**Design of experiments driven optimization of alkaline pretreatment and saccharification for sugarcane bagasse**

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**ABSTRACT**

To maximize the sugar release from sugarcane bagasse, a high-resolution Fractional Factorial Design (FFD) was combined with a Central Composite Orthogonal (CCO) design to simultaneously evaluate a wide range of variables for alkaline pretreatment (NaOH: 0.1–1 mol/L, temperature: 100–220 °C, and time: 20–80 min) and enzymatic saccharification (enzyme loading: 2.5–17.5%, and reaction volume: 550–850 µL). A total of 46 experimental conditions were evaluated and the maximum sugar yield (423 mg/g) was obtained via enzymatic hydrolysis under optimized conditions (0.25 mol/L NaOH at 202 °C for 40 min, with 12.5% of enzyme loading). Biomass compositional analyses showed that the pretreatments strongly removed lignin (up to 70%), silica (up to80%) and promoted cellulose enrichment (25–110%). This robust design of experiments resulted in maximizing enzymatic hydrolysis efficiency of sugarcane bagasse and further indicated that this combined approach is versatile for other lignocellulosic biomasses.

**Keywords:** Biomass pretreatment; Central Composite Orthogonal Design; Fractional Factorial Design; lignocellulose; alkaline pretreatment

**1. Introduction**

First-generation bioethanol utilizes sucrose from sugarcane (*Saccharum* spp.) and starch from maize as the main sugars for bioethanol production via fermentation of glucose. Brazil produced 32.4 billion liters of bioethanol in 2019 and accounted for 30% of the global production (Renewable Fuels [Association, 2019](#_ENREF_3)). The scale of production of sugarcane-derived sugar and bioethanol in Brazil means that bagasse, the lignocellulosic fraction of sugarcane after sucrose extraction, accumulates at sugar mills at a rate of 160,000 tons/year. Although the current costs of production and engineering hurdles make it more expensive than oil-derived fuels, cellulosic bioethanol (or second-generation bioethanol) represents a further step in the transition from fossil fuels to more sustainable energy sources, ([Yang et al., 2020](#_ENREF_40); [Zabed et al., 2017](#_ENREF_41)). In spite of this, using sugarcane bagasse for cellulosic ethanol is one of the most realistic routes to second generation biofuels because it is available in large quantities in sugarcane mills, avoiding the logistic problems involved in the use of other feedstocks ([Amorim et al., 2011](#_ENREF_2); [Zabed et al., 2017](#_ENREF_41)). In a sugar-to-bioethanol conversion process, a yield of 80 wet ton/ha/year of sugarcane would produce a maximum theoretical yield of 3,000 L of ethanol/ha from bagasse. This is a significant amount taking into account that, from the same wet ton yield, the average first-generation bioethanol production is 6,900 L of ethanol/ha ([Somerville et al., 2010](#_ENREF_36)).

Lignocellulosic material is primarily composed of plant secondary cell walls containing cellulose, hemicellulose and lignin organized in an interconnected network of polymers ([Marriott et al., 2016](#_ENREF_24)). Cellulose and hemicellulose fractions account for up to 75% of the total dry cell wall, which can be broken down into simple monosaccharides ([Gomez et al., 2008](#_ENREF_14)). By contrast, the presence of lignin hinders enzymatic saccharification and its removal requires high thermochemical inputs ([Lima et al., 2014](#_ENREF_23); [Oliveira et al., 2020b](#_ENREF_30)). A strategy to mitigate this recalcitrance is the optimization of conditions for the production of fermentable sugars from lignocellulosic biomass for reducing bioethanol production costs ([Aditiya et al., 2016](#_ENREF_1); [Mota et al., 2018](#_ENREF_27)).

The conversion of lignocellulosic biomass to fermentable sugars requires pretreatment processes to reduce biomass recalcitrance and thereby improving enzymatic saccharification ([Mota et al., 2019](#_ENREF_28)). In this scenario, alkaline pretreatment technologies utilize different chemical catalysts such as sodium hydroxide (NaOH), ammonia (aqueous and gaseous), sodium carbonate, potassium hydroxide, calcium hydroxide (lime), or the simultaneous or sequential combination of these catalysts ([Kim et al., 2016](#_ENREF_18); [Woiciechowski et al., 2020](#_ENREF_39)). Major reactions during alkaline pretreatment include the extensive removal of lignin and hemicelluloses from lignocellulose. NaOH pretreatment reduces the degree of polymerization of lignocellulose components and alters the physical properties of the biomass such as surface area, porosity and crystallinity ([Kim et al., 2016](#_ENREF_18); [Lima et al., 2014](#_ENREF_23); [Rezende et al., 2011](#_ENREF_34)). NaOH also plays an important role in the removal of ferulic and *p*-coumaric acids, which are ester-linked to hemicellulose and the lignin polymer, thus breaking covalent bonds between hemicelluloses and lignin ([de Oliveira et al., 2015](#_ENREF_8); [Lima et al., 2018](#_ENREF_22); [Oliveira et al., 2019](#_ENREF_31)).

