**Improved hydrolysis yields** **and silica recovery by design of experiments applied to acid-alkali pretreatment in rice husks**

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**Abstract**

In this work, a two-step pretreatment using acid and alkali was optimized for rice rusks (*Oryza sativa*) using a 25-1 fractional factorial design (FFD), followed by a central composite design (CCD) to further optimization of enzymatic saccharification. The effect of five variables was simultaneously evaluated: H2SO4 concentration (from 0 to 5.4% w/w); NaOH concentration (0 to 6% w/w); temperature (85 to 125 °C); time (20 to 100 min) and solid to liquid ratio (S/L=5 to 12.5% w/w). The best pretreatment conditions were: 1.8 % w/w of H2SO4 in the first step and 6 % w/w of NaOH at 85 °C for 100 min at a S/L=12.5% (w/w) in the alkaline step, which resulted in 58.7 mg of glucose/g substrate, 8-fold increase compared to the sample *in natura* (7 mg/g). In rice husks, differently from the results commonly found in literature, NaOH extracts mainly silica instead of lignin, while H2SO4 has an important role in lignin removal. High purity silica (97%) was isolated at high yields (70%) from the alkaline liquor by a simple and scalable process, which could contribute to make ethanol production from this biomass economically viable.

**Keywords:** Experimental design; acid pretreatment; alkali pretreatment; rice husk; silica.

**Abbreviations**

FFD, fractional factorial design; DOE, design of experiments; CCD, central composite design; S/L, solid to liquid ratio; HPLC, high performance liquid chromatography; XRF, x-ray fluorescence spectroscopy; ANOVA, analysis of variance; FPU, filter paper units.

**1. Introduction**

Lignocellulosic biomass is an abundant, cheap and less polluting raw material to produce fuels and other chemicals, when compared to non-renewable fossil derivatives (Kumar et al., 2009; Zabed et al., 2017). Biomasses from wood, herbaceous, aquatic, animal and human waste sources can be considered for this purpose (Tursi, 2019). Agricultural wastes are particularly interesting for energy conversion since they do not compete with food production and their valorisation mitigate the negative impacts associated with the accumulation and disposal of agricultural residues in the environment (Somerville et al., 2010; Zabed et al., 2017). In addition, the use of agricultural residues may have positive impacts at a regional level by creating new jobs and promoting development on rural communities (Tursi, 2019).

Rice husks, a biomass residue from herbaceous source, are available at low cost and in large quantities (Dagnino et al., 2017; Wu et al., 2018). Although rice production is mostly located in Asian countries, this is a crop well distributed across the globe, with an annual production that exceeded 780 million tons worldwide in 2018 (Faostat, 2020). Considering that 20% of the weight of the grains are husks, Abbas *et al.* estimated that the potential global production of bioethanol from rice husks would be enough to satisfy *ca*. 20% of the global demand for ethanol to be blended with gasoline at a 10% volume ratio (Abbas and Ansumali, 2010). At present, rice husks are used in a diverse range of applications such as burning in rice processing plants, generation of electricity, or bedding for farm animals.

Although rice husks are a cellulose-rich biomass, they are also composed of other structural polymers, such as hemicellulose and lignin, extractives and up to 20% w/w of silica (Ang et al., 2013; Dagnino et al, 2013). Such a high percentage of silica constitutes a problem for biomass processing because it acts as a physical barrier for enzymatic degradation and forms insoluble incrustations that damages reactors and filtration systems. Conversely, silica is also a valuable compound that after extraction can be used in different applications, such as catalysis, anti-sticking agents, raw material to produce silicon and as an adsorbent for heavy metals and organic contaminants in soil amendment and wastewater treatment plants (Le et al., 2015; Shen, 2017). Minu *et al.* studied the recovery of silica from black liquor resulting from bioethanol production from rice straw (Minu et al., 2012), showing that lignin and silica were isolated by precipitation under pH reduction.

Ethanol production from lignocellulosic biomass relies on the hydrolysis of polysaccharides and, although at present it is not economically viable, it can become a commercial reality by optimizing biomass pretreatments. Indeed, inefficient pretreatments increase enzyme costs and produce poor hydrolysis yields (Kumar et al., 2009; Ang et al., 2013). A multitude of pretreatments have been tested in different lignocellulosic biomasses, including physical methods based on milling, extrusion, ultrasound, or irradiation (with microwaves, electron-beam, gama-rays, etc.); physico-chemical methods using acids, alkalis, solvents, ionic liquids, liquid hot water or explosion with steam, carbon dioxide or ammonia; and finally, biological methods using fungi, bacteria, and enzymes (Cheah et al., 2020; Sun and Cheng, 2002; Das et al., 2021; Rezende et al., 2011).

The ideal pretreatment to produce cellulosic ethanol should improve hydrolysis yields, minimizing cellulose loss and the production of inhibitors of hydrolytic enzymes (Kumar et al., 2019; Sun and Cheng, 2002). It should also be cost effective and environmentally sound (saving energy and allowing the recycling of water and chemicals), in addition to permit the maximum use of the biomass components, within a biorefinery approach (Cheah et al., 2020; Galbe and Wallberg, 2019). This integral approach aiming at the valorisation of the co-products is key to make ethanol production more sustainable and economically feasible.

