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Article

Pretreatment and Enzymatic Saccharification of Water Hyacinth, Sugarcane Bagasse, Maize Straw, and Green Coconut Shell Using an Organosolv Method with Glycerol and $FeCl_3$

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A method to pretreatment of biomasses using glycerol as a green solvent was developed. Optimization of organosolv pretreatment was performed using a 2^3 factorial design, investigating synergism among the variables particle size (from < 0.85 to > 2.0 mm), $FeCl_3$ concentration (0.025-0.175 mol L^{-1}), and temperature (160-220 °C). Although the effects of the variables differed according to the type of lignocellulose, it was nonetheless possible to find an optimal condition in common (< 0.85 mm; 0.025 mol L^{-1}; 220 °C), which was economically and environmentally sustainable, employing a small amount of catalyst. The biomasses pretreated using this combination of factors exhibited enzymatic digestibility exceeding 93% after 48 h, reflecting efficient reduction of recalcitrance as a result of high removal of hemicellulose (ca. 90%), provided by the use of $FeCl_3$, together with substantial delignification. There was low formation of inhibitors in the hydrolysate, indicating that it could be directly fermented. The lignin removed to liquors could be easily recovered by centrifugation. Tests carried out under the optimal condition revealed that after the pretreatment the biomass could be directly used in enzymatic hydrolysis, without washing and wet. Furthermore, after simple treatment, crude glycerol was as effective as commercial glycerol.

Keywords: biomass, ferric chloride, reducing sugars, bioethanol, lignin, experimental design

Introduction

In the production of cellulosic ethanol, the recalcitrance of the biomass requires it to be submitted to a pretreatment step to break down the lignocellulose, in order to obtain good yields in the hydrolysis and fermentation steps.[1,2] However, the costs and environmental impacts associated with pretreatment can make the production of this second-generation biofuel unfeasible. Therefore, it is necessary to develop pretreatment methods that are more efficient, economically viable, and environmentally sustainable, enabling the development of a biorefinery where the biomass is converted into bioethanol and other value-added chemical products, minimizing the generation of waste and maximizing profits, while contributing to the implementation of a bioeconomy.[1,3]

The organosolv process is one of the possible pretreatment methods, involving the application of organic solvents for the fractionation and subsequent use of the biomass components.[4-6] The use of glycerol as a solvent is attractive, since it is economically and environmentally sustainable, while the possibility of using crude glycerol from the production of biodiesel further contributes to the sustainability of the process. Glycerol is a nontoxic organic solvent with a high boiling point, so it can be used at atmospheric pressure, making the process safer and avoiding the need for sophisticated equipment. Therefore, it is necessary to expand research efforts aimed at ensuring the feasibility of using crude glycerol.[7-10]

The use of metallic salts ($AlCl_3$, $FeCl_3$, $Fe_2(SO_4)_3$, and others) to substitute conventional catalysts (NaOH, HCl, and H_2SO_4) has been investigated for the pretreatment of various biomasses. In particular, $FeCl_3$ can be highlighted for its capacity to reduce the recalcitrance of biomasses.[11-14]

Various types of lignocellulosic biomass have been studied to determine their energy potentials in the production of cellulosic ethanol and other added-value chemicals. Several such biomasses may be highlighted. The water hyacinth (*Eichhornia crassipes*), an aquatic

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plant that has a high growth rate and is difficult to control, is considered an invasive weed in many regions worldwide, because it can degrade ecosystems, obstruct navigation routes, and damage irrigation channels and hydroelectric installations.[1,15-17] Sugarcane bagasse is an abundant agroindustrial residue generated in the sugar and alcohol industry.[18-21] Maize straw is a waste produced in large quantities from the processing of maize, an important agricultural product in many regions of the world.[13,22,23] Green coconut shell is a waste that has become increasingly abundant and problematic as coconut production and consumption has expanded. After extraction of the edible fraction, the shell represents 80% of the initial mass and takes around eight years to degrade.[24-28]

These biomasses are good candidates for the production of cellulosic ethanol, due to their high contents of carbohydrates, the fact that they are renewable resources, and the need to resolve environmental problems related to their disposal. Hence, the main objective of this work was to optimize an organosolv pretreatment method employing glycerol, in the presence of $FeCl_3$, applied in the deconstitution of water hyacinth, sugarcane bagasse, maize straw, and green coconut shell, evaluating the effect of using different types of lignocellulose. Investigation of the application of crude glycerol was also made, aiming at amplifying the use of this material.

Experimental

Biomass

Water hyacinth was collected at the Açude Macela reservoir, located in the municipality of Itabaiana (Sergipe State, Brazil). Sugarcane bagasse, maize straw, and green coconut shells were purchased at an open-air market in the city of Aracaju (Sergipe State, Brazil). The compositions of these materials are shown in Table 1.

After drying by exposure to solar irradiation, the biomasses were processed to obtain three particle sizes: smaller than 0.85 mm, 0.85-2.0 mm, and larger than 2.0 mm (2.0-4.0 mm). In the case of the water hyacinth, all parts of the plant were triturated together, while in the case of the coconut shell, the epicarp was triturated together with the mesocarp.

Reagents

Commercial analytical grade glycerol (99.5%) was purchased from Dinâmica Química Contemporânea Ltda. (São Paulo, Brazil). Crude glycerol from cotton biodiesel production was provided by the Center for Strategic Technologies of the Northeast (CETENE, Caetés, Pernambuco, Brazil). Iron(III) chloride was obtained in the form of analytical grade $FeCl_3.6H_2O$ (97-102%, Dinâmica, Indaiatuba-SP, Brazil). The Cellic CTec3 cellulase and hemicellulase enzyme complex was obtained from Novozymes Latin America Ltda. (Araucária, Paraná, Brazil). Carbohydrate standards, HMF (hydroxymethylfurfural), and furfural (purity ≥ 99.0%), as well as high-performance liquid chromatography (HPLC) grade acetic acid, were acquired from Sigma-Aldrich (São Paulo, Brazil). Other reagents used were anhydrous citric acid (99.5%, Synth, São Paulo, Brazil), hydrochloric acid (37%, Química Moderna, Barueri-SP, Brazil), sulfuric acid (95-99%, Vetec, Rio de Janeiro, Brazil), phosphoric acid (85%, Neon, São Paulo, Brazil), acetonitrile (≥ 99.9%, LiChrosolv, Merck, USA), sodium hydroxide (98.0%, Synth, São Paulo, Brazil), 3,5-dinitrosalicylic acid (99.8%, Neon, São Paulo, Brazil), and potassium sodium tartrate tetrahydrate (99.5%, Neon, São Paulo, Brazil).

Organosolv pretreatment with glycerol

A 2^3 factorial experimental design was used to optimize the process. The variables considered were particle size (< 0.85, 0.85-2.0, and > 2.0 mm), temperature (160-220 °C), and $FeCl_3$ concentration (0.025-0.175 mol L^{-1}). The combinations of levels of the variables used in the assays (design matrix) are shown in Table 2, together with the responses. The responses evaluated were the solids yield after pretreatment and the percentage of sugars released in the enzymatic hydrolysis. Statistical analysis was performed using Statistica v. 7.0 software.[29]

All the pretreatment assays employed 1.5 g of the dried and triturated *in natura* biomass. Considering the

Table 1. Compositions of the four *in natura* biomasses evaluated in this work

Biomass	Composition / %					
	Cellulose	Hemicellulose	Lignin	Ash	Moisture	Extractives
Water hyacinth	25.5 ± 0.9	13.8 ± 0.5	12.0 ± 1.5	17.4 ± 0.12	8.3 ± 0.2	14.8
Sugarcane bagasse	32.8 ± 0.5	25.2 ± 1.5	18.9 ± 2.4	1.4 ± 0.01	6.6 ± 0.1	11.9
Maize straw	35.1 ± 0.8	29.5 ± 1.9	7.0 ± 0.2	2.3 ± 0.07	7.1 ± 0.2	11.2
Coconut shell	26.3 ± 0.3	14.1 ± 1.0	27.2 ± 0.4	5.0 ± 0.01	6.4 ± 0.1	17.3

Table 2. Factorial design (2^3) matrix with the responses: solids yield and total sugars released in 24 h of enzymatic hydrolysis

