses along with the cellulose conversion into glucose. Still, the fermentation of pentoses into ethanol would demand microorganisms other than the usual *Saccharomyces cerevisiae* [46], which should be considered. In addition, the partial removal of hemicellulose to the liquid fraction will require a proper separation from the lignin, also solubilized in the alkaline liquid fraction, to enable its application [47].

Regardless of the acid step, alkaline treatments effectively removed lignin up to 90% (Table S5). Lignin can be precipitated from the alkaline liquid fraction by acid addition [48] and used to produce lignin nanoparticles [49], polyols by depolymerization [50], or carbon materials [51]. Rice straw also has a significant quantity of extractives, commonly composed of several high-value-added molecules, including sterols, fatty acids, and terpenes, which can be fractionated before acid or alkaline steps, enhancing biomass use and the revenue process, as was previously shown in similar biomasses, such as elephant grass leaves and stems and maize [52, 53].

Morphological Characterization

Significant morphological changes in the solid substrates followed the chemical changes that increased the glucose release. Figure 5 shows FESEM images of the substrates before treatments and the silica mapping of amplified areas. *In natura* rice straw has a surface covered by silica structures, as shown in the secondary electron images (Fig. 5A, C, E) and on the silicon maps (Fig. 5B, D, F). The two main silica structures identified in rice straw were papillae (indicated as P, Fig. 5B) and dumbbell-shaped bodies of silica (DBS, Fig. 5B) [54]. DBS are solidly silicified cells (Fig. 5B, F). In contrast, papillae are tiny outgrowths of silica structures (Fig. 5B, D) [55]. Indeed, 75–91% of the rice straw ashes are Si [3], vital in improving rice growth, providing mechanical strength, and protecting the plant from pathogens. In rice, silica is presented preferentially in the epidermal cell wall, negatively affecting cellulase action. As determined by the ash quantification after pretreatment, the extent of silica removal from rice straw depends on the alkalinity of the pretreatment.

After the treatments, biomass morphology was changed by removing hemicellulose, lignin, and silica, resulting in more exposed and separated cellulose fibers. After the acid step, morphological changes were subtle, as shown for sample SH2, treated with 3.6% wt% H_2SO_4 (Fig. 6A, B). The slight differences in chemical composition (Fig. 4) and substrate morphology explain the slight increase in the HY only after acid treatments (Table 2). However, it is noticeable that the acid treatment caused a disorder in silica structures compared to in natura rice straw. DBS are less oriented and more rounded. In addition, unlike in natura substrate (Fig. 5), silica is more distributed (lower contrast) on the fiber surface (Fig. 6B), probably due to the partial silica removal and redeposition.

Regarding alkaline treatments, the mildest method used in this study (S1) did not significantly change the biomass structure (Fig. 6C, D), which is related to the slight change in the chemical composition (Fig. 4). On the other hand, substrate S16 (acid-alkaline) (Fig. 6E), which resulted in higher glucose releases, was significantly modified by the pretreatments, showing open bundles of cellulose fibers more exposed to enzymatic action. This morphological effect of bundle separation is similar to that obtained for other biomasses, such as sugarcane bagasse [27] and elephant grass [11], and it is assigned to lignin removal from the interfibrillar regions. In addition, no silica structures were observed in the EDS analysis of sample S16 (Fig. 6E), showing the apparent effect of the alkali pretreatment on the silica domains of the plant cell wall.