Unfortunately, in practice, there is no such a perfect pretreatment attending to all these aspects, and comprehensive reviews compare the advantages and the drawbacks of the most common pretreatments, highlighting challenges and future perspectives (Conde-Mejía et al., 2012.; Das et al., 2021; Cheah et al., 2020; Rezania et al., 2020). Furthermore, while the general effect of the different pretreatments on lignocellulosic substrates is acknowledged in most cases, their results on hydrolysis yields differ from one biomass to another. For instance, the two-step pretreatment approach applied to rice husks in this work include both a dilute acid and a dilute alkali step. The acid step is known to hydrolyse mainly the hemicellulose fractions of the lignocellulosic substrates, while pretreatments with diluted base act by removing lignin, hemicellulose, and silica (Das et al., 2021; Rezende et al., 2011). However, the same acid-alkali pretreatment resulted in different hydrolysis efficiencies in different grasses (sugarcane bagasse, elephant grass and corn biomass) and the experimental conditions leading to the best hydrolysis yields were also different in each case (Mota et al., 2021, Rezende et al., 2018; Camargos et al., 2019). Pretreatments should thus be tailored to the particular characteristics of each biomass and can be designed to fractionate and valorise as much as possible of the biomass components, minimizing process residues (Attard et al., 2020; Ubando et al., 2020).

In the last five years, bioethanol generation from rice husks has been tested under physical and chemical pretreatments comprising: extrusion and ultrasound (Zhang et al., 2020); cryo-crushing (Castoldi et al., 2017); deep eutectic solvents (Okur and Koyuncu, 2020); hydrothermal and saturated steam (Khamis et al., 2019); subcritical water (Abaide et al., 2019); ammonium carbonate (Ebrahimi et al., 2017a); alkaline peroxide (Favaro et al., 2019; Bazargan et al., 2020); sodium hydroxide (Shahabazuddin et al., 2018) and acid-alkali (Novia et al, 2019). Shahabazuddin et al. (2018) used a Box Behnken design to optimize the conditions of a single-step alkali treatment, considering the variables: biomass loading (S/L = 10-25% w/w); particle size (0.25 to 1 mm); NaOH concentration (0.5 to 2% w/w); and reaction time (20 to 60 min). Other authors (Novia et al., 2019) applied an acid-alkali pretreatment to rice husks focusing on the design of an adequate pretreatment reactor, not in the final hydrolysis yields. These authors used hydrodynamic simulation of the pretreatment and computational fluid dynamics (CFD) and varied the concentrations of sulfuric acid and of sodium hydroxide (both from 1 to 5% w/v), while keeping the temperature and the time constants.

In the present work, we used design of experiments (DOE) to optimize an acid-alkali pretreatment in rice husks, aiming to improve the release of sugars to produce bioethanol. DOE is a valuable multivariate technique for pretreatment optimization, providing meaningful information with a reduced number of experiments. It also identifies interactions between the different experimental conditions tested, which would not be possible using the traditional one-factor-at-a-time approach (Bruns et al., 2005). Here, this technique was used in two ways. First, a 25-1 fractional factorial design (FFD) was applied to evaluate the sugar release as a function of the variables: H2SO4 concentration in step 1, NaOH concentration in step 2, Temperature, Time and the Solid/Liquid ratio in the step 2. In a second approach, a rotatable central composite design (CCD, α = and five replicates at the center points) was used to improve the model, based on the data selected in the previous FFD. Under different pretreatment conditions, the substrates became enriched in cellulose and their hemicellulose, lignin and silica contents were transferred to the pretreatment liquors. Compositional analysis determined the specific effects of pretreatments on sample composition and its relation to hydrolysis yields. The optimized condition for sugar release also resulted in efficient silica solubilization to the alkaline pretreatment liquor, where it was subsequently precipitated under selective pH and recovered, allowing the valorisation of the non-polysaccharide fraction.

1. **Materials and methods**

**2.1 Biomasses and materials**

Rice husks (*Oryza sativa*) from the variety IRGA 424 were purchased from a local rice mill (Porto Ferreira-SP, Brazil). Prior to pretreatments, rice husks showed 7.5 to 8% of moisture content; 14.8 ± 0.2 % of ash content and a cellulose to lignin ratio of 0.86. They were dried in a convection oven (Tecnal TE-394/3, Brazil) at 60 °C for 8 h, then knife milled (SOLAB - SL 31) until passing through a 2 mm sieve and stored.