Lignocellulosic feedstocks have intrinsic compositional and structural features that require the optimization of the pretreatment conditions to maximize saccharification yields ([Choi et al., 2013](#_ENREF_7); [Zabed et al., 2017](#_ENREF_41)). Traditionally, optimization of pretreatment conditions involves varying one independent factor at a time (e.g. concentration of catalyst, pretreatment temperature, time, solid loading, etc.), while the other factors are fixed. This approach requires a large number of experiments and does not allow evaluating the interactions among the individual factors ([Kim et al., 2016](#_ENREF_18); [Rezende et al., 2018](#_ENREF_33)). One strategy to overcome these challenges is the application of approaches based on the design of experiments (DOE) to assess the simultaneous effects of different experimental factors and their interactions with a reduced number of experiments ([Bruns et al., 2006](#_ENREF_6)).

DOE approaches are applied to optimize biomass pretreatments and enzymatic saccharification ([Duque et al., 2013](#_ENREF_10); [Kataria et al., 2017](#_ENREF_17); [Rezende et al., 2018](#_ENREF_33); [Singh et al., 2011](#_ENREF_35)). For instance, a 23 central composite design was used to evaluate a steam explosion pretreatment in elephant grass enhancing the cellulose saccharification yield by 55% ([Kataria et al., 2017](#_ENREF_17)). A 23 factorial DOE was also conducted to evaluate the influence of extrusion temperature in barley straw, the NaOH:dry biomass ratio and the enzyme loading on pretreated biomasses achieving 5-fold increase of enzymatic saccharification ([Duque et al., 2013](#_ENREF_10)). We previously used a 2ν5−1 Fractional Factorial Design (FFD) to determine the effect of five independent factors (milling time, catalyst concentration, pretreatment time, temperature, and requirement of the first acid treatment) in two different pretreatment methodologies (acid-alkali and acid-organosolv) ([Rezende et al., 2018](#_ENREF_33)). Improved sugar yields in elephant grass (205 mg/g substrate against 40 mg/g in sample *in natura*) were obtained using an alkali methodology with NaOH concentration of 4.5% w/v, 85 °C and 100 min after ball milling the sample.

In the present work, a 2ν5−1 FFD was used to evaluate the effect of three variables for pretreatment (NaOH concentration, pretreatment temperature and pretreatment time) and two for enzymatic saccharification (enzyme loading and reaction volume) on sugarcane bagasse at laboratory scale. The results obtained in this high-resolution FFD with a reduced number of experiments drove the selection of the conditions that were used in a subsequent DOE step using a Central Composite Orthogonal (CCO) design. In CCO design, the optimal experimental conditions were refined by evaluating three variables for pretreatment (NaOH concentration, pretreatment temperature and pretreatment time) and one for enzymatic saccharification (enzyme loading). This original approach, combining FFD and CCO design, allowed the optimization of biomass pretreatment and enzymatic saccharification using fewer experiments to reduce optimization time and energy input.

**2. Materials and methods**

***2.1 Plant material***

Sugarcane bagasse (SCB) was kindly provided by Natems Sugars Private Limited, Hyderabad, Telangana, India. Plant material was washed, dried in a convection oven at 60 °C to constant weight, and then knife-milled (625 µm × 188 µm avg.) and stored at room temperature.

***2.2 Alkaline pretreatment of sugarcane bagasse***

Dry material (400 mg) of SCB was pretreated in 20 mL Parr pressure vessels containing 16 mL of NaOH solution. Pretreatment conditions were performed according to the FFD or to the CCO design varying NaOH concentration, pretreatment temperature and pretreatment time (see details in the “Experimental design for alkaline pretreatment and enzymatic hydrolysis” section). The remaining solid residues were rinsed three times with distilled water, centrifuged (4,000×g for 15 min) and dried at 45 °C for 72 h.