Assay	Variable (coded values in parentheses)			Response							
	Particle size (1) / mm	$FeCl_3$ concentration (2) / (mol L^{-1})	Temperature (3) / °C	Solids yield / %				Sugars released (24 h) / %			
				Water hyacinth	Sugarcane bagasse	Maize straw	Coconut shell	Water hyacinth	Sugarcane bagasse	Maize straw	Coconut shell
1	< 0.85 (–1)	0.025 (–1)	160 (–1)	62.5	49.1	44.6	52.6	14.2	35.6	43.9	16.9
2	> 2.0 (+1)	0.025 (–1)	160 (–1)	87.3	85.4	46.6	86.9	14.7	32.2	40.9	8.7
3	< 0.85 (–1)	0.175 (+1)	160 (–1)	28.3	36.0	23.6	27.2	44.0	56.0	66.6	24.3
4	> 2.0 (+1)	0.175 (+1)	160 (–1)	66.5	50.0	33.4	48.5	35.4	43.6	55.7	24.8
5	< 0.85 (–1)	0.025 (–1)	220 (+1)	37.0	35.0	27.6	34.0	54.1	50.5	56.3	27.2
6	> 2.0 (+1)	0.025 (–1)	220 (+1)	44.6	37.6	33.1	57.0	33.3	49.3	56.3	25.8
7	< 0.85 (–1)	0.175 (+1)	220 (+1)	15.5	11.7	14.9	13.4	60.8	37.0	64.5	14.0
8	> 2.0 (+1)	0.175 (+1)	220 (+1)	16.0	19.1	15.9	16.0	42.7	27.5	51.0	21.0
9	0.85-2.0 (0)	0.1 (0)	190 (0)	38.8	39.1	28.6	33.7	39.3	48.8	57.4	21.7
10	0.85-2.0 (0)	0.1 (0)	190 (0)	37.4	35.4	27.5	27.3	35.5	45.7	63.6	22.8
11	0.85-2.0 (0)	0.1 (0)	190 (0)	39.0	37.1	29.9	32.2	41.7	45.6	59.6	22.9

optimization performed in a previous study,[30] the reaction time used was 10 min (after thermal stabilization) and the solid/liquid ratio (m/m) was 6% (1.5 g biomass *per* 20.0 mL glycerol).

The reaction medium was placed in a round-bottom flask coupled to a reflux system and was heated in an oil bath, using a heating plate with a magnetic stirrer. The oil bath was preheated to the desired temperature and the stirring speed was kept at 300 rpm. The reaction time was measured from when the reaction medium reached the required temperature. After the reaction, the flask was cooled in a water bath at ambient temperature. The liquid fraction was separated from the solid fraction by cloth filtration, with 50 mL of tap water used to ensure complete transfer of the solid residue from the flask. The pH of the liquid fraction (liquor) was measured. The solid fraction (pretreated biomass) was washed three times with 100 mL volumes of tap water and was then dried at 35 °C, followed by measuring the mass.

After determining the condition considered optimal, evaluation was made of (*i*) submitting the biomass directly to the enzymatic hydrolysis process, without washing and wet; (*ii*) performing the pretreatment reaction without catalyst ($FeCl_3$); (*iii*) halving the quantity of catalyst ($FeCl_3$); and (*iv*) substituting $FeCl_3$ by HCl.

Crude glycerol application

Crude glycerol was employed in reactions with water hyacinth, under the condition considered optimal, according to the 2^3 factorial design. The glycerol was used as received (pH 8.6), without any type of treatment, as well as after being submitted to different simple treatments. The first of these was evaporation, with the crude glycerol being placed in a beaker that was immersed in an oil bath at 120 ± 5 °C for 3 h. The second treatment was precipitation, with the pH of the crude glycerol being reduced to 3.0 by the addition of 85% phosphoric acid, followed by transfer to a separation column for 24 h, resulting in the separation of glycerol (lower phase) from the precipitated insoluble fatty acids (upper phase). The third treatment method employed both procedures, with precipitation being followed by evaporation.

The reactions were firstly performed under reflux, using the as-received glycerol, the evaporated glycerol, and the precipitated and evaporated glycerol. The reactions were then performed with the reactor open (without coupling the flask containing the reaction medium to the reflux system), using the as-received glycerol and the precipitated glycerol. Evaluation was also made of using the biomass pretreated with the precipitated crude glycerol (keeping the reactor open) directly in the enzymatic hydrolysis process, without passing through the washing and drying steps.

Lignin recovery

The lignin removed to the liquor was recovered by centrifugation for 10 min at 3,500 rpm (5810 R centrifuge, Eppendorf), followed by washing and drying in an oven at 40 °C. The washing was performed with the centrifuge using two steps, in order to ensure the removal of excess glycerol. The total volume of water used was equivalent to 40% of the volume of the liquor that was centrifuged. In each washing step, half of the water required was added to

the Falcon tube containing the solid (lignin), with vigorous stirring for homogenization, followed by centrifugation for 10 min.

Enzymatic hydrolysis

The enzymatic hydrolysis was performed with the Cellic CTec3 enzyme complex at proportions in the range 10-20 FPU (filter paper unit) $g_{biomass}^{-1}$. Erlenmeyer flasks were filled with 0.125 g of pretreated biomass, 15 mL of 50 mmol L^{-1} citrate buffer solution (pH 4.85), and 5 µL of Cellic CTec3. The flasks were incubated for 48 h at 48.5 °C, with stirring at 100 rpm. Aliquots of the reaction medium were removed at regular time intervals (20, 24, 28, and 48 h) and filtered through 0.22 µm nylon filters. The percentage of reducing sugars (RS) released in the enzymatic hydrolysis was calculated using equation 1:

$$\%RS = 100 \frac{C_{RS}(g\ L)^{-1}}{C_{biomass}(g\ L^{-1})} \quad (1)$$

where, C_{RS} (g L^{-1}) is the concentration of reducing sugars released in the hydrolysis, and $C_{biomass}$ (g L^{-1}) is the ratio between the quantity (in grams) of pretreated biomass and the volume of buffer used in the enzymatic hydrolysis.

Methods of analysis

Moisture and ash content determinations

The methodologies employed for determination of the moisture and ash contents of the biomasses were adapted from the procedures used by the National Renewable Energy Laboratory (NREL): NREL/TP-510-42621[31] and NREL/TP-510-42622,[32] respectively.

Determination of reducing sugars and composition analysis

The total reducing sugars were determined by the 3,5-dinitrosalicyclic acid (DNS) method, using a methodology adapted from Bernfeld.[33] The lignin, hemicellulose, and cellulose contents of the *in natura* and pretreated biomasses were determined according to NREL procedures,[34] with adaptation only of the analytical methodology used for sugar quantification. The total content of hexoses was quantified as cellulose, while the total content of pentoses was quantified as hemicellulose, applying the appropriate anhydrous corrections (0.88 and 0.90 for C-5 and C-6 sugars, respectively).

The pentoses and hexoses contents were determined by HPLC, using a Prominence instrument (Shimadzu, Kyoto, Japan) equipped with an evaporative light scattering detector (ELSD-LTII, Shimadzu, Kyoto, Japan) and a Phenomenex Luna NH_2 3µ 100 Å column (150 × 2.0 mm). The mobile phase consisted of ultrapure water (eluent A) and HPLC grade acetonitrile (eluent B), in gradient elution mode (95% B: 0.00-0.01 min; 80% B: 0.01-15 min; 95% B: 15-20 min), at 0.8 mL min^{-1} and 38 °C. The ELSD conditions were as follows: gain of 12; 35 °C; 350-360 kPa (N_2).

The recoveries of the components (cellulose, hemicellulose, and lignin), degree of removal, cellulose digestibility, and glucose yield were calculated using equations 2-6:

$$\text{Component recovered (\%)} = \frac{\text{Component in pretreated biomass (\%)} \times \text{Solid yield (\%)}}{\text{Component in native biomass (\%)}} \quad (2)$$

$$\text{Solids yield (\%)} = \frac{\text{Dry biomass mass after pretreatment}}{\text{Mass of in natura biomass before pretreatment}} \times 100 \quad (3)$$

$$\text{Degree of removal(\%)} = 100 - \text{component recovered} \quad (4)$$

$$\text{Cellulose digestibility (\%)} = \frac{\text{Glucose produced via enzymatic hydrolysis (g)} \times 0.9}{\text{Cellulose in pretreated biomass (g)}} \times 100 \quad (5)$$

$$\text{Glucose yield (\%)} = \frac{\text{Glucose produced via enzymatic hydrolysis(g)} \times 0.9}{\text{Cellulose in native biomass (g)}} \times 100 \quad (6)$$

Determinations of HMF, furfural, and acetic acid

HMF, furfural, and acetic acid were determined by HPLC, using a Prominence instrument (Shimadzu, Kyoto, Japan) equipped with a diode array detector (DAD) and a Shim-Pack VP-ODS column (4.6 × 250 mm) maintained at 35 °C. Isocratic elution mode was employed, with a mobile phase consisting of acetonitrile (ACN):H_2O (20:80 v/v) acidified to pH 3.0 with phosphoric acid, at a flow rate of 0.8 mL min^{-1}. The run time was 10 min and the injection volume was 10 µL. Acetic acid was detected at 210 nm, while HMF and furfural were detected at 280 nm.

Fourier transform infrared spectroscopy (FTIR) analyses

FTIR spectra were obtained using an IRPrestige-21 instrument (Shimadzu, Kyoto, Japan), in the range 4000-400 cm^{-1}, with a total of 32 scans and resolution of 8 cm^{-1}. The samples were prepared by dispersion in potassium bromide.