* 1. **Acid-alkali pretreatment and experimental design analysis**

Rice husks were pretreated using an acid and an alkali step in sequence, as previously described for sugarcane bagasse (Rezende et al., 2011) and elephant grass (Rezende et al., 2018). In the acid step, milled rice husks were treated with aqueous H2SO4, using a 1:10 (g/mL) solid to solution (S/L) ratio for 40 min at 120 °C. Pretreated solids were then separated by filtering in cotton tissue (150 thread count), rinsed with tap water until neutral pH and oven dried at 60 °C for 7 h. In the following pretreatment, samples were treated with NaOH solutions, using S/L ratios, times and temperatures as described in DOE (see below). At the end of this step, the solids were filtered, rinsed and dried as previously (Rezende et al., 2011). Acid and alkali pretreatments were carried out at 85, 105, 120 and 125 °C. The autoclave (Phoenix AV-75, Araraquara-SP, Brazil) was used when the pretreatment temperature was above 100 ℃, while the water bath (Fisaton model 550, São Paulo-SP, Brazil) was used when the temperature was below 100 ℃. The autoclave takes 15 min to reach the pretreatment temperature and 80 min to cool to room temperature, and the temperature in the water bath was controlled to follow the same heating/cooling times of the samples treated in autoclave to ensure a similar contact of the biomass with the pretreatment liquids in all the temperatures. The pretreatment times reported throughout the paper are considered as the time during which the reaction was kept at the constant pretreatment temperature. A detailed temperature profile as a function of time for the pretreatments at 85 and 125 °C is presented in Figure S1 (Supplementary Material).

DOE was carried out in two steps: a 25-1 FFD was first applied as a screening to evaluate the sugar release as a function of the variables: H2SO4 concentration in step 1 (acid step); NaOH concentration in step 2 (alkali step); Temperature; Time and the S/L ratio in step 2, with conditions specified in Tables 1 and 2. Based on the most relevant variables determined in FFD, and by analysing the regression coefficients and the response surface, we established the direction of higher responses to be further optimized in CCD. A rotatable CCD (α = and five replicates at the center points) was established in the shifted region in order to provide degrees of freedom to estimate high order coefficients for the regression model. The acid step conditions other than concentration in CCD were: temperature = 121 °C, S/L = 10%, and time = 40 min, while the alkali conditions were: temperature = 85 °C, S/L = 12.5% w/w and time = 100 min (Table 4 and 5). The combination of the levels of the CCD is graphically shown in Figure S2, using the coded variables.

* 1. **Compositional and morphological analysis**
     1. **Analysis of matrix polysaccharides, cellulose, and lignin**

Prior to the compositional analysis, all biomasses were ground to a fine powder in a ball mill (TissueLyser II, Qiagen (Hilden, Germany) for 30 s at 30 Hz. Matrix polysaccharides (hemicellulose fraction), cellulose and lignin in FFD were quantified as previously described (Rezende et al., 2018), while in CCD samples they were determined following the NREL protocol (Sluiter and Sluiter, 2011). Both sets of methods were compared in a significant number of rice husk samples and the compositional values obtained were equivalent.

* + 1. **Determination of moisture and ash contents**

Moisture contents were determined in triplicate, using a heating balance (Mettler Toledo, Switzerland) and the ash contents were determined in duplicate by total calcination of 1g of solid biomass samples in muffle oven (EDG F-1800 10P, São Carlos, Brazil) at 600 °C for 24 h.

* + 1. **Scanning electron microscopy (SEM)**

Rice husk surface morphology before and after pretreatments was analysed in a JEOL 6360 LV scanning electron microscope (JEOL, Japan), operating at 10 kV. Prior to analysis, dried samples were coated with an iridium film, using a BALTEC MED 020 sputter coater (Oerlikon-Balzers, Liechtenstein), using a current of 11.3 mA for 120 s.

* 1. **Enzymatic saccharification**

Hydrolysis was carried out in a shaking incubator using an enzyme cocktail with a 4:1 ratio of Celluclast and Novozyme 188 (both from Novozymes, Bagsvaerd, Denmark) in a minimum of 4 replicates. Hydrolysis conditions were 50 °C, pH 4.5 (25 mM sodium acetate buffer) for 12h, with enzyme loading of 8 FPU/g biomass, biomass weight of 4 mg and total liquid volume of 850 µL (S/L=0.47%), following previous works (Gomez et al., 2010; Mota et al., 2021). Prior to incubation, biomass substrates underwent a 2 h hydration step in the buffer at room temperature. Enzymatic assays were carried out both manually and automatically. Automated saccharification was performed based on Gomez et al. (2010) and the determination of reducing sugars in this case was performed using 3-methyl-2-benzothiazolinone hydrozone (Gomez et al., 2010; Anthon and Barret, 2002). In the case of hydrolysis assays set up manually, sugars released in hydrolysis were determined in a high performance liquid chromatography (HPLC) equipment (Agilent 1200), coupled with a refractive index detector, using a Biorad HPX87H column at 45 °C and H2SO4 5 mmol.L-1 as mobile phase.

The sample presenting the highest glucose release in the hydrolysis (RB2), was also submitted to enzymatic hydrolysis for 72 h, keeping the other conditions the same as in the previous procedure. Hydrolysis yields (HY) were calculated considering the total of glucose released in hydrolysis (RG) in mg/g of substrate and the cellulose content (mg/g) in the hydrolysed substrate, according to Equation 1, where 1.1 is a correction factor to the addition of water molecules to the anhydroglucose residues as they are hydrolysed from cellulose (Ebrahimi, 2017b).