***2.3 Experimental design for alkaline pretreatment and enzymatic hydrolysis***

Pretreatment and enzymatic saccharification conditions were optimized together in two steps of experimental design: 1) an initial screening of the data using a 2V5-1 FFD, with triplicates in the central point and a Multiple Linear Regression (MLR) fitting model; and 2) an optimization of the data by means of a response surface model (RSM) based on a CCO design (star distance 1.54), also with triplicates in the central point and MLR fitting model. The main response evaluated in both cases was the reducing sugars released in enzymatic saccharification in mg/g substrate. Modelling procedures were carried out using Modde 12 software (Umetrics, Sweden).

The coefficient plot of the effects was used to select the significant factors that influence sugar release, and included in a model together with the coefficients required to keep the hierarchy of this model. Analysis of Variance (ANOVA) was used to test the Regression Significance and Model Lack of Fit by means of F-tests (Box et al., 2005; Bruns et al., 2006). Graphs of residuals and predicted *vs* actual values were used as auxiliary diagnostics tools. Response surfaces described the behavior of the response over the experimental domain and select the conditions that lead to the maximization of sugar release.

**FFD:** Five independent factors were evaluated (3 in the alkali pretreatment and 2 in enzymatic hydrolysis): 1) NaOH concentration (0.4 to 1 mol/L); 2) Pretreatment temperature (180 to 220 °C); 3) Pretreatment time (40 to 80 min); 4) Enzyme loading (2.5 to 12.5%; enzyme loading refers to the percentage of enzyme in the substrate (g enzyme/ 100 g substrate) for hydrolysis) and 5) Reaction volume in hydrolysis (from 550 to 850 µL and 4 mg of SCB). **Table 1** shows the levels of the experimental conditions, sample name (F1 to F19, where F indicates FFD), and the amount of reducing sugars released (mg/g substrate).

**CCO design:** Four independent factors were evaluated (three in the alkali pretreatment and one in enzymatic hydrolysis): 1) NaOH concentration (0.1 to 0.4 mol/L); 2) Pretreatment temperature (100 to 180 °C); 3) Pretreatment time (20 to 60 min); and 4) Enzyme loading (7.5 to 17.5%). Reaction volume in the hydrolysis was kept constant at 850 µL, since the conditions used correspond to low solid to liquid ratio. **Table 2** shows the levels of the experimental conditions, sample names (C1 to C27, where C indicates CCO design), and the amount of reducing sugars released (mg/g substrate).

***2.4 Automated enzymatic saccharification analysis***

Enzymatic saccharification analysis was performed using the automated system as previously described ([Gomez et al., 2010](#_ENREF_15)). Dry SCB (~4 mg with the precise weight recorded for each sample) was weighed into four replicates in 96‐well plates and expressed as the average of saccharification. Enzymatic saccharification and reducing sugars determinations were performed in a liquid handling robotic platform (Tecan Evo 200; Tecan Group Ltd. Männedorf, Switzerland), containing the enzyme cocktail Cellic® Ctec3 (Novozymes, Bagsvaerd, Denmark) from 2.5 to 17.5% (g enzyme/ 100 g substrate) at 50 °C in 25 mmol/L sodium acetate buffer pH 4.5 for 18 h of enzymatic hydrolysis. The enzyme loading and total volume of reaction used for the saccharification hydrolysis were varied following the FFD and CCO design (see section **2.3** and **Table 1** and **Table 2**). Automated determination of the reducing sugars released after hydrolysis was performed by colorimetric assay using 3-methyl-2-benzothiazolinone hydrazone (MBTH).

***2.5 Determination of lignin content by acetyl bromide method***

Lignin content was determined by the acetyl bromide method in the samples before and after alkaline pretreatment ([Fukushima & Hatfield, 2001](#_ENREF_12)). The absorbance was measured at 280 nm in triplicates using the extinction coefficient for grasses (17.75 L/g.cm) and expressed as percentage of lignin on a dry weight basis ([Foster et al., 2010](#_ENREF_11)).

***2.6 Determination of silica and ash content***

Dry material of SCB *in natura* (SCB-IN) and pretreated SCB was used to determine the silica content by X-ray fluorescence spectroscopy (XRF), using silica powder as standard to generate the calibration curve, as previously described ([Reidinger et al., 2012](#_ENREF_32)).

Ash content was determined in the SCB-IN, and in the conditions F16 (from FFD) and C20 (from CCO design) and expressed as the percentage of ash on a dry weight basis. The measurements were conducted in duplicate by total calcination of 500 mg of dry material in muffle oven at 600 °C for 24 h.