Results and Discussion

Optimization of the pretreatment

The compositions of the *in natura* biomasses (Table 1) indicated that they were all suitable for the production of second generation ethanol since they contained high levels of carbohydrates. The sugarcane bagasse and maize straw stood out as having the highest levels of cellulose and hemicellulose, while the coconut shell

had the highest amount of lignin, giving the material its characteristic rigidity. The water hyacinth presented a high ash content, which was probably related to the ability of this aquatic plant to remove minerals (including heavy metal contaminants) from water bodies.

A 2^3 factorial experimental design (Table 2) was used, because it was well suited to the study proposal, enabling the optimization of three variables with a small number of experiments, making it possible to identify the interactions and evaluate the experimental error. The influence of particle size has generally received little attention in the literature. However, all research related to the production of second-generation ethanol has highlighted the need to reduce the particle size of the biomass, with different sizes having been used in the published studies. The variables temperature and $FeCl_3$ concentration were optimized in a previous study,[30] but only for water hyacinth biomass at a particle size of 0.85-2.0 mm. Furthermore, the best $FeCl_3$ concentration was defined after the temperature had already been optimized. Therefore, in order to evaluate other particle sizes and expand the study to other biomasses, it was important to determine how these three variables behaved together, considering the influence that each one exerted on the others.

The solids yields of the pretreated biomasses (Table 2) provided the first indication of the effect of the pretreatment on the structures of the *in natura* biomasses. A high solids yield indicates little structural change (low removal of lignin and hemicellulose) and, consequently, low reduction of recalcitrance. Meanwhile, a low solids yield can indicate excessive degradation, with undesired removal of cellulose. In both cases, the result is a low sugars yield in the hydrolysate, so it is necessary to find an intermediate condition.

It can be seen from Table 2 that for all the biomasses, the solids yield varied substantially according to the pretreatment condition. The smallest mass loss was observed in experiment 2, while experiment 7 showed the greatest mass reduction. These results could be explained by the different levels of the variables. Experiment 2 was performed using the mildest conditions of temperature and $FeCl_3$ concentration, as well as the largest particle size. Experiment 7 was conducted using the most severe conditions of temperature and quantity of catalyst, together with the smallest particle size, with the highest surface area favoring structural alterations.

In terms of the percentage of sugars released (Table 2), experiment 7 showed a higher yield than experiment 2, although the difference was only significant for water hyacinth. The other pretreatment conditions provided sugar release equivalent to or better than that of experiment 7.

Figures 1a-1d show the influence of the pretreatment condition on the release of sugars in the enzymatic saccharification process, from 20 to 48 h. The conditions of experiment 7 only led to the best release of sugars in the case of water hyacinth (Figure 1a), with the release

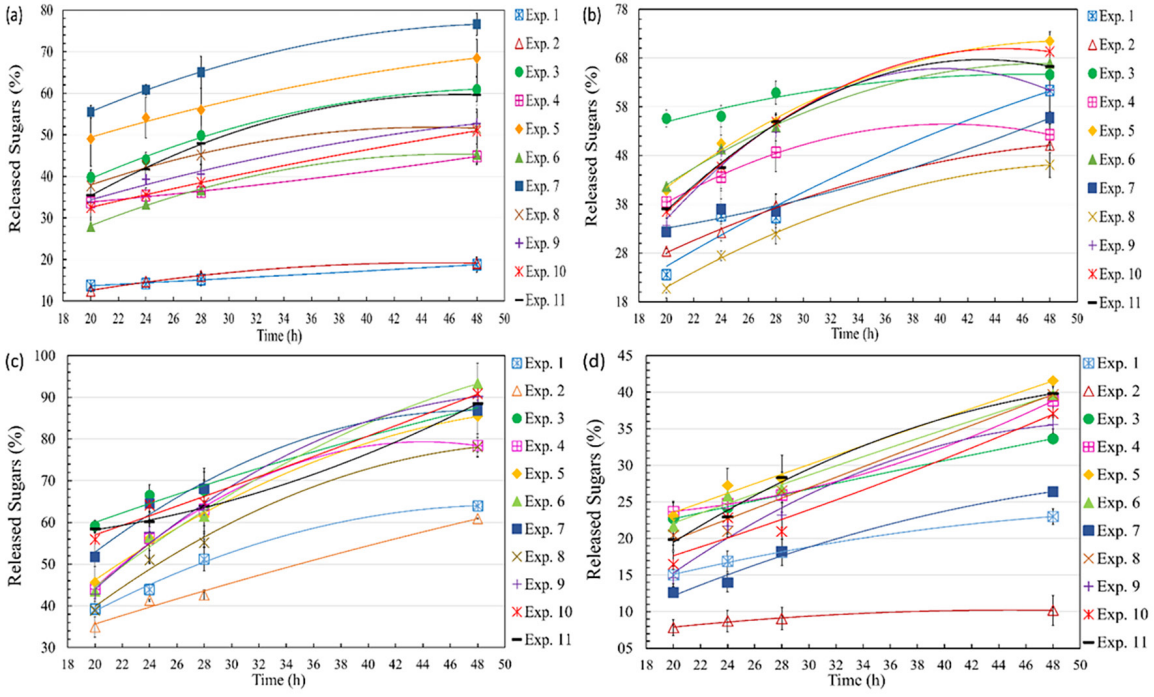

Figure 1. Percentage of sugars released, as a function of enzymatic hydrolysis time, for the four biomasses: (a) water hyacinth; (b) sugarcane bagasse; (c) maize straw; (d) coconut shell.

being similar to that obtained in experiment 5, considering the standard deviations. Hence, the low solids yield was probably accompanied by an excessive loss of cellulose.

Although the studied biomasses had different amounts of cellulose, the effect of biomass recalcitrance could not be mitigated by a greater amount of enzyme, since the enzyme acts on the cellulose fraction and the success if its action depends on access to it. Hence, if there was difficulty in accessing the cellulose, due to factors such as crystallinity or coating with lignin, among others, an excess of enzyme would not make a difference, since the barrier preventing its action would continue to exist and would not be eliminated by use of a greater amount of enzyme. Throughout the development of the work, tests were carried out for the same pretreated biomass sample, varying the amount of enzyme (10, 15, and 20 FPU g^{-1}). It was observed (Figure S1, Supplementary Information (SI) section) that an increase in the amount of enzyme did not necessarily provide greater release of sugars, since the use of 15 FPU g^{-1} provided better release in 48 h of enzymatic hydrolysis. Given this result, together with the fact that it is common to use values in the range 10-20 FPU g^{-1}, a concentration of 15 FPU g^{-1} was adopted in the present work.

Hence, the action of the enzyme depends on the cellulose content and the recalcitrance of the biomass. By fixing the solids/enzyme ratio, it is possible to identify the pretreatment condition that provides the greatest reduction of recalcitrance, together with the best preservation of cellulose, consequently maximizing the release of sugars. Previous studies reported in the literature[8,20,26-28] concerning the production of second-generation ethanol have usually maintained a fixed solids/enzyme ratio.

As shown in Figures 1a-1d, the conditions of experiment 2 led to low efficiency, with small structural changes. This inefficiency was equivalent to that observed for water hyacinth in experiment 1 (Figure 1a), with similar values for sugarcane bagasse in experiments 1, 7, and 8 (Figure 1b), and was greatest for maize straw (Figure 1c) and coconut shell (Figure 1d).

Statistical analysis

Statistical analysis of the effects was used to obtain a better understanding of the influence of each variable. For this, the total sugars yield obtained at the midpoint time (24 h) of the enzymatic saccharification was used (Table 2).

The estimated effects of the variables on the release of sugars are shown in Figure 2, in the form of a Pareto diagram to facilitate visualization of the significant effects. The heights of the bars indicate the $t_{calculated}$ values. The $t_{tabulated}$ values, related to the degrees of freedom of the residuals, indicate the limit from which the effects are significant for the response, considering a significance level of 5% ($p \leq 0.05$).