(Equation 1)

* 1. **Silica recovery**

Silica was recovered from the liquor of the alkaline pretreatment of rice husks (condition RB2 in Table 5 showing high ash removal and the highest glucose release). For this, the liquor pH was lowered from 12-13 to 7 using 2% (v/v) of H2SO4 (Minu et al., 2012), and kept for 12 hours at room temperature for silica precipitation. Subsequently, the liquor was vacuum filtered and solid was dried at 105 ºC until constant weight. Finally, the material obtained was calcined in a muffle oven (EDG F-1800 10P, São Carlos, Brazil) at 800 ºC for 4 h and the silica content was determined by x-ray fluorescence spectroscopy (XRF). XRF was carried out in a Shimadzu XRF-1800 fluorimeter using Rh as the radiation source operating at 40 kV and 95 mA, in semi-quantitative mode and using all the channels (Cl, S, P, Si, Al, Mg, Na, F, Ti-U and K, Ca, Sn-Cs).

1. **Results and discussion**

**3.1 Design of experiments (DOE)**

Two DOE approaches were carried out in this work to optimize the sugar release of rice husk samples. A 25-1 fractional factorial design was firstly applied as a screening to evaluate the sugar release as a function of five variables. After defining that only two of these variables were significant, a central composite design was used to refine the optimal experimental conditions based on a quadratic model.

**3.1.1 25-1 Fractional factorial design**

Table 2 shows the sugar release for the samples prepared following FFD and Figure 1 shows the half normal plot of the factors, where significant effects are those that deviate from the straight line centred in zero. Concentration of H2SO4 in step 1 (Factor A) and concentration of NaOH in step 2 (Factor B) presented the highest effects on sugar release, and interaction between these factors (AB) could also be noticed. Both effects are positive indicating that higher levels of these factors provide higher values of sugar release. The other factors and their interactions play a smaller role in changing the sugar release.

Analysis of variance (ANOVA) of the model containing the significant coefficients is shown in Table 3. The calculated F value of MSRegression/MSresidual is equal to 3.55, whereas the tabulated F value (8, 10, 95% confidence level) is 3.07. The calculated F value of MSlack of fit/MSpure error is 83.59, whereas the tabulated F value (8, 2, 95% confidence level) is 19.37. The second result clearly indicates lack of fit of the linear model, which will be confirmed by the residual graphs shown in Figure 2. Because the MSlack of fit is high, it inflates the MSresidual causing the calculated F value for MSRegression/MSresidual to be low (3.55). This might give a false impression that the factors do not significantly influence the response when in fact, the linear model is not suitable to represent this data set. The diagnostics graphs of residuals *versus* predicted values and predicted *versus* actual experimental values are shown in Figures 2(a) and 2(b).

The responses varied over a broad range (from 5.7 to 50.7 mg/g), indicating that the factors studied have a strong impact on the sugar release (Figure 2). On the other hand, residuals are not random, and the centre points are clearly far from the rest of the points (Figures 2A and 2B). The response surface shown in Figure 3 also indicates that there is a curvature in the middle of the experimental domain, since the experimental centre points are above the surface.

**Table 1.** Levels of the factors in the 25−1 fractional factorial design.

|  |  |  |  |
| --- | --- | --- | --- |
| **Factors** | | | |
|  | **Low level (-1)** | **High level (+1)** | **Central (0)** |
| **A- [H2SO4] (% w/w)** | none | 3.6 | 1.8 |
| **B- [NaOH] (% w/w)** | 0.5 | 4.5 | 2.5 |
| **C- Temperature (°C)** | 85 | 125 | 105 |
| **D- Time (min)** | 20 | 100 | 60 |
| **E- S/L (% w/w)** | 5 | 12.5 | 8.75 |

**Table 2.** Sample identification with the corresponding experimental conditions and the response of sugar release in the 25−1 fractional factorial design with 3 replicates at the centre point.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Sample**  **RA** | **[H2SO4] step 1 (% w/w)** | **[NaOH] step 2 (% w/w)** | **Temperature step 2 (°C)** | **Time**  **step 2 (min)** | **S/L**  **step 2 (% w/w)** | **Sugar release (mg/g)**  **SD\*: ± 1.2 mg/g** |
| 1 | 0 | 0.5 | 85 | 20 | 12.5 | 5.7 |
| 2 | 3.6 | 0.5 | 85 | 20 | 5.0 | 26.6 |
| 3 | 0 | 4.5 | 85 | 20 | 5.0 | 21.9 |
| 4 | 3.6 | 4.5 | 85 | 20 | 12.5 | 40.8 |
| 5 | 0 | 0.5 | 125 | 20 | 5.0 | 14.3 |
| 6 | 3.6 | 0.5 | 125 | 20 | 12.5 | 15.4 |
| 7 | 0 | 4.5 | 125 | 20 | 12.5 | 20.6 |
| 8 | 3.6 | 4.5 | 125 | 20 | 5.0 | 43.5 |
| 9 | 0 | 0.5 | 85 | 100 | 5.0 | 11.3 |
| 10 | 3.6 | 0.5 | 85 | 100 | 12.5 | 22.4 |
| 11 | 0 | 4.5 | 85 | 100 | 12.5 | 23.1 |
| 12 | 3.6 | 4.5 | 85 | 100 | 5.0 | 40.7 |
| 13 | 0 | 0.5 | 125 | 100 | 12.5 | 7.3 |
| 14 | 3.6 | 0.5 | 125 | 100 | 5.0 | 25 |
| 15 | 0 | 4.5 | 125 | 100 | 5.0 | 14.2 |
| 16 | 3.6 | 4.5 | 125 | 100 | 12.5 | 50.7 |
| 17 | 1.8 | 2.5 | 105 | 60 | 8.75 | 41.9 |
| 18 | 1.8 | 2.5 | 105 | 60 | 8.75 | 44.1 |
| 19 | 1.8 | 2.5 | 105 | 60 | 8.75 | 43.8 |