***2.8 Determination of matrix polysaccharide composition and crystalline cellulose content***

Dry material (4 mg) was hydrolyzed with 0.5 mL of 2 mol/L trifluoroacetic acid (TFA) for 4 h at 100 °C under argon atmosphere and the monosaccharides released were analyzed by High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) as previously reported ([Jones et al., 2003](#_ENREF_16)). The analysis of matrix polysaccharide composition was conducted in triplicates and expressed as mg monosaccharide/g substrate. The remaining fractions of glucose, xylose and arabinose were calculated considering the quantification of monosaccharides in the solids after pretreatments. The initial amounts of monosaccharides in SCB-IN were considered as 100% to calculate the remaining percentages in the pretreated samples.

The remaining solid fraction was hydrolyzed with 90 μL of 72% H2SO4 (w/w) at 25 °C for 4 h under argon atmosphere ([Updegraff, 1969](#_ENREF_37)). Next, 1890 μL of deionized water was added and incubated at 120 °C and glucose content was determined by the colorimetric anthrone-sulfuric acid assay ([Viles & Silverman, 1949](#_ENREF_38)). Crystalline cellulose content was determined in triplicate and expressed as the percentage on a dry weight basis.

**3. Results and discussion**

***3.1 Alkaline pretreatment and enzymatic saccharification using high-resolution Fractional Factorial Design (FFD)***

DOE were carried out in two steps in this work, including independent variables of both alkaline pretreatment and enzymatic saccharification of sugarcane bagasse (SCB). In the FFD, the first DOE step, the factors analyzed were: NaOH concentration, pretreatment temperature, pretreatment time, enzyme loading and total volume of reaction in hydrolysis (**Table 1**).

**Table 1** also shows the reducing sugars (in mg/g substrate) released after 18 h of enzymatic hydrolysis at 50 °C, which was used as the main response to determine the relevance of the factors. These hydrolysis conditions were previously established in the automated system as indicators of saccharification potential ([Gomez et al.,](#_ENREF_37) 2010). Overall, pretreated samples presented higher sugar release than SCB-IN and distinct responses to different pretreatment conditions. The best conditions in terms of sugar release (> 300 mg/g substrate) for FFD were those of the samples with the highest reaction volume (850 μl) and enzyme loading (12.5%). The majority of samples subjected to pretreatment conditions in FFD also had improved release of reducing sugars, and F16 showed the highest sugar release (376.9 mg/g substrate) and the highest hydrolysis yield (68%**~~,~~** ~~E-supplementary data of this work can be found in e-version of this paper online~~).

**Fig. 1a** shows the coefficient plot of the factors affecting sugar release after pretreatment and enzymatic saccharification of SCB (only significant coefficients with values larger than the error bars are shown). The effects related to enzymatic hydrolysis (enzyme loading and reaction volume) had large influence on the release of reducing sugars, and they were both positive, indicating that greater amounts of sugars should be achieved using higher enzyme loadings and higher total volume of reaction in hydrolysis, within the respective experimental ranges tested. The effects related to pretreatment conditions (NaOH concentration, pretreatment temperature and time) were also relevant to a lower extent, and they contributed negatively to the total amount of reducing sugars released during enzymatic saccharification. There are also several significant binary interactions in this system: [NaOH] × Temperature; [NaOH] × Time; [NaOH] × Enzyme loading; Temperature × Time; Temperature × Enzyme loading; Temperature × Reaction volume in hydrolysis; Time × Enzyme loading; Enzyme loading × Reaction volume in hydrolysis, which highlight the importance of varying all these factors simultaneously to study this system. The inclusion of these significant coefficients in the linear model and the exclusion of non-significant terms resulted in a robust and reliable model (R2 = 0.999; Q2 = 0.993; model validity = 0.791; and reproducibility = 0.998). The graph of observed *vs.* predicted experimental responses (**Fig. 1b**) shows that the data fitted the linear model with a high correlation.

This initial screening indicated that the release of reducing sugars should improve if the NaOH concentration, pretreatment temperature and time were decreased (negative coefficients in **Fig. 1a**). Consequently, values lower than: 0.4 mol/L NaOH, 180 °C and 40 min were used in the following DOE approach. In contrast, FFD suggested that the enzymatic saccharification parameters (enzyme loading and reaction volume in hydrolysis) should be increased to more than 12.5% of enzyme loading or more than 850 µL of reaction volume in the hydrolysis.