Considerations of Enzymatic Hydrolysis with Different Enzymes

Samples S8, S15, and S16, which resulted in the best results in DOE, were also hydrolyzed using the enzymatic cocktail Cellic CTec2 for 24 h. This assay was carried out with two main goals: compare two different sets of enzymes and provide a proof of concept using higher solid/ratio conditions in enzymatic hydrolysis aiming at increasing glucose concentration to enable fermentable conditions more economically

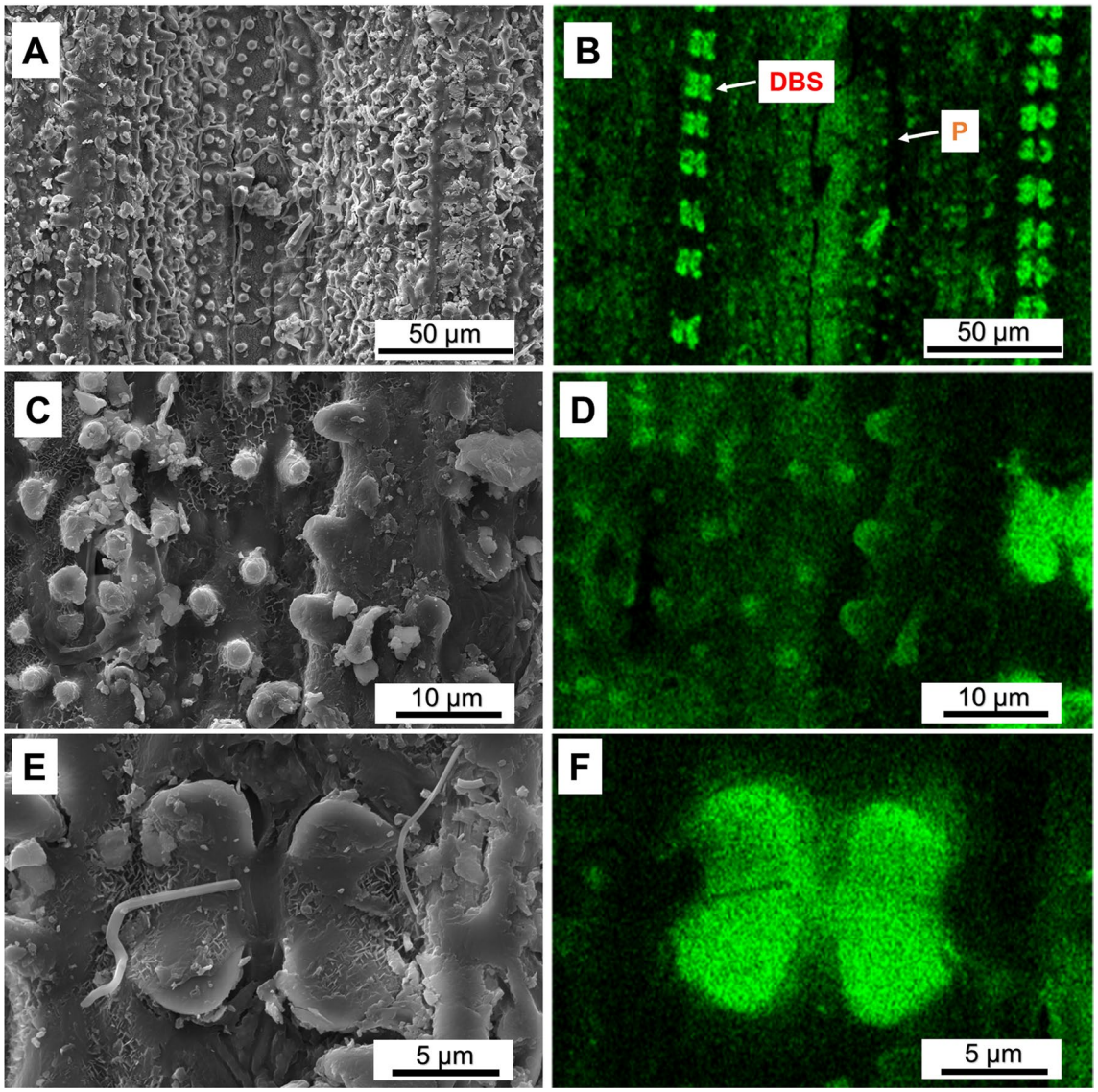


Fig. 5 Scanning electron microscopy images of rice straw in natura (**A**, **C**, **E**) and Si mapping obtained by EDS (**B**, **D**, **F**) in the same areas of **A**, **C**, and **E**, respectively. Si is indicated as green in the images. DBS is the code for dumbbell silica bodies, and P is for papillae. The ash content in this sample is 7.7 ± 0.2%. (Color figure online)

viable. Firstly, we kept the enzymatic dosage in 8 FPU for assays at a solid content of 0.47% (same as the one used in DOE) and then evaluated higher solid contents (2.5 and 5%). Also, we evaluated higher enzyme dosages (20 FPU) for conditions using higher solid contents.