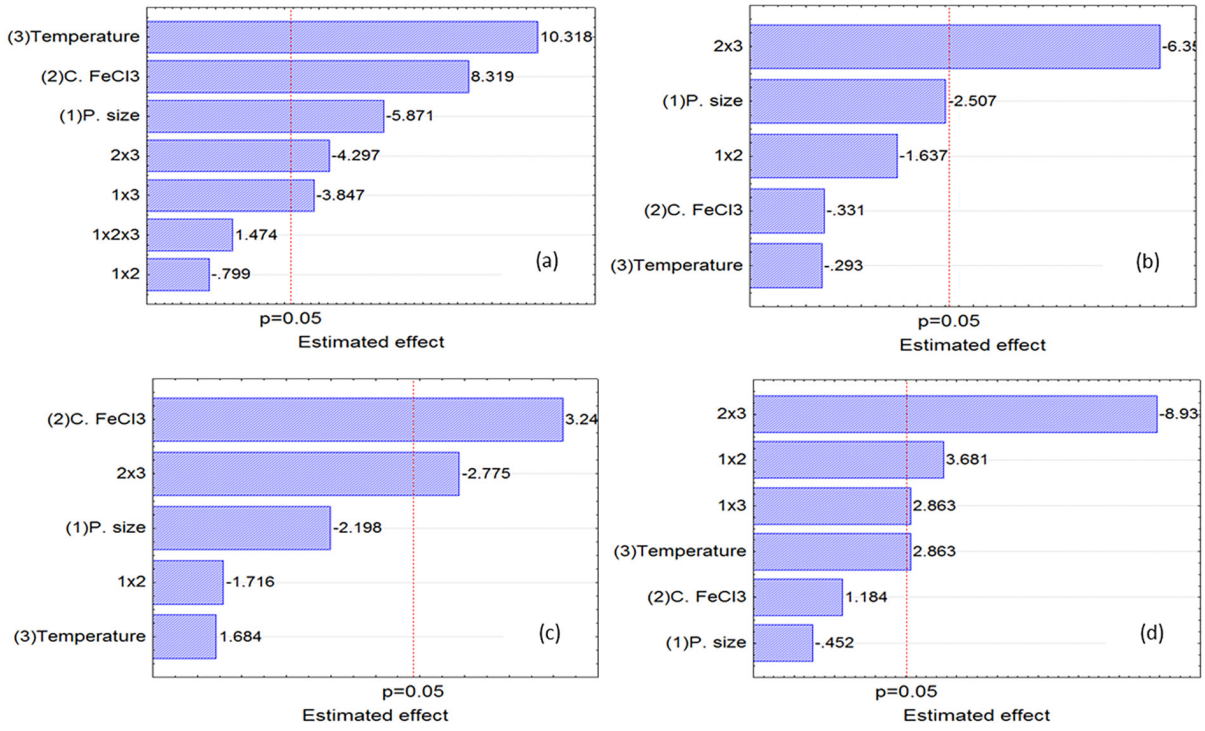

Figure 2. Pareto diagrams showing the significant effects on sugars release (5% significance level, $p \leq 0.05$), for (a) water hyacinth; (b) sugarcane bagasse; (c) maize straw; (d) coconut shell.

For water hyacinth (Figure 2a), the main effects of temperature (3), $FeCl_3$ concentration (2), and particle size (1) were significant (in that order of relevance). Significant effects were also observed for the interactions $FeCl_3$ concentration × temperature (2 × 3) and particle size × temperature (1 × 3).

The temperature had a strong influence on pretreatment of the water hyacinth, in agreement with our previous work.[30] This was the variable with the greatest positive effect on the release of sugars, showing that the structural alteration of the water hyacinth was facilitated at high temperature (220 °C). The $FeCl_3$ concentration also showed a positive effect on the percentage of sugars released, with better yields at the highest concentration (0.175 mol L^{-1}). In contrast, particle size had a substantial negative effect, showing that the pretreatment reaction was favored by increase of the surface area, which was achieved by reducing the particle size.

Experiment 7, which showed the highest sugars release for water hyacinth (Figure 1a), was performed with these three variables kept at the optimal levels indicated by the statistical analysis (220 °C, 0.175 mol L^{-1}, and < 0.85 mm). However, the interaction effects between temperature and $FeCl_3$ concentration, as well as between temperature and particle size, which were both negative, indicated that it would be possible to obtain good results using other combinations.

It can be seen from Figure 1a that in experiment 5, performed with the $FeCl_3$ concentration reduced to a low level (0.025 mol L^{-1}), the yield was not much lower than in experiment 7, since the temperature was kept at the high level (220 °C) and the particle size at the low level (< 0.85 mm).

For the sugarcane bagasse, the Pareto diagram (Figure 2b) showed that only the interaction between $FeCl_3$ concentration and temperature (2 × 3) was significant for the response. Different to the water hyacinth, the main effects of temperature and $FeCl_3$ concentration were the least significant. Both were negative, indicating that changing from the lower level to the upper level led to a decrease of the response. However, since the interaction of these variables was significant, their effects should be interpreted together.

The greatest release of sugars in the enzymatic hydrolysis was observed for the experiments in which these variables were equilibrated (Figure 1b). When the temperature and the $FeCl_3$ concentration were set at low or high levels, the yield decreased (experiments 2 and 8). Hence, in the case of the sugarcane bagasse, it was necessary to control the severity of the pretreatment. When the temperature level was increased, the $FeCl_3$ concentration level should be kept low, and *vice versa* (experiments 3 and 5). The central point condition was also favorable.

For the maize straw, the Pareto diagram (Figure 2c) showed that the main effect of the $FeCl_3$ concentration and the effect of interaction between this variable and temperature (2 × 3) were significant. Given the positive effect of the $FeCl_3$ concentration, the experiments performed with this factor at the upper level showed sugar releases that were similar and satisfactory (experiments 3, 4, 7, and 8). In addition, the significant 2 × 3 interaction effect enabled good results to be achieved in the assays where the $FeCl_3$ concentration was kept at the low level, if the temperature was kept at the high level (experiments 5 and 6). The central condition (experiments 9, 10, and 11) also provided a favorable balance (Figure 1c).

For the coconut shell, the Pareto diagram (Figure 2d) showed that the most significant effect for the release of sugars was the interaction between the $FeCl_3$ concentration and temperature (2 × 3), followed by the effects of interaction between particle size and $FeCl_3$ concentration (1 × 2), and particle size and temperature (1 × 3), as well as the main effect of temperature, which was significant and positive. Hence, it could be seen that for coconut shell, an appropriate combination among the variables was essential for achieving satisfactory release of sugars in the enzymatic saccharification process.

The negative effect of the interaction between $FeCl_3$ and temperature (2 × 3) indicated that the simultaneous increase of the levels of these two variables would excessively increase the severity of the reaction, leading to a low yield due to high degradation. In addition, the positive effects of the 1 × 2 and 1 × 3 interactions indicated that with increases of the $FeCl_3$ concentration (2) and temperature (3), the particle size (1) should also be maintained at the upper level (> 2.0 mm), in order to avoid excessive structural alteration.

It was evident that the low yield of experiment 7 (Figure 1d) was due to a severe unfavorable combination of the three factors, while the greatest release provided by the conditions of experiment 5, followed by experiments 6, 4, 3, and the assays for the central condition, was due to favorable balances among the levels of the variables.

The experimental design was important for obtaining a better understanding of the effects of the variables in the pretreatment reactions, notably because it enabled identification of interactions among them, showing that they were interdependent, with the ideal level for one being dependent on the level of the other. For all the biomasses, the interaction between the $FeCl_3$ concentration (2) and temperature (3) was significant for the response. For better clarity, Figures 3a-3d show the effects of this interaction for the four biomasses.

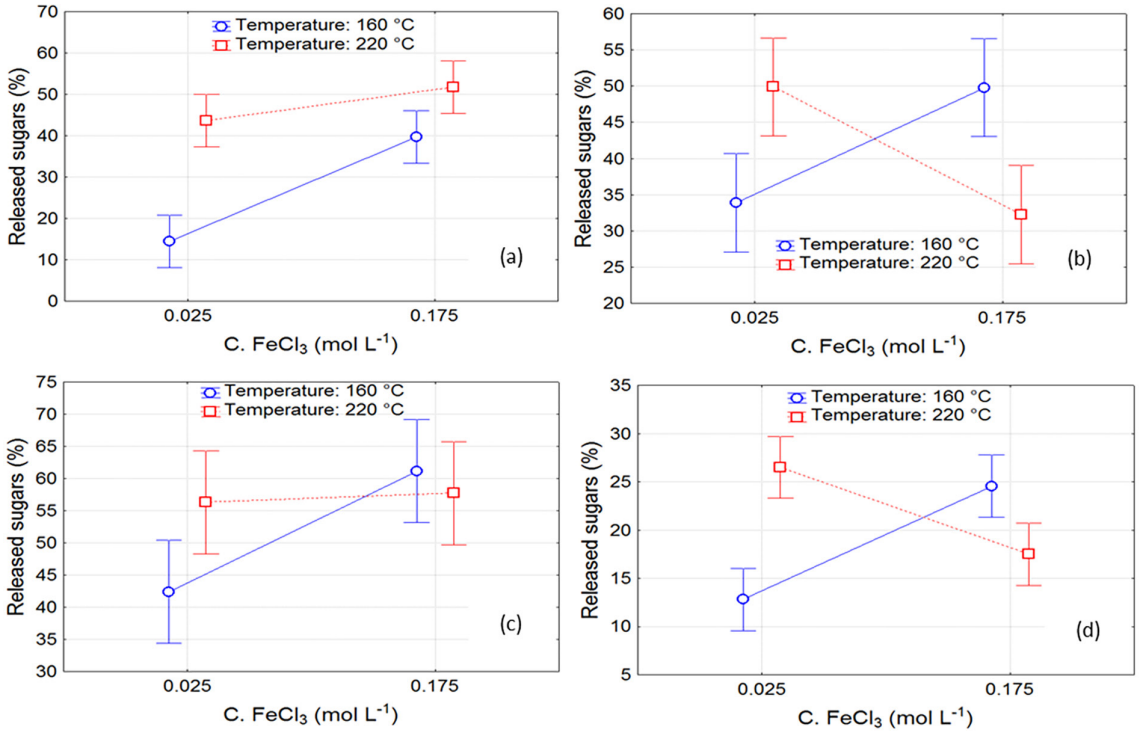

Figure 3. Illustration of significant interaction between the variables $FeCl_3$ concentration and temperature: (a) water hyacinth; (b) sugarcane bagasse; (c) maize straw; (d) coconut shell.