\* Standard deviation (SD) calculated for triplicated measurements in the central point.

**[Figure 1]**

**Table 3.** ANOVA table of the model describing the sugar release as a linear function of the selected coefficients, based on 25−1 fractional factorial design results.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Source** | **Sum of Squares** | **df** | **Mean Square** | **F-value** | **p-value** |
| **Model** | 2709.5 | 8 | 338.69 | **3.55** | 0.0325 |
| A-[H2SO4] step 1 | 1345.06 | 1 | 1345.06 | 14.09 | 0.0038 |
| B-[NaOH] step 2 | 1016.02 | 1 | 1016,02 | 10.64 | 0.0085 |
| D-Time step 2 | 2.18 | 1 | 2.18 | 0.0228 | 0.883 |
| E- S/L step 2 | 8.27 | 1 | 8.27 | 0.0866 | 0.7746 |
| AB | 127.13 | 1 | 127.13 | 1.33 | 0.2754 |
| AD | 22.8 | 1 | 22.8 | 0.2388 | 0.6356 |
| BE | 106.61 | 1 | 106.61 | 1.12 | 0.3155 |
| DE | 81.45 | 1 | 81.45 | 0.8532 | 0.3774 |
| **Residual** | 954.69 | 10 | 95.47 |  |  |
| Lack of Fit | 951.84 | 8 | 118.98 | **83.59** | 0.0119 |
| Pure Error | 2.85 | 2 | 1.42 |  |  |
| **Cor Total** | 3664.19 | 18 |  |  |  |

**[Figure 2]**

Due to the lack of fit of the linear model, a central composite design (CCD) was performed to provide the degrees of freedom to estimate quadratic coefficients. CCD was planned in a shifted region in relation to the original experimental domain, as shown in Figure S3: [H2SO4] = 1.8 to 5.4 % w/w (or 1 to 3% v/v) and [NaOH] = 2 to 6 % w/w, since the results of FFD indicated this could be a promising region. The response surface in Figure 3 shows that the higher responses (close to 50 mg/g) are obtained towards higher values of H2SO4 and NaOH concentrations.

**[Figure 3]**

Since temperature, time and the solid to liquid ratios did not influence the sugar release, they could be kept at their more convenient values in terms of costs and time saving. Temperature in the second pretreatment step was kept at its lower level (85°C), while solid to liquid ratios were maintained at 12.5% w/w (high solid content), to minimize the liquid hydrolysates that are produced as residues of the process. Time in step 2, although itself is not one of the significant individual factors, it is involved in one important secondary interaction, [H2SO4] x Time ((AD). If all the optimal conditions are kept as follows: ([NaOH] = 4.5 % w/w, [H2SO4] = 3.6 % w/w, Temperature = 85°C; S/L = 12.5% w/w), but the time in step 2 is reduced to 20 min, a sugar release of 40.8 ± 2.7 mg/g is obtained (conditions of sample RA4), showing that improved results can be achieved using time at its higher level (100 min). However, at industrial scale, the cost-benefit ratio needs to be evaluated since the sugar improvement (40.8 to 50.7 mg/g) may not be worthwhile considering that it implies a 5-fold increase in the reaction time.

**3.1.2 Central composite design**

Table 5 shows the sugar released from the samples prepared according to the central composite design (α = with 5 replicates at the centre point). The results indicate that the range of variation was not as broad as in the previous design (minimum = 31.0 and maximum = 58.7 mg/g) and the variation at the centre point (authentic replicates) was 43.7 – 49.2.