Figure 7 shows the HY and glucose concentration for S8, S15, and S16. Cellic CTec2 (8 FPU, 0.47% of solids) resulted in lower HY (between 67 to 85%) compared to the conditions using Celluclast 1.5L/Novozyme 188. When Cellic CTec2 was used, sample S15 presented a higher HY between the samples. The comparison indicates that Celluclast 1.5L/Novozyme 188 was more efficient for hydrolyzing the analyzed samples, probably because it employs a combination of two enzymes. Still, it is noteworthy that increasing residence time for Cellic CTec2 could increase yields closer to those achieved in DOE since previous use of this enzymatic cocktail for 72 h resulted in HY closer to 100%, when it was applied to elephant grass that underwent acid-alkaline or only alkaline treatments [11].

Fixing the enzyme dosage at 8 FPU and increasing the solid content to 5 wt% resulted in increased glucose concentration (from about 3 g/L to values closer to 30 g/L). This is an interesting result about achieving high glucose concentrations since HY are important but should be considered together with the glucose concentrations, which facilitates further conversion into ethanol. Finally, a test using a higher

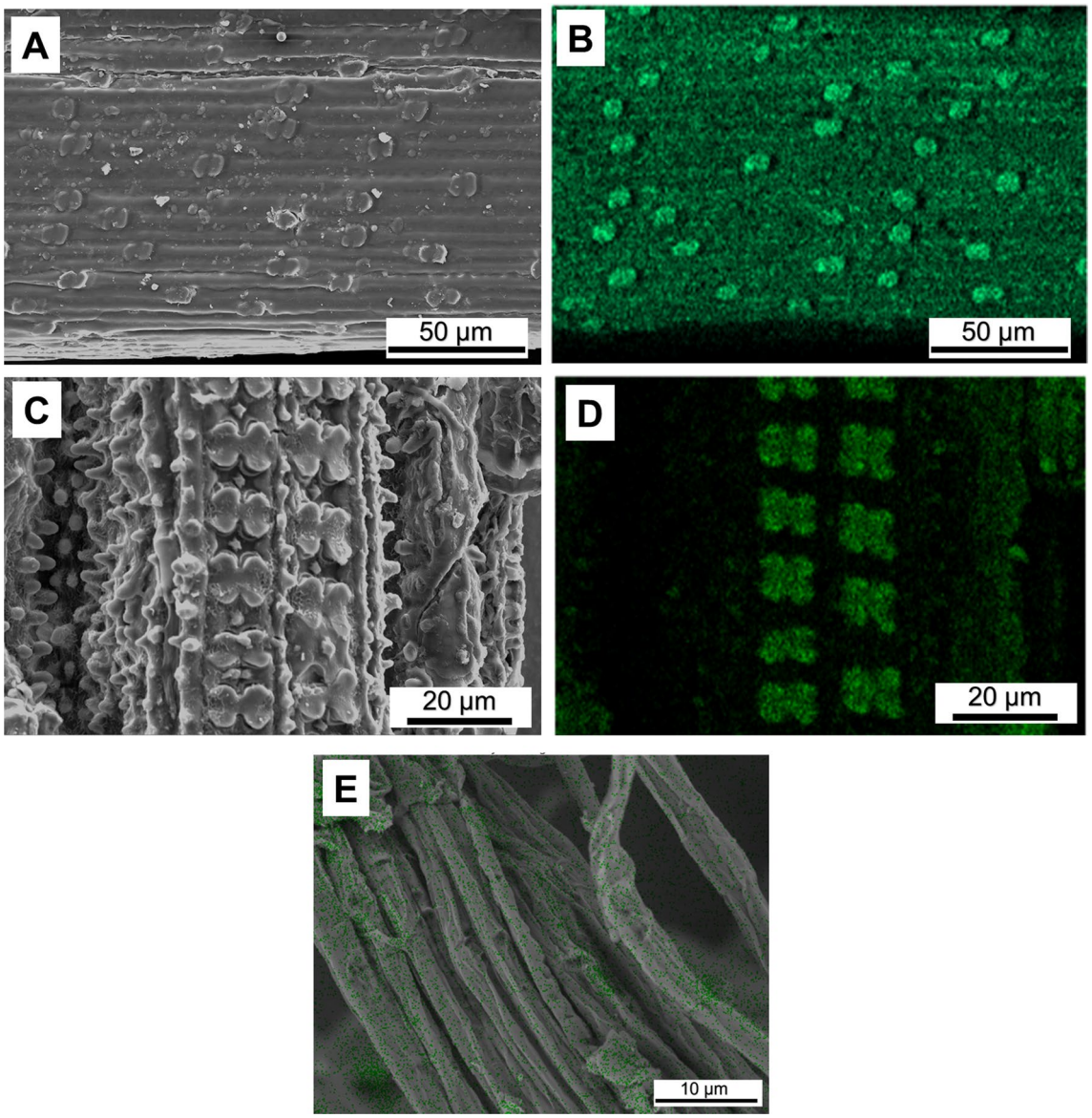


Fig. 6 Scanning electron microscopy and Si map of samples SH2 (**A** and **B**), S1 (**C** and **D**), and S16 (**E**)