For water hyacinth (Figure 3a), when the temperature was set at the low level (160 °C), increase of the $FeCl_3$ concentration resulted in a substantial increase of sugars release. However, at high temperature (220 °C), there was only a slight effect of increasing the $FeCl_3$ concentration. Similar effects were observed for the maize straw (Figure 3c).

For the sugarcane bagasse (Figure 3b) and coconut shell (Figure 3d), the use of a pretreatment temperature of 160 °C resulted in the sugars release in the enzymatic hydrolysis being favored by $FeCl_3$ at the highest level. However, when the biomass was pretreated at 220 °C, use of the highest concentration of the catalyst was unfavorable and led to excessive degradation.

The results showed that for the four biomasses, it was preferable to use a pretreatment temperature of 220 °C and to substantially reduce the amount of catalyst (by 86%), which increased both economic viability (due to lower reagent consumption) and environmental sustainability (since the final effluent was less contaminated).

For water hyacinth, experiment 7 (Figure 1) provided the best release of sugars, but it employed the catalyst at the high level. Consequently, the conditions of experiment 5 were more viable, since a much smaller amount of catalyst was used, without any significant decrease of the yield. In addition, the low solids yield of experiment 7 (Table 2) was unfavorable, considering the release of sugars from the entire pretreated biomass. Therefore, the pretreatment conditions of experiment 5 were considered ideal for the water hyacinth.

From comparison of the optimized conditions of the water hyacinth pretreatment achieved by means of the 2^3 experimental design (220 °C; 0.025 mol L^{-1}; > 0.85 mm) with the optimization obtained in our previous work,[30] it was evident that the process had become more sustainable. Approximately the same sugars yield was achieved in 24 h of enzymatic hydrolysis (54 ± 5% in the present work, compared to 51 ± 3% in the previous work),[30] using a 75% lower $FeCl_3$ concentration. It should be noted that the only factor responsible for this lower consumption of catalyst was the smaller particle size. Hence, for water hyacinth, the consequent increase of the surface area facilitated the structural alteration and enabled reduction of the quantity of catalyst.

The pretreatment condition of experiment 5 was also most suitable for the sugarcane bagasse. It can be seen from Figure 1b that the conditions of experiment 6 and those at the central point (9, 10, and 11) resulted in sugars releases that were very similar to that of experiment 5. However, for the central condition, it was preferable to increase the temperature by 30 °C, in order to achieve a 75% reduction of $FeCl_3$ (the condition of experiment 5). In the case of experiment 6, the only difference was in the particle size. Since the solids yield was similar, it could

therefore be seen that it was possible to obtain similar results using a wide range of particle sizes, since this factor was not significant for the response.

For the maize straw, the conditions of experiment 5 were again optimal, considering the sustainability of the process in terms of minimizing the amount of catalyst used, with sugars release similar to the amounts observed for other less viable conditions (Figure 1c). The conditions of experiment 6 were also shown to be satisfactory, with sugars release and solids yield equivalent to those of experiment 5. As in the case of the sugarcane bagasse, this indicated the possibility of using a wide range of particle sizes, since this factor was not significant for the response.

For the coconut shell, the conditions of experiment 5 also provided the best pretreatment, with the highest sugars yield (Figure 1d) and satisfactory sustainability of the process. Experiment 6 could also be highlighted, presenting sugars release very similar to that of experiment 5, but a considerably higher solids yield. As already mentioned, the only difference between these experiments was the particle size, which was not a significant variable for the response. Hence, a wide range of particle sizes could also be employed for coconut shell, considering the pretreatment procedure adopted.

The analysis of the effects (Figure 2) revealed that the inherent structural characteristics of the different biomasses had a major influence on the effects of the variables and their importance in the pretreatment reactions. Nonetheless, it was possible to identify a condition (experiment 5) that was suitable in all cases and could be considered the optimized condition for all the biomasses.

The results obtained here showed that the particle size should not be neglected, since it can influence the outcome of the pretreatment. It should be noted that there was no relationship with the content of lignin (the biomass component that confers resistance), since the coconut shell, which contained the greatest quantity of lignin, did not need to be substantially reduced in size. In contrast, the water hyacinth, which had much lower lignin content, required a considerable reduction of particle size.

As the effects of the variables differed among the biomasses, the fitted models were also different. However, use of analysis of variance (Table S1, SI section) enabled the fitting of linear models for all the biomasses (equations S1-S4, SI section). In all cases, $F_{calculated}$ was higher than $F_{tabulated}$ and the R^2 (determination coefficient) values explained 85.2-98.8% of the total variance.

Comparison of the model predictions with the experimental results for the optimized condition (experiment 5) showed excellent fits of the theoretical model to the experimental data (Table S2, SI section). Since the analysis of variance confirmed that the data fitted the model, it was possible to generate response surfaces (Figure S2, SI section).

Optimal condition

Table 3 shows the compositions of the biomasses pretreated using the optimal condition (experiment 5), together with the calculated percentage recoveries of cellulose, hemicellulose, and lignin. Also shown are the percentage removals of hemicellulose and lignin, and the cellulose digestibility.

The pretreated biomass that showed the highest recovery of cellulose was that of water hyacinth, with a value of 87.3%, which could be considered very satisfactory, since cellulose is the component of greatest interest for the production of 2G ethanol. Sugarcane bagasse showed the second highest recovery of this component (65.6%), followed by maize straw (54.6%) and coconut shell (51.1%). For the last two materials, the loss of cellulose could be considered high.

Table 3. Compositions of the biomasses pretreated under the optimal condition, percentage recovery and removal values of the components, and digestibility of the cellulose

Pretreated biomass	Composition / %			Recovery / %			Hemicellulose removal / %	Lignin removal / %	Cellulose digestibility / %
	Cellulose	Hemicellulose	Lignin	Cellulose	Hemicellulose	Lignin			
Water hyacinth Exp. 5 2^3	60.2 ± 0.9	3.1 ± 1.1	22.3 ± 0.3	87.3	8.3	68.8	91.7	31.2	76.2 (24 h) 93.3 (48 h)
Sugarcane bagasse Exp. 5 2^3	61.5 ± 0.8	4.8 ± 0.7	4.6 ± 1.5	65.6	6.7	8.5	93.3	91.5	66.7 (24 h) 98.3 (48 h)
Maize straw Exp. 5 2^3	69.4 ± 0.7	5.7 ± 0.8	15.7 ±1.6	54.6	5.3	61.5	94.7	38.5	75.5 (24 h) 99.6 (48 h)
Coconut shell Exp. 5 2^3	39.5 ± 0.8	5.0 ± 1.4	25.7 ± 2.1	51.1	12.1	32.1	87.9	67.9	54.6 (24 h) 95.6 (48 h)

The optimal pretreatment condition enabled high removal of hemicellulose (ca. 90%) for the four biomasses (Table 3). This reflected reduced recalcitrance and indicated that it would be feasible to use the sugars released in the liquor, due to the almost complete removal of the hemicellulosic fraction, as well as the presence of glucose from the cellulose fraction that was degraded. Other studies[35,36] have also reported high removal of hemicellulose achieved using $FeCl_3$, further evidencing that the use of this catalyst favors the decomposition of this fraction. The $FeCl_3$ salt dissolves in glycerol, producing Fe^{3+} and Cl^- ions. Ionic iron in the trivalent form can easily act as a Lewis acid, weakening and causing breakage of glycosidic bonds. As reported in our previous work,[30] the small size of the Fe^{3+} ion leads to short distances between the metal and the ligand, resulting in relatively strong interactions between the metal and the electron donor sites of the biomass, resulting in increased rupture of C–O–C and C–O bonds, among others.

The removal of lignin varied according to the biomass, with the sugarcane bagasse and coconut shell, which originally had the highest lignin contents (Table 1), presenting the greatest delignification. It was evident from the cellulose digestibility percentages after both 24 and 48 h (Table 3) that the pretreatment was effective in reducing the recalcitrance of the biomasses, since high conversion of cellulose to glucose was achieved after 24 h of enzymatic saccharification, with digestibility above 90% reached after 48 h (values close to 100% were obtained for the sugarcane bagasse and the maize straw). This indicated that despite the removal of lignin being a factor of great importance in reducing recalcitrance and improving enzymatic digestibility, as reported by Toscan *et al.*,[37] who extracted lignin from elephant grass by organosolv pretreatment using imidazole and evaluated the effect on enzymatic saccharification, other factors such as hemicellulose removal may be of similar importance. Hence, high enzymatic digestibility can be achieved according to a complex process involving the removal of lignin and hemicellulose, or even the creation of disorder of these components in the lignocellulosic structure.