The selected model is presented in Equation 2:

*Sugar release (y) = 45.54 + 5.93 [NaOH] + 2.65 [H2SO4] -1.95 [NaOH]2 + 2.35 [H2SO4]2 – 8.03 [NaOH]2\*[H2SO4].* (Equation 2)

**Table 4.** Levels of the factors in the central composite design (α = ).

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Factors** | | | | | |
|  | **Low level**  **(-1)** | **High level (+1)** | **-α** | **Central (0)** | **+α** |
| **A- [H2SO4] (% w/w)** | 1.8 | 5.4 | 1.1 | 3.6 | 6.1 |
| **B- [NaOH] (% w/w)** | 2 | 6 | 1.2 | 4 | 6.8 |

**Table 5.** Sample identification with the corresponding experimental conditions and the responses of sugar release according to the central composite design (α = ) with 5 replicates at the center point.

|  |  |  |  |
| --- | --- | --- | --- |
| **Sample**  **RB** | **[H2SO4] step 1**  **(% w/w)** | **[NaOH] step 2**  **(% w/w)** | **Sugar release (mg/g)**  **SD\*: ± 2.2 mg/g** |
| 1 | 1.8 | 2.0 | 47.7 |
| 2 | 1.8 | 6.0 | 58.7 |
| 3 | 5.4 | 2.0 | 36.6 |
| 4 | 5.4 | 6.0 | 48.3 |
| 5 | 3.6 | 1.2 | 31.0 |
| 6 | 3.6 | 6.8 | 48.5 |
| 7 | 1.1 | 4.0 | 44.6 |
| 8 | 6.1 | 4.0 | 52.1 |
| 9 | 3.6 | 4.0 | 44.2 |
| 10 | 3.6 | 4.0 | 44.7 |
| 11 | 3.6 | 4.0 | 49.2 |
| 12 | 3.6 | 4.0 | 45.9 |
| 13 | 3.6 | 4.0 | 43.7 |

\* Standard deviation (SD) calculated for 5 replicates measurements in the central point.

The ANOVA data for this model is shown in Table 6, where NaOH shows the most significant influence on the response (p = 0.0004), followed by the term [NaOH]2\*[H2SO4] (p = 0.0035). The remaining coefficients were kept for hierarchical purposes. The model indicates a complex response surface (Figure 4). Nevertheless, fit was observed with MSlack of fit/MSpure error = 2.0, a lower value than the tabulated F value of 6.59 (3, 4, 95% confidence level). The regression was also significant, considering that the calculated F value of MSRegression/MSresidual is 14.4, while the tabulated F value (5, 7, 95% confidence level) is 3.97. Furthermore, the graph of residuals *vs.* predicted values presents a random pattern (Figure 5A) and the predicted values are in good agreement with the actual values (Figure 5B).

**Table 6.** ANOVA table for the selected model based on central composite design results.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Source** | **Sum of Squares** | **df** | **Mean Square** | **F-value** | **p-value** |
| **Model** | 499.56 | 5 | 99.91 | **14.40** | 0.0014 |
| A-NaOH | 281.42 | 1 | 281.42 | 40.57 | 0.0004 |
| B-H2SO4 | 28.13 | 1 | 28.13 | 4.05 | 0.0839 |
| A² | 26.49 | 1 | 26.49 | 3.82 | 0.0916 |
| B² | 38.38 | 1 | 38.38 | 5.53 | 0.0509 |
| A²B | 128.85 | 1 | 128.85 | 18.57 | 0.0035 |
| **Residual** | 48.56 | 7 | 6.94 |  |  |
| Lack of Fit | 29.15 | 3 | 9.72 | **2.00** | 0.2561 |
| Pure Error | 19.41 | 4 | 4.85 |  |  |
| **Cor Total** | 548.12 | 12 |  |  |  |

**[Figure 4]**

**[Figure 5]**

The higher response for sugar release was 58.7 mg/g (RB2 Sample), corresponding to a hydrolysis yield (for glucose) of 8.5%, which is 3.8 times the hydrolysis yield of the *in natura* rice husk used in this work. Sample RB2 was also submitted to longer enzymatic hydrolysis for 72h and the sugar release was 114 mg/g, a 16.2% conversion of cellulose to glucose. Ebrahimi et al. (2017b), using a pretreatment with acidified glycerol carbonate on rice husks, obtained a hydrolysis yield four times higher than in *in natura* rice husk, but a higher enzyme loading (10 FPU/g biomass for 72 h) was used in their work. Increased enzyme loadings should further improve the hydrolysis values.

Three additional points varying the concentrations of acid and alkali were evaluated towards the direction of increased sugar release in Figure 4. All the other experimental conditions were fixed (85 °C; 100 min and S/L = 12.5%) and the results after 12h of hydrolysis were:

* [H2SO4] =1.8% w/w; [NaOH] = 8% w/w: sugar release = 47.9 mg/g
* [H2SO4] =0.9% w/w; [NaOH] = 6% w/w: sugar release = 47. 9 mg/g
* [H2SO4] =0.9% w/w; [NaOH] = 8% w/w: sugar release = 50.9 mg/g

No significant increase in the response was achieved, indicating that the system has probably reached its maximum of sugar release at this point. Therefore, the best conditions to achieve the maximum sugar release (58.7 mg/g) from rice husks samples according to our optimization were those of sample RB2: [H2SO4] = 1.8% w/w (1% v/v) in the first step, and [NaOH] = 6% w/w at 85 °C for 100 min at a S/L=12.5% w/w in the second step, which corresponds to sample RB2. Although the absolute values of sugar release in rice husks are not outstanding as compared to other biomasses, the optimization performed here certainly contributed to substantially increase (8 times) the quantity of sugars that would be obtained from the sample *in natura* (7mg/g).