***3.2 Optimization of alkaline pretreatment and enzymatic saccharification using Central Composite Orthogonal (CCO) design***

A second set of experiments was performed following a CCO design (star distance 1.54). Three pretreatment factors were evaluated here at values lower than the first experimental DOE set (NaOH concentration, pretreatment temperature and pretreatment time), while one factor (enzyme loading) was evaluated at higher levels.

**Table 2** shows the levels of the experimental conditions, sample names (where the C in sample names indicates CCO design) and the corresponding sugar release. The samples with higher saccharification (> 400 mg/g substrate) for the CCO design were C20 and C24, both using NaOH concentration (0.25 mol/L), and pretreatment time (40 min) at the central levels. **Fig. 1c** shows the significant coefficients of the factors influencing the release of reducing sugars from SCB in CCO design, together with coefficients to keep the hierarchical order in the model. The effects of pretreatment temperature and pretreatment time, and the enzyme loading in saccharification contributed positively to increasing sugar released upon saccharification. NaOH concentration as an independent factor was not a significant factor within the ranges used here, but it contributed as a quadratic term (NaOH × NaOH). Pretreatment time was also included as a quadratic term (Time × Time) to improve the prediction of the model. In addition, binary interactions such as [NaOH] × Temperature; Temperature × Enzyme loading; and Time × Enzyme loading had a smaller effect in this system. The quadratic model obtained was valid and presented a high prediction capability (R2 = 0.951; Q2 = 0.878; model validity = 0.740; and reproducibility = 0.964). The observed *vs*. predicted experimental responses (**Fig. 1d**) shows that the data fitted the non-linear model.

Two response surfaces are shown in **Fig. 2**, where the highest values of reducing sugars released were obtained using NaOH concentration of 0.25 mol/L (within 0.23 and 0.27 mol/L) and 45 min (43 to 47 min) pretreatment time . In each plot, the other factors not displayed were kept constant at their medium levels (**Fig. 2a** temperature = 140 °C and enzyme loading = 12.5% and **Fig. 2b** NaOH concentration = 0.25 mol/L and time = 40 min). Release of reducing sugars higher than 380 mg/g substrate would also be achieved using different combinations of temperature and enzyme loadings. For an enzyme loading of 15%, for instance, sugar releases higher than 380 mg/g would be achieved at temperatures higher than 170 °C, while for a higher enzyme loading, lower temperatures (140 °C) would be enough to achieve this. Higher enzyme loadings would thus compensate for the use of lower temperatures and vice-versa. The highest theoretical predicted value of reducing sugars released was 400 mg/g substrate (C24 in **Fig. 2b**) using the following experimental conditions: NaOH concentration = 0.25 mol/L, temperature = 140 °C, time = 40 min, and enzyme loading = 20.2%. The prediction of the model showed good agreement with the experimental value obtained in this sample (410 ± 34 mg/g substrate, **Fig. 1d**) with 63% of hydrolysis yield. The maximum experimental value obtained for the release of reducing sugars was 423 mg/g substrate in the sample C20 (0.25 mol/L NaOH; 201 °C; 40 min and a 12.5% enzyme loading) (**Table 2)**. This value was higher than the predicted from the model for this sample (384 mg/g substrate), with 62% of hydrolysis yield (sample C20 had the higher deviation between the predicted to observed value, **Fig. 1d)**.

Although high temperature (201 °C) led to the maximum release of sugars within this study (423.4 mg/g substrate in sample C20), decreasing pretreatment temperature to 78 °C resulted in a sugar release that was 20% lower than the maximum (337.8 mg/g substrate in sample C19). Considering that large scale pretreatment with NaOH is associated with large CAPEX due to the expensive high-end steel required at high temperature, our data indicate that low temperature pretreatments could be an alternative to consider in process design. **Table 2** also shows a reduction of just 18% in sugar release (410.9 mg/g in C24 to 337.8 mg/g in C19) when the enzyme loading was reduced 38% (from 20.2% to 12.5% of enzyme loading). Currently, enzymes account for 18% of production cost in a lignocellulosic biorefinery ([Brown et al., 2020](#_ENREF_5)) and a 38% of reduction in the enzyme loading could be a significant cost reduction. On the other hand, modifying NaOH concentration or pretreatment time led to a reduction in sugar release (C17, C18, C21 and C22), indicating that these variables should be kept at their central levels.