It should be highlighted that the HPLC analysis of the carbohydrates present in the hydrolysate obtained under the optimal conditions revealed that only hexoses were present. Hence, given the high removal of hemicellulose (Table 3), it could be concluded that the hexoses content of the hydrolysate was derived from cellulose and consisted only of glucose.

Assays under optimal conditions

Additional tests were performed using the optimal pretreatment condition, in order to confirm its effectiveness and find a more favorable solution to substitute the washing process after pretreatment. The sugars releases obtained in these tests were compared with that obtained under the optimized condition (experiment 5), as shown in Figures 4a-4d.

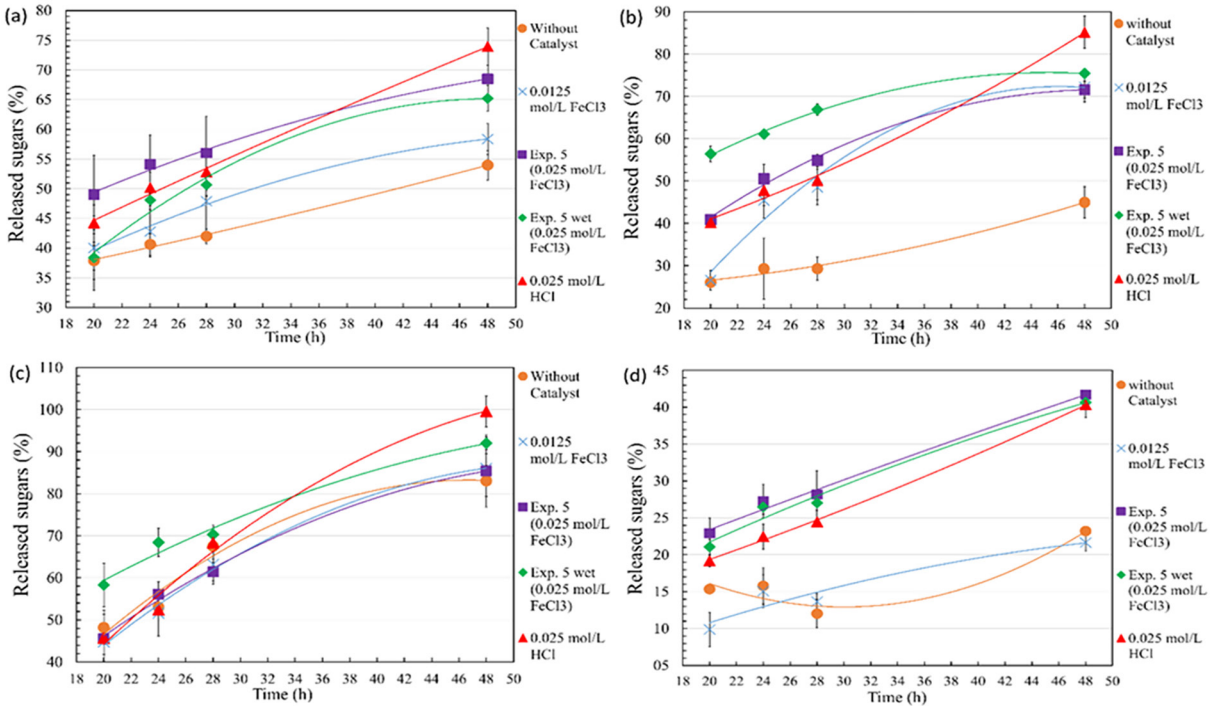

Figure 4. Percentages of sugars released in the enzymatic hydrolysis processes for the pretreated biomasses in tests under the optimal condition: (a) water hyacinth; (b) sugarcane bagasse; (c) maize straw; (d) coconut shell.

For the water hyacinth, sugarcane bagasse, and coconut shell (Figures 4a, 4b, and 4d), it was evident that the presence of the $FeCl_3$ catalyst in the pretreatment reaction increased the yield of sugars released in the enzymatic hydrolysis process. For the maize straw (Figure 4c), the biomass pretreated without catalyst provided a total sugars yield equivalent to that obtained under the optimum condition. However, the analysis of this hydrolysate by HPLC revealed the presence of pentoses, which corresponded to 36% of the total sugars.

Hence, even for the maize straw, the addition of the catalyst was advantageous, since it enabled production of a hydrolysate selective for the presence of glucose, consequently facilitating the fermentation process. In the case of the sugarcane bagasse, the reaction without catalyst was less efficient and pentoses were present in the hydrolysate (39% of total sugars). This confirmed that the catalyst acted on the hemicellulosic fraction, facilitating its removal.

For water hyacinth, reduction of the amount of $FeCl_3$ by 50% (Figure 4) resulted in sugars release lower than under the optimum condition. For the sugarcane bagasse and maize straw, it was possible to reduce the amount of catalyst and maintain the same yield obtained under the optimal condition, with HPLC analysis of the hydrolysates revealing the presence of very small amounts of pentoses, which could not be reliably quantified. For the coconut shell, the use of 0.0125 mol L^{-1} of $FeCl_3$ resulted in inefficiency equivalent to that of the reaction without catalyst.

When the $FeCl_3$ was replaced by HCl (Figures 4a-4d), the results for the coconut shell and water hyacinth obtained after 48 h of enzymatic saccharification were equivalent to that of experiment 5, considering the standard deviations. For the sugarcane bagasse and maize straw, the use of HCl resulted in a small increase in the percentage of sugars released. However, for these two biomasses, the amount of $FeCl_3$ could be reduced by half. This demonstrated that the use of $FeCl_3$ to replace conventional catalysts (such as HCl) is a promising strategy.

The wet biomasses were employed directly in the enzymatic saccharification process (experiment 5, wet), after being separated from the liquor, without being submitted to the washing and drying processes. For all the biomasses, the sugars release after 48 h of hydrolysis (Figure 4) was equivalent to that obtained using the washed and dried biomass (considering the standard deviations).

This result was very promising, since it showed that for the organosolv pretreatment process with glycerol, there was no requirement to wash the biomass after the pretreatment reaction. This provided considerable economization of water, further contributing to economic and environmental sustainability. The absence of the requirement for drying also provided savings in terms of time, since the biomass could be sent directly for hydrolysis after the pretreatment, as well as of energy (if the drying step was performed in an oven).

Determinations of HMF, furfural, and acetic acid

Table S3 (SI section) shows the concentrations of HMF, furfural, and acetic acid determined in the hydrolysates and liquors obtained under the optimal condition and in the corresponding assays. The determination of these byproducts was important for evaluation of the potential of the hydrolysate and the pretreatment liquor to produce a satisfactory ethanol yield when used in a fermentation process, considering the inhibitory effects that these compounds have on the microorganisms responsible for the fermentation.

In the studies of Bellido *et al.*[38] and Toquero and Bolado,[39] evaluation was made of the inhibitory effect of acetic acid on the yeast *Pichia stipitis*, which showed promise in the fermentation of pentoses, but was also capable of fermenting hexoses. It was found that ethanol production decreased by an average of 20% in the presence of 0.5 g L^{-1} acetic acid, while complete inhibition was observed at 3.5-4.0 g L^{-1}. For furfural and HMF, the inhibitory action was relevant from 2.0 and 0.5 g L^{-1}, respectively. In the case of HMF, the inhibition was less pronounced and the presence of the compound at concentrations of around 0.1 g L^{-1} had a positive effect on the fermentation process.[38]

The results (Table S3) showed that the hydrolysates obtained using the optimum condition (experiment 5) could be employed in the fermentation process without any additional treatment, since the acetic acid concentrations were below 0.5 g L^{-1}, with the exception of the maize straw hydrolysate (0.87 g L^{-1}). Furthermore, HMF and furfural, when detected, were present at levels well below those necessary to cause inhibitory effects, as also observed for the other hydrolysates.

Compared to the use of $FeCl_3$, the pretreatment with HCl led to greater quantities of acetic acid in the hydrolysates of the water hyacinth, sugarcane bagasse, and coconut shell, while the amounts were similar in the case of the maize straw hydrolysate. These concentrations (Table S3) were sufficient to cause substantial inhibitory effects. Therefore, it could be concluded that pretreatment using $FeCl_3$ was preferable. It should also be noted that pretreatment of the biomasses without use of a catalyst resulted in greater production of acetic acid, compared to pretreatment using $FeCl_3$.