**3.2. Effect of pretreatments on saccharification**

The effect of pretreatment conditions on the chemical composition of the treated samples, and the relation between chemical composition and saccharification were evaluated in all the samples separated according to the experimental designs. Figure 6 shows the reducing sugars obtained after enzymatic saccharification (12h of hydrolysis at 50 °C), and also lignin, cellulose and ash contents for the solid samples of FFD. Chemical composition is presented in detail in Tables S1 (FFD samples) and S2 (CCD samples), together with the pretreatment yield for every sample. As previously discussed, a significant difference in sugar release (grey bars) in these samples is due to the acid-alkali pretreatment. Untreated rice husks (RIN) released *ca.* 7 mg/g substrate and the acid step alone can increase this amount to *ca.* 25 mg/g (RH1 and RH2). A sugar release of around 40 mg/g substrate can be obtained in samples RA4, RA8, RA12, all of which had a first 3.6% H2SO4 step, followed by an alkali step with higher NaOH concentration (4.5%). Also, the central points (RA17, RA18, and RA19) presented sugar release of around 40 mg/g and the most efficient sample (RA16 with 50 mg/g substrate) is the one with all the parameters at high levels (3.6% w/w H2SO4; 4.5% w/w NaOH; 125 °C; 100 min; 12.5% w/w S/L in Table 2).

**[Figure 6]**

Rice husks present high ash contents, which can reach 15% w/w in samples *in natura* (RIN) and up to 20% in samples treated with acid only (RH1 and RH2). Silica represents the main inorganic fraction (ash) in rice samples (Figure S4). Acting as a physical barrier to cellulase action, silica is a hurdle in industrial processes in general, due to the formation of insoluble precipitates. Effective pretreatments to remove silica, which is solubilized in alkali medium (pH>9), are fundamental to obtain practical hydrolysis yields in silica-rich biomasses, such as rice husks (Le et al., 2015).

Silica and ash contents in these samples are determined by the NaOH concentration applied in the pretreatments: NaOH at 2.5 and 4.5% w/w removes the inorganic fraction almost completely, resulting in percentages lower than 1% (RA3, RA4, RA7, RA8, RA11, RA12, RA15 to RA19 in Figure 6). In samples treated with low NaOH concentration (0.5% w/w), the acid step produces higher ash content (compare RA5 and RA6 or RA9 and RA10, for instance). As in the case of RH1 and RH2, acid pretreatments do not seem to dissolve silica from rice husks and can increase its percentage due to the removal of other cell wall components.

Silica removal in the alkali step is followed by morphological changes on rice husks, as can be observed in Figure 7. Figure 7A shows the typical morphology of a rice husk surface, formed by a corrugated outer epidermis with ridges punctuated with prominent and regularly spaced globular protrusions (Nascimento et al, 2016; Park et al., 2003). The linear organization of the ridges is better observed in Figure 7B, which shows the sample morphology after the acid treatment (sample RH1 in Table 2). The morphology of the post-acid sample is remarkably similar to the surface of the non-treated husks, showing no evident effects of the acid step. Conversely, after the alkaline treatment with 6% w/w NaOH (sample RB2 in Figure 7C and 7D), rice husk outer surface appears damaged, ruptured, and discontinuous due to the alkali action. According to Park et al. (2003) silica is present in rice husks as particles (grains) concentrated just below the thick-walled epidermis, so that the morphological changes observed in Figure 7C and 7D are perfectly in accordance with the removal of these silica grains and with the decrease in the ash content in these samples (fromca. 15 to 1.4 % w/w). Differently, ash contents in the post-acid samples remain high (ca. 15-20% w/w) in line with the surface unmodified morphology.

**[Figure 7]**

Ash affects sugar release in rice husks, but there is no straightforward correlation. Low ash contents can be associated with high sugar yields in some cases (RA4, RA8, RA12, RA16 to RA19), but also with inefficient saccharification in others (such as in RA3, RA7, RA11, and RA15). Besides, samples with high silica contents can also present high saccharification (RH1, RH2, RA2, RA10, RA14). This indicates that the effect of ash on sugar release depends on other factors. In Figure S5, the Pearson’s correlation coefficient R=-0.59 for sugar release and ash content, but there is a clear segregation of results in two groups (low or high ash content). It is important to highlight that although the coefficient R can identify possible correlations, it may not indicate a causal relationship.

Saccharification in rice husks shows a clear association with the cellulose amounts in the substrate (R=0.87 in Figure S6), and higher sugar yields occur in samples with increased cellulose contents. The interaction between saccharification and lignin content in rice husks was also complex, and low correlation between these biomass components was observed (R=0.66 in Figure S7), indicating mixed effects of pretreatment factors. Lignin content show small variations (between 25 and 34% w/w), while sugar release varies significantly from 5 to 50 mg/g substrate. The higher sugar yields in samples containing slightly increased lignin contents (RA4, RA8, RA12, for instance) go against the concept of lignin being the main barrier to hydrolysis. In rice husks, the increased saccharification seems correlated to the drastic silica decrease. Since rice husks are rich in silica, its removal leads to significant increases in the percentual distribution of the other components.