***3.3 Effects of pretreatments on lignin content***

All the samples pretreated using FFD and CCO design were analysed to determine the chemical composition of SCB for lignin and cellulose (**Fig. 3**), matrix polysaccharide composition (**Figs. 4 and 5**), silica and the hydrolysis yield. SCB-IN has 24% of lignin, and this percentage decreased to less than 11% in all the samples of the FFD (**Fig. 3a**). In some FFD samples lignin levels as low as 4.0% and 4.4% were achieved, both using harsh experimental conditions. Previous studies have shown a negative correlation between lignin content and enzymatic saccharification, indicating the key role of lignin for limiting the access of cellulases to the substrate, minimizing the saccharification efficiency ([Masarin et al., 2011](#_ENREF_25); [Mota et al., 2019](#_ENREF_28); [Oliveira et al., 2020b](#_ENREF_30); [Rezende et al., 2018](#_ENREF_33); [Rezende et al., 2011](#_ENREF_34)). In FFD samples, however, all the lignin values are similarly low, and the correlations between lignin content and the release of reducing sugars were not significant (*P* ≥ 0.05). The more severe conditions applied in FFD (higher NaOH concentration, pretreatment temperature and time) reduced the lignin to minimal levels, exposing other factors at play that determine the release of sugars.

In the CCO design, where lignin removal was less severe (**Fig. 3b**), lignin amounts were typically higher than in FFD and a negative correlation was observed between enzymatic saccharification and lignin content in most samples. Given that different enzyme loadings were applied in the CCO design for SCB saccharification, the correlation analysis between lignin content and sugar release was performed by groups of samples hydrolyzed under the same enzyme loadings (C1–C8; C9–C16; and C17–C27 as indicated in **Table 2**). Samples C23 and C24 were excluded in this analysis because they were digested with different enzyme loadings. Negative correlations with significant differences (*P* < 0.05) were observed between sugar release and lignin content in the sample groups C1 to C8 (Pearson correlation r = –0.88, *P* = 0.0035) and C17 to C27 (Pearson correlation r = –0.68, *P* = 0.0422), whereas the group C9 to C16 had no significant correlation (Pearson correlation r = –0.42, *P* = 0.2939). These results suggest that, if the enzyme loading is limiting, the amount of lignin affects directly the recalcitrance and the correlations between the amount of lignin and saccharification may be useful to identify the role of lignin in biomass recalcitrance. However, there are multiple factors besides lignin content that determine sugar release ([Marriott et al., 2016](#_ENREF_24); [McCann & Carpita, 2015](#_ENREF_26); Mota et al., 2020; [Oliveira et al., 2020b](#_ENREF_30)). ~~(E-supplementary data of this work can be found in e-version of this paper online).~~

The effect of the pretreatment conditions on the amount of lignin can be observed in **Fig. 3b**, where lignin decreases as the NaOH concentration, pretreatment time and temperature increase. These results confirm the CCO result that the three pretreatment parameters are important for lignin removal. In samples where all the pretreatment conditions were at high levels (0.4 mol/L NaOH, 180 °C and 60 min, as shown in **Table 2**), lignin amounts reached the minimum values (7.6 and 7.8%, respectively), representing a reduction in lignin content of 68% in C8 and C16 samples in comparison to SCB-IN. Medium pretreated conditions (C23 to C27, 0.25 mol/L NaOH, 140 °C and 40 min) reduced lignin to approximately 14%. In samples at the star points (C17 to C22), higher or lower lignin amounts were obtained depending on the severity of the NaOH concentration, pretreatment time and temperature.

***3.4 Effects of pretreatments on cellulose and silica contents***

The cellulose content of samples pretreated using FFD conditions varied between 38% and 54% w/w, while SCB-IN showed 26% of cellulose (**Fig. 3a**). Samples F6 and F14 contained the highest cellulose content (50–54%), and both samples were pretreated with high levels of NaOH concentration and time (1 mol/L NaOH and 80 min, **Table 1**). Using experimental conditions of CCO design, cellulose contents varied from 33% (0.017 mol/L NaOH, 140 °C and 40 min) to 54% (0.4 mol/L NaOH, 180 °C and 60 min) (**Fig. 3b**). This enrichment in cellulose is a direct consequence of the removal of lignin and matrix polysaccharides. There is no statistically significant correlation (*P* > 0.05) between release of reducing sugars and cellulose amounts in FFD samples. High pretreatment severity produces drastic structural and compositional changes on lignocellulose, making it more difficult to identify correlations between the lignocellulosic components ([Li et al., 2016a](#_ENREF_20); [Oliveira et al., 2020b](#_ENREF_30)).