HMF and furfural showed slightly higher concentrations in the liquors obtained using the optimum condition, compared to the concentrations in the hydrolysates, although the values were much lower than required to cause inhibition. However, high concentrations of acetic acid (1.86-3.00 g L^{-1}) were observed, which were sufficient to cause strong inhibition. Consequently, it was not feasible to use the liquors in a fermentation process, without prior treatment to reduce the negative effect of acetic acid. From the perspective of a biorefinery, one option would be to recover this byproduct by distillation, consequently providing it with added value.

According to Kamireddy *et al.*,[35] the formation of acetic acid is related to the ester and acetyl functional groups attached to the monosaccharides in hemicellulose. The pretreatment disrupts these bonds, forming acetic acid. Hence, the high concentration of this byproduct in the liquors could be attributed to the high removal of hemicellulose provided by the organosolv pretreatment with glycerol.

Other studies using $FeCl_3$ have reported high removal of hemicellulose and the presence of acetic acid at high concentrations in the liquor. For example, the pretreatment of maize straw with aqueous solutions of $FeCl_3$ at concentrations of 0.075 and 0.125 mol L^{-1}, at 160 °C, led to the formation of 2.91 and 3.30 g L^{-1} acetic acid, respectively.[35] Elsewhere,[36] organosolv pretreatment of sugarcane bagasse using ethanol and 0.025 mol L^{-1} $FeCl_3$, at 190 °C, produced 2.4 g L^{-1} acetic acid in the liquor, and it was concluded that a higher temperature led to greater formation of this byproduct.

In the present work, the formation of HMF and furfural was lower than in previous studies.[35,36] This could have been due to the use of glycerol, since Zhang *et al.*[40] found that glycerol may prevent dehydration and cleavage of glycosidic bonds. The reduced formation of these compounds can be advantageous, considering that a mixture of inhibitors increases the prejudicial effects in fermentation processes.[38,39]

Amounts of glucose produced by enzymatic hydrolysis

Table 4 shows, for the four biomasses evaluated, the amounts of glucose produced in the hydrolysate (in milligrams *per* gram of pretreated biomass and in grams *per* hundred grams of *in natura* biomass). Also shown are the maximum theoretical amounts of glucose that could be produced, considering the cellulose contents of the *in natura* biomasses, as well as comparison with previous studies reported in the literature.[8,20,23,26,41]

For water hyacinth, the work of Guragain *et al.*[8] was most comparable to the present study, since it investigated the use of organosolv pretreatment with glycerol (for 1 h at 230 °C) applied to this biomass. As shown in Table 4, the amount of sugars obtained was slightly higher than in the present study. However, no information was provided about the biomass composition, either before or after the pretreatment. Hence, it was not possible to

Table 4. Quantities of glucose produced in the hydrolysate after 48 h, maximum theoretical amounts, and yields obtained in studies published in the literature

	Glucose production		
Pretreated biomass	Hydrolyzed (48 h)	Theoretical	Other studies
Water hyacinth Exp. 5 2^3	623 mg glucose *per* g pretreated biomass 23 g glucose *per* 100 g *in natura* biomass	28 g glucose *per* 100 g *in natura* biomass	Guragain *et al.*[8] 719 mg RS *per* g pretreated biomass (487 mg were glucose)
Sugarcane bagasse Exp. 5 2^3	671 mg glucose *per* g pretreated biomass 24 g glucose *per* 100 g *in natura* biomass	36 g glucose *per* 100 g *in natura* biomass	Zhang *et al.*[20] 26 g glucose *per* 100 g *in natura* biomass
Maize straw Exp. 5 2^3	767 mg glucose *per* g pretreated biomass 21 g glucose *per* 100 g *in natura* biomass glucose yield = 54%[a]	39 g glucose *per* 100 g *in natura* biomass	Wei *et al.*[23] glucose yield = 90%[a]
Coconut shell Exp. 5 2^3	419 mg glucose *per* g pretreated biomass 14 g glucose *per* 100 g *in natura* biomass	29 g glucose *per* 100 g *in natura* biomass	Nogueira *et al.*[26] 430 mg RS *per* g pretreated biomass (247 mg were glucose) Gundupalli and Bhattacharyya[41] 12 g glucose *per* 100 g *in natura* biomass

[a]Considering the amount of cellulose in the *in natura* biomass. RS: reducing sugars.

determine whether the difference could have been due to variations in the biomass composition. In addition, the procedure employed by Guragain *et al.*[8] was not selective for the production of glucose, indicating the presence of a substantial quantity of hemicellulose in the pretreated biomass, while the enzymatic loading was extremely high.

It should be noted that in the present work, it was possible to optimize glucose production, in relation to our previous investigation[30] using water hyacinth. The increase in the rate and effectiveness of the reaction, achieved by reducing the particle size, enabled a substantial reduction in the amount of $FeCl_3$ used, leading to a pretreated biomass with higher solids yield (increase from 22.9 to 37.0%), due to greater recovery of cellulose (increase from 48.2 to 87.3%). Consequently, there was a 64% improvement in the amount of glucose produced *per* 100 g of *in natura* biomass, which increased from 14 g *per* 100 g to 23 g *per* 100 g (after 48 h of enzymatic hydrolysis). The lignin was also removed to a lesser extent, contributing to the higher solids yield, although the cellulose digestibility remained high.

For the sugarcane bagasse, the amount of glucose produced in this work (Table 4) could be compared to that obtained by Zhang *et al.*,[20] who performed pretreatment (at 160 °C for 10 min) using aqueous 0.025 mol L^{-1} $FeCl_3$ solutions in a pressurized reactor system. The amount of glucose obtained in the hydrolysate was very similar to that obtained here. However, the bagasse employed contained around 8% more cellulose.

In the present work, there was a greater loss of cellulose for the pretreatment liquor (experiment 5), accompanied by 91.5% delignification, due to the use of glycerol at high temperature (220 °C). This substantial removal of lignin, together with removal of 93.3% of the hemicellulose, resulted in almost 100% conversion of the cellulose recovered in the pretreated biomass to glucose in 48 h. In the work of Zhang *et al.*,[20] the recalcitrance was reduced to a lesser extent since there was almost no removal of lignin. Hence, the cellulose recovered (at a higher percentage than in the present work) was not completely converted to glucose. A strategy used by Zhang *et al.*[20] was the addition of surfactant (Tween 80) to the enzymatic hydrolysis process, which led to higher glucose production than shown in Table 4, but also made the process more expensive.

For maize straw, Wei *et al.*[23] performed an organosolv pretreatment using dimethyl sulfoxide (DMSO), catalyzed by $FeCl_3$ (120 °C; 45 min; 0.05 mol L^{-1} $FeCl_3$). A high yield of glucose in the hydrolysate was achieved, due to high recovery of cellulose in the pretreated straw (91.9%), together with high removals of hemicellulose (93.1%) and lignin (29.8%), resulting in almost all of the recovered cellulose being converted to glucose.

In the present work, the organosolv pretreatment with glycerol, catalyzed by $FeCl_3$, also provided high removal of hemicellulose (94.7%) and substantial delignification (38.5%), resulting in almost 100% cellulose digestibility. A lower glucose yield in the hydrolysate was due to lower cellulose recovery (54.6%).

The results obtained here were competitive with those of Wei *et al.*,[23] assuming adequate fermentation of the sugars present in the pretreatment liquor (after removal of acetic acid). The liquid fraction obtained by Wei *et al.*[23] also presented inhibitors (with substantial levels of furfural and acetic acid), while other points to note are that they used a concentration of $FeCl_3$ two-fold higher than in the present work, while the reaction time was more than four times longer.

The use of glycerol rather than DMSO is also advantageous, since glycerol has been devalued in the market due to its formation as a byproduct of biodiesel production, in addition to the possibility of using the glycerol in its crude form. DMSO, which has a relatively high boiling point and an acceptable toxicity level, is used extensively as a solvent in a wide range of other applications. Consequently, its use for the pretreatment of biomass would increase costs, making the process economically unfeasible.

For the coconut shell, the amount of glucose obtained in the hydrolysate was in line with values reported elsewhere.[26,41] This indicated that solubilization of much of the cellulose in the liquor may be easily achieved using different types of pretreatment. Nogueira *et al.*[26] employed an alkaline pretreatment process with dilute NaOH (2% m/v), in an autoclave at 121 °C for 10 min, which resulted in the production of 430 mg of reducing sugars in the hydrolysate *per* g of pretreated coconut biomass. The method was not selective for glucose production (247 mg g^{-1}). Gundupalli and Bhattacharyya[41] pretreated *in natura* coconut using sulfuric acid in a pressurized system (8.2 min; 200 °C; 0.21% m/m of acid), resulting in a yield of 12 g of glucose *per* 100 g of coconut, which was slightly lower than achieved here, despite the use of biomass with higher cellulose content. The particle size used was similar in the two studies.