The cellulose content in rice husk samples is influenced by the same factors as the sugar release (H2SO4 and NaOH concentration, Table S3), thus explaining the high positive correlation between these two responses.

Considering the silica amount in RA samples as a response, the only relevant factor is NaOH concentration (Table S4). Acid concentration is not significant for silica removal, thus explaining the moderate negative correlation with sugar release. This reinforced the profile previously discussed for elephant grass samples: although acid and alkali hydrolysis are theoretically possible for silica removal, only alkali is effective in the conditions used in these pretreatments (Rezende et al., 2018).

Finally, considering the lignin amount in RA samples, the relevant factors for this response are H2SO4 concentration and its interaction with NaOH concentration (AB) (Table S5). NaOH concentration is not significantly relevant as an individual factor for lignin removal in rice husk samples, which is a surprising result since alkaline hydrolysis is often applied with this purpose. The influence of only one factor (H2SO4 concentration) on sugar release and lignin content in RA sample is consistent with R=0.66 for these responses. For RA samples, it is thus possible to conclude that both H2SO4 and NaOH concentrations are important for their final cellulose content and sugar release, but while the first factor acts on lignin removal, the latter contributes by removing silica from the substrate. Figure S8 shows that the acid step has also an important role for hemicellulose removal, as previously reported (Ang et al., 2013; Rezende et al., 2018).

The relationship between compositional changes and saccharification on the samples of the central composite design (samples RB1-RB13) was also evaluated (Figure 8). The amount of reducing sugars released in RB samples are all in the range of 30 to 60 mg/g biomass, and the lignin percentages are virtually constant (varying from 30 to 34 %). A similar situation is observed in ash contents, which are all below 2%, except for RB5 that was treated with the lowest NaOH concentration (1.17% w/w) and had 9.6 % of ashes. In this sample, saccharification was less efficient, which could be a consequence of the high silica content and of the relatively low cellulose content. Cellulose percentage is practically constant for all the samples and is also difficult to find a general correlation rule between its content and the sugar released. For example, the highest value of sugar released was obtained for sample RB2 (58.7 mg/g), which has one of the highest contents of cellulose (62.9%). Conversely, the lowest sugar release (31.0 mg/g for RB5) was also obtained in a sample with one of the highest cellulose contents (51.7%).

**[Figure 8]**

Correlations between reducing sugar release and ash content, cellulose, and lignin for RB samples are shown in Figures S9 to S11. As observed for the samples of the fractional factorial design, the correlation between sugars and ash is negative and moderate (R=-0.67, Figure S9). The correlation between sugar release and cellulose is positive but not high (R=0.56, Figure S10) and no correlation is observed between the sugar released and the lignin content (R= 0.21, Figure S11).

In summary, the analysis of pretreatment effects in sample composition and saccharification, showed that both, acid and alkali pretreatments are important to improve saccharification results, but for different reasons. In the first FFD, when NaOH concentration increased (0.5 to 4.5 % w/w), improved sugar release is achieved, mainly due to an enrichment in cellulose and to a decrease in the silica content. On the other hand, the contribution of increasing acid concentrations (from 0 to 3.6% w/w) to saccharification is more related to the acid effect in decreasing the lignin and the hemicellulose amount, together with an increase in cellulose content. In the range of variables tested in CCD (H2SO4 from 1.8 to 5.4% w/w and NaOH from 2 to 6% w/w), saccharification results are further improved (ca. 16%) compared to the FFD, but poor correlation is observed between sample composition and hydrolysis efficiency.

**3.3. Silica recovery**

To maximize the use of co-products obtained in this two-step pretreatment, silica was precipitated from the liquor of the alkaline step of sample RB2, which is the optimal condition for glucose release and for silica removal. Around 70% of the silica content present in rice husks *in natura* could be recovered from the alkaline liquor of sample RB2 by this procedure, and it was 97% pure silica (Figure 9).

**[Figure 9]**

Figure S12 shows a detailed composition analysis of the solid and liquid fractions obtained after the acid and alkaline treatments used to prepare sample RB2 from *in natura* biomass. Following the pretreatment steps, it is possible to track the distribution of sugars, lignin and ash in the different fractions until the final optimized sample (RB2).

This is a very economical, one-step and scalable method for silica recovery, which can contribute to a profitable use of this pretreatment co-product. Other procedure allows the recovery of up to 90% of the silica contained in rice husks and *Arundo donax*, in its amorphous and pure (99% pure) form, but it requires previous lignin isolation, centrifugation steps and high temperature (Barana et al., 2016).

1. **Conclusion**

There is a variety of pretreatment methodologies available to increase the enzymatic digestibility of plant biomasses to produce cellulosic ethanol. Pretreatments are essential to make these conversion processes viable, but methods with well-known effects in some biomasses can have a different result in others, since they are highly dependent on the sample chemical composition and morphology. Therefore, future direction