enzyme dosage (20 FPU) was evaluated, resulting in glucose concentrations between 35 and 37 g/L, while HY ranged from 72 to 82%. It suggests that increasing enzyme dosage and solid contents should be considered together, aiming at both HY and glucose concentration.

Comparing between the evaluated samples, higher glucose concentrations can be achieved using both acid-alkaline (S8 and S16) or only alkaline treatments (S15). It is important to mention that this assay was carried out as proof of concept to demonstrate the optimized pretreatment effectiveness. Sequential studies can be performed at high-solids conditions to consider specific questions due to the so-called high-solids effect [56]. It includes a reduction in glucose production due to inhibition of enzymes because of high product concentration, high concentration of degradation products, and unproductive binding of enzymes, to cite a few, which are very dependent on process parameters, such as the type of reactors.

The results achieved at high solid concentrations are similar to those reported when rice straw was treated with sequential acid-alkaline, but here we generally achieved higher HY in a reduced residence time of enzymatic hydrolysis. Rice straw treated sequentially with H_2SO_4 (1 wt%) and NaOH (1.5 wt%) and hydrolyzed with Zytex-Supercut (10 FPU/g) and in-house β-Glucosidase 100 IU enzymes resulted in HY 83.2% after 48 h [57]. Similarly, rice straw treated with H_2SO_4 (1 wt%) followed by NaOH (1.25 wt%)