Silica is an important component of the inorganic fraction of lignocellulosic biomass of grasses. Biomasses with lower levels of silica in general present higher release of reducing sugars ([Glazowska et al., 2018](#_ENREF_13); [Lima et al., 2014](#_ENREF_23); [Rezende et al., 2018](#_ENREF_33)). SCB-IN had 1.5% of silica, whereas, silica varied between 0.3% and 0.4% in samples pretreated under FFD, and from 0.25% to 1.2% in CCO samples, a reduction of up to 83%. In general, higher reducing sugars releases were observed in samples with lower silica (samples C20 and C23–C27, **Table 2**), which also correspond to samples pretreated under medium NaOH concentration (0.25 mol/L) and pretreatment time (40 min). SCB-IN showed 3.8% of ash, while the conditions F16 and C20 had 1.6% and 1.1%, respectively ~~(E-supplementary data of this can be found in e-version of this paper online)~~. Besides lignin removal, these results indicate that using NaOH concentrations equal or higher than 0.25 mol/L, could also efficiently remove silica from SCB. The data also indicates that besides lignin removal, silica removal by NaOH pretreatment contributes to improve the enzymatic saccharification.

***3.5 Effects of pretreatments on matrix polysaccharide content and composition***

Analysis of the matrix polysaccharide content and composition, and its remaining fraction in SCB pretreated under FFD and CCO design conditions are shown in **Fig. 4** and **5**, respectively. The matrix polysaccharide total amount in SCB-IN was 111 mg/g substrate, while under FFD conditions, samples F8 and F16 (pretreatment replicates) showed the largest decrease in total matrix polysaccharides, reaching *ca.* 30 mg/g substrate, which is mostly due to decreases in xylose and arabinose amounts, and also the removal of minority monosaccharides (galactose, fucose, rhamnose, mannose, galacturonic acid, and glucuronic acid) (**Fig. 4a, b**). A gradual decreasing profile was observed in **Fig. 4a** as the pretreatment severity increased (NaOH concentration, pretreatment temperature and time in **Table 1**) (F1 to F8 and F9 to F16).

Similarly, using CCO design conditions, samples C8 (49.5 mg/g substrate) and C16 (47.5 mg/g substrate) had the lowest total content of matrix polysaccharides (**Fig. 5a**), though the gradual decrease was not observed here. Previous studies have reported that partial removal of lignin and matrix polysaccharides may improve the access of cellulases to cellulose and reduces the non-productive protein adsorption on lignin ([Ko et al., 2015](#_ENREF_19); [Li et al., 2016b](#_ENREF_21); [Mota et al., 2019](#_ENREF_28); [Oliveira et al., 2020a](#_ENREF_29)).

Compositional analysis of the matrix polysaccharides revealed that the major monosaccharides were xylose, glucose and arabinose, with small amounts of galactose, fucose, rhamnose, mannose, galacturonic acid and glucuronic acid (**Fig. 4a** and **5a)**. F1 and F9 (duplicates submitted to pretreatment under less severe conditions in **Table 1**: 0.4 mol/L NaOH, 180 °C and 40 min) had the smallest reductions in xylose and arabinose. In contrast, F8 and F16 showed largest reductions in xylose and arabinose, using pretreatment conditions at higher levels in FFD (1 mol/L NaOH, 220 °C, 80 min), whereas the amount of glucose in F8 and F16 (~16 mg/g substrate) were quite similar to that in SCB-IN (16.7 mg/g substrate) (**Fig. 4a**).

In CCO, samples from C1 to C18 exhibited similar lower levels of xylose, arabinose and glucose (**Fig. 5a**). Notably, the pretreatment conditions applied in most samples of this group (C1–C16) were at low (0.1 mol/L NaOH, 100 °C, 20 min) or high levels (0.4 mol/L NaOH, 180 °C, 60 min). Samples from C19 to C27, which were pretreated with 0.25 mol/L NaOH, showed similar amounts of arabinose (14.2–17.4 mg/g) and glucose (14.7–21.4 mg/g), when compared to SCB-IN (arabinose: 16.5 mg/g; glucose: 16.7 mg/g). In addition, these samples presented lower amounts of xylose (45.9–52.2 mg/g) compared to SCB-IN (59.3 mg/g) (**Fig. 5a**). Moreover, reductions in xylose and arabinose in NaOH-pretreated SCB suggest an overall reduction in arabinoxylans, the main hemicellulose in SCB ([de Souza et al., 2012](#_ENREF_9); [Lima et al., 2014](#_ENREF_23)). These results indicate that different pretreatment conditions remove different cell-wall monosaccharides.