Lignin recovery

Tests showed that it was not necessary to add acid to the liquor in order to precipitate the lignin, in contrast to previous work,[9] especially when the liquor is obtained using an alkaline process.[42,43] In the present case, the liquor was naturally acidic (Table S4, SI section), so the acid-insoluble lignin was already precipitated, only requiring separation, which was achieved by centrifugation. There

was no need to reduce the pH, even for only slightly acidic liquors (pH near 7). This was a highly favorable finding, since it simplified the lignin recovery process and avoided the additional cost associated with acid use, consequently increasing the sustainability of the organosolv method using glycerol in the presence of $FeCl_3$. In addition, the lignin washing process (after separation) was designed so as to consume the smallest possible amount of water.

The liquors derived from the coconut shell pretreatment (Table S4) contained the highest amounts of precipitated lignin, since this biomass had the highest lignin content, among those studied. Furthermore, 88% of the lignin content of coconut shell is acid-insoluble lignin. Under the optimum condition (experiment 5), 93.4% of the lignin removed during the coconut shell pretreatment was recovered as a solid.

The infrared spectra acquired for the recovered lignins (Figure S3, SI section) were similar to those obtained in other studies.[9,42,43] Bands in the region 3600-3200 cm^{-1} were associated with hydroxyl groups and phenolic compounds, while bands at around 1500 cm^{-1} were related to vibrations of the aromatic rings present in lignin.

Crude glycerol application

The effectiveness of crude glycerol as a solvent in the organosolv pretreatment process was evaluated by using it in the pretreatment reaction of water hyacinth, under the optimum condition (experiment 5). The first test was performed using the as-received crude glycerol generated during cotton biodiesel production. However, as shown in Figure 5, the results were not promising, mainly because the reaction temperature did not reach 220 °C, due to the presence of residual water and alcohol from the biodiesel production process.

As for all the other reactions performed in this work, the reaction medium was placed in a round-bottom flask coupled to a reflux system, so that the vapors returned to the reaction medium. The presence of water and alcohol could be seen by the continuous formation of condensation, which only allowed a temperature of 188 °C to be reached. As discussed above, this temperature was insufficient to obtain good results for the water hyacinth.

Therefore, a possible solution was to subject the crude glycerol to an evaporation process. However, this made the material even thicker and more viscous, due to the presence of other impurities from the production of biodiesel, such as fatty acids (soap), mono-, di-, and triglycerides, and esters, making its use unfeasible.

The next strategy was to perform a simple purification using acid treatment, commonly known as neutralization. The main aim of this treatment was to remove the fatty acids (and the residual basic catalyst) that formed the soap and resulted in the crude glycerol having an extremely viscous and dark appearance. The reaction of the acid with the soap produced insoluble free fatty acids, salt, and water. The precipitated fatty acids could then be separated by decantation, since they rose to the top, while the glycerol remained in the lower part.[44]

After precipitation of the fatty acids, the glycerol was denoted "precipitated crude glycerol". This was also submitted to the evaporation process for removal of excess water and alcohol, with the product being denoted "precipitated and evaporated crude glycerol". When the biomass pretreatment was performed with this material, the release of sugars was higher than achieved using the as-received crude glycerol, but the yield was still low (Figure 5). It could be seen that the evaporation procedure did not provide efficient removal of water, as there was still considerable dripping into the reaction medium, so the maximum temperature achieved was 204 °C.

Given this persistent interference of water, it was decided to perform the reaction with the reactor open, in order to allow the vapors to escape. Firstly, the reaction

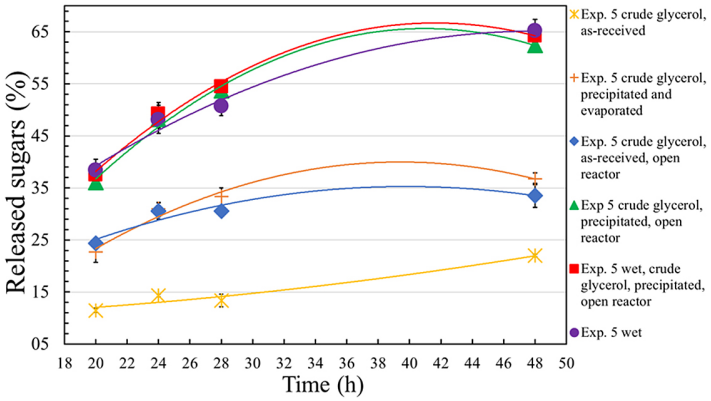

Figure 5. Percentage of sugars released in the enzymatic hydrolysis for the assays using crude glycerol (as-received and after different treatments) in the organosolv pretreatment of water hyacinth under the optimal condition. The results are compared with the use of commercial glycerol.

using this strategy was performed with the as-received crude glycerol. There was intense bubbling and constant release of vapors during the reaction. The temperature reached 220 °C, but the subsequent filtration and washing steps were greatly hindered by the extreme viscosity of the material associated with the presence of the soap. In addition, as shown in Figure 5, the result was not promising.

Therefore, the next reaction was performed using the precipitated crude glycerol, with the reactor kept open. The temperature reached the ideal level (220 °C), and the sugars release was the same as under the optimal condition employing the commercial glycerol (Figure 5), indicating that the soaps were also causing interference. In addition, the use of the precipitated crude glycerol, in an open reactor, facilitated the subsequent steps since the viscosity of the material was lower.

This reaction was repeated, with the pretreated water hyacinth (wet and without washing) being directly submitted to the enzymatic hydrolysis process (experiment 5: wet, crude glycerol, precipitate, open reactor). The results (Figure 5) showed that the dry biomass and the wet biomass without washing achieved the same yield as the reaction using the commercial glycerol with the wet and unwashed biomass (experiment 5: wet). Hence, it could be concluded that after simple acid treatment, the crude glycerol from cotton biodiesel production could be successfully applied in the organosolv pretreatment of water hyacinth.

In the hydrolysate obtained from pretreatment of the water hyacinth under the conditions of experiment 5 (wet biomass, precipitated crude glycerol, and open reactor), the formation of HMF (0.0003 g L^{-1}) and furfural (0.0017 g L^{-1}) was below the inhibitory range. Acetic acid was formed at a concentration (0.56 g L^{-1}) that could cause slight inhibition. The liquor showed low concentrations of HMF (0.0030 g L^{-1}) and furfural (0.0771 g L^{-1}). However, the quantity of acetic acid produced (7.69 g L^{-1}) was much higher than obtained with the commercial glycerol. Consequently, this important chemical product should be suitably recovered, allowing the liquor to be fermented for use of the solubilized sugars. The high formation of acetic acid could be attributed to the acidity of the precipitated crude glycerol (pH 3.0), which increased removal of the acetyl groups.

Studies concerning the pretreatment of biomass using crude glycerol are scarce. Sun and Chen[10] performed the pretreatment of wheat straw (at 200 °C for 3 h), concluding that a lower yield (21%) obtained with crude glycerol was due to the presence of lipophilic compounds that hindered the process. Guragain *et al.*[8] used crude glycerol in the pretreatment of water hyacinth (at 230 °C for 1 h), concluding that there was no influence of lipophilic compounds, with the yield being 14% lower using crude glycerol than using pure glycerol. As mentioned by Guragain *et al.*,[8] these results may have been influenced by the origin of the crude glycerol (with differences in the production method and the catalyst used, among other factors). In both studies, the glycerol was used as-received and in a reflux system.

Conclusions

The implementation of the 2^3 factorial design for the four biomasses clearly showed that the effects of the variables depended on the specific characteristics of each type of lignocellulose. Nonetheless, it was possible to find a common condition that could be considered optimal in all cases. Consequently, the proposed process was effective for the pretreatment of various types of lignocellulosic waste, representing an advance in the development of diversified bio-based industrial systems.

The results demonstrated that particle size is a variable that should not be neglected, and that glycerol at high temperature, used together with $FeCl_3$, has excellent potential for the removal of lignin and especially hemicellulose, consequently greatly reducing the recalcitrance of the biomass, as evidenced by the high cellulose enzymatic digestibility.

It was clear from the optimization experiments that the use of a suitable combination of variables could greatly improve the sustainability of the process. A highly important feature of the optimization, achieved without loss of yield, was the absence of any requirement to wash the biomass after the pretreatment, so it was possible to perform the hydrolysis directly, since glycerol and $FeCl_3$ (at low concentrations) are not harmful to enzymes. This greatly contributed to the economic and environmental sustainability of the proposed methodology.

In addition, measurements of different fermentation inhibitors showed that they were present at low concentrations in the hydrolysate obtained using $FeCl_3$, while the use of HCl resulted in higher concentrations of these compounds. Therefore, the use of $FeCl_3$ was preferable since the sugars release was similar for the two catalysts.

Finally, the most important finding of this study was that after performing a simple treatment, crude glycerol could be used in biomass pretreatment, providing the same yield as commercial glycerol. Furthermore, biomasses pretreated using crude glycerol could be used in enzymatic hydrolysis processes, without the need for washing and drying steps.

Supplementary Information

Supplementary data are available, free of charge, at http://jbcs.sbq.org.br as a PDF file.

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