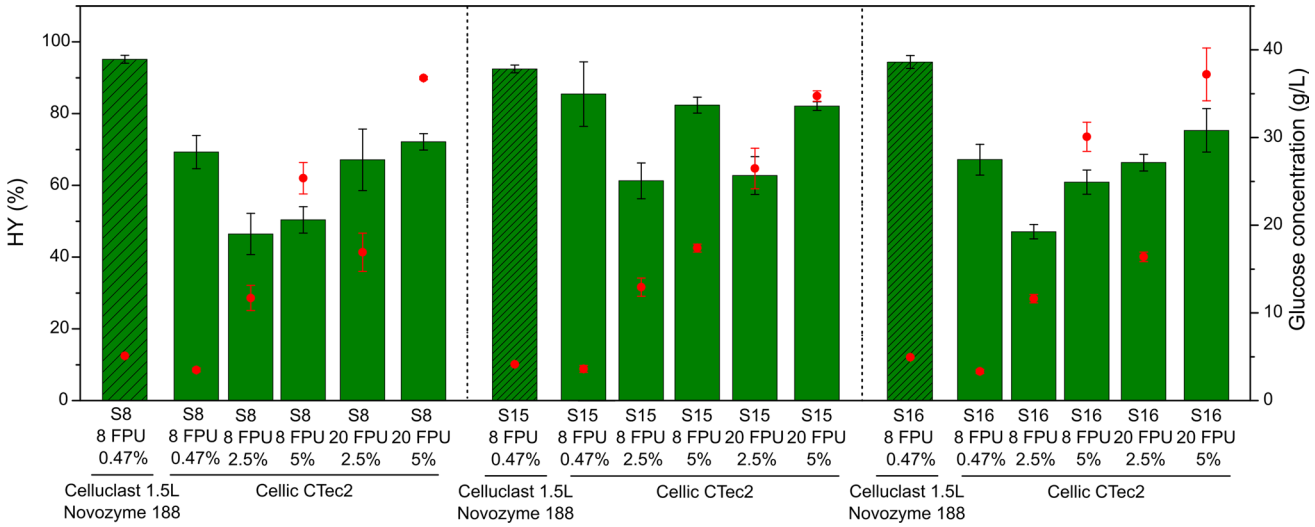


Fig. 7 HY and glucose concentration obtained from samples S8, S15, and S16 hydrolyzed with the enzymatic cocktails Celluclast 1.5 L/Novozyme 188 or Cellic CTec2 for 24 h. Green bars indicate HY and red circles indicate glucose concentration. (Color figure online)

resulted in HY of 70.9% after enzymatic hydrolysis with 25 FPU/g Accellerase® for 72 h [26].

Conclusions

Acid-alkaline or only alkaline treatments applied to rice straw resulted in improved glucose conversion from 18% (in natura sample) to more than 90% under optimized pretreatment conditions, using a short enzymatic reaction time (24 h) and low enzyme charge (8 FPU/g) of a mixture of enzymatic cocktails: Celluclast 1.5 L and Novozyme 188. Optimized conditions were also evaluated using a different enzymatic cocktail (Cellic CTec2), resulting in cellulose conversion of up to 82% glucose and concentration up to 37 g/L at 5% of solid content. These outstanding results were achieved due to the pretreatment optimizations using DOE tools, which allowed a simultaneous evaluation of the two types of pretreatments. The optimization showed that the acid step was not crucial for achieving higher hydrolysis yields, but that it can be helpful for hemicellulose use in a biorefinery approach. Chemical characterization showed the effective removal of hemicellulose in the acid step, and lignin and ashes in the alkaline step, which was responsible for the pretreatment effectiveness. Morphological analyses corroborated enzymatic hydrolysis and chemical composition and allowed us to observe the effect of the pretreatments on silica structures. The use of DOE for simultaneous optimization of the pretreatments, the analysis of additional enzymatic hydrolysis conditions, and the correlations between chemical and morphological changes in the substrate should contribute to a better understanding of the most relevant parameters for the use of rice straw as a valuable lignocellulosic substrate in the biofuel and chemical production.

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Author Contributions All authors contributed to the study conception and design. Biomass treatments, enzymatic hydrolysis, and data collection for characterizations were performed by Bruna R. Moreira and Eupidio Scopel; analyses were performed by all authors. The first draft of the manuscript was written by Bruna R. Moreira and Eupidio Scopel, and all authors commented on previous versions of the manuscript. Marcia C. Breitkreitz and Camila A. Rezende supervised the project. Funding was provided by Camila A. Rezende. All authors read and approved the final manuscript.

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Data Availability The datasets generated during and/or analyzed during this study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors have no relevant financial or nonfinancial interests to disclose.

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