**Fig. 4b** and **5b** show the remaining fractions of glucose, xylose and arabinose from matrix polysaccharides in FFD and COO design samples, determined in the solids remaining after NaOH pretreatments and expressed as percentages compared to SCB-IN. In FFD samples, glucose in the remaining fractions was higher than xylose and arabinose in all the samples (F1 to F19) (**Fig. 4b**), indicating that the NaOH-based pretreatment was more efficient in removing xylose and arabinose rather than glucose. The alkaline pretreatment resulted in larger removal of arabinoxylans from lignocellulose than glucans, because NaOH can cleave the ester bonds between lignin and hemicelluloses ([de Souza et al., 2012](#_ENREF_9); [Kim et al., 2016](#_ENREF_18); [Lima et al., 2014](#_ENREF_23); [Masarin et al., 2011](#_ENREF_25)).

In CCO design samples (**Fig. 5b**), the remaining fractions of glucose, xylose and arabinose were reduced by 40–50% across most samples, reflecting an overall reduction in total matrix polysaccharides. Interestingly, **Fig. 6** shows that the highest sugar released was achieved in the sample C20 (423.4 mg/g), and increased the amount of glucose by 15% together with a reduction of 18% in xylose and 78% in silica. In summary, these results show that the pretreatment conditions strongly removed silica and xylose from the lignocellulose. It is worth noting that xylose, arabinose and glucose are differentially removed upon specific pretreatment conditions, and therefore would represent an advantage for the rational design of enzyme cocktails to optimize the cellulose-to-glucose conversion.

**4. Conclusions**

The two-step DOE applied here represent an approach to optimize biomass conversion at laboratory scale that can assist in scaling up efforts. Pretreatment conditions showed the efficiency of optimizing the parameters analyzed reaching maximum experimental values of 423 mg/g sugar release, against 145 mg/g from the sample *in natura*, reducing the lignin content to just 8.7% and with a large effect in cellulose amount, which was doubled when compared to SCB-IN. This method might be applicable to other lignocellulosic feedstocks and this detailed study enables a better understanding of biomass pretreatment and enzymatic saccharification of SCB for application in sugarcane industries.

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**Supplementary data**

E-supplementary data for this work can be found in e-version of this paper online.

**CRediT authorship contribution statement**

Thatiane R. Mota: Conceptualization, Investigation, Methodology, Validation, Writing - original draft, Writing - review & editing. Dyoni M. Oliveira: Methodology, Investigation and Writing - original draft. Rachael Simister: Methodology. Caragh Whitehead: Methodology. Alexandra Lanot: Methodology. Wanderley D. dos Santos: Supervision and Writing - review & editing. Camila A. Rezende: Conceptualization; Formal Analysis, Writing - original draft, Writing - review & editing. Simon J. McQueen-Mason: Conceptualization, Writing - review & editing, Supervision, Funding acquisition. Leonardo D. Gomez: Conceptualization, Writing - review & editing, Supervision, Funding acquisition.

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**FIGURE LEGENDS**

**Figure 1.** **a** FFD coefficient plot, **b** experimental values *vs.* predicted values of sugar release for FFD, **c** CCO design coefficient plot and **d** experimental values *vs.* predicted values of sugar release for CCO design**.** NaOH concentration (NaOH); Pretreatment temperature (Temp); Pretreatment time (Time); Enzyme loading (Enz); and Reaction volume (RV).

**Figure 2.** CCO design response surfaces showing the amount of sugar released (color scale on the right with values in mg/g): **a** NaOH concentration and pretreatment time and **b** pretreatment temperature and enzyme loading in hydrolysis.

**Figure 3.** Reducing sugars released from samples treated with NaOH according to **a** FFD and **b** CCO design, and their percentages of lignin and cellulose. Sugarcane bagasse *in natura* (SCB-IN) was included for comparison.

**Figure 4. a** Matrix polysaccharide composition in FFD samples and **b** percentages of glucose, xylose and arabinose remaining in the solid substrates of sugarcane bagasse *in natura* (SCB-IN) and samples treated with NaOH (F1˗F19).

**Figure 5. a** Matrix polysaccharide composition in CCO design samples and **b** percentages of glucose, xylose and arabinose remaining in the solid substrates of sugarcane bagasse *in natura* (SCB-IN) and samples treated with NaOH (C1˗C27).

**Figure 6.** Flowchart summarizing the FFD and CCO design steps and the comparison between sugar release and compositional components from sugarcane bagasse *in natura* and the sample C20 with the maximum sugar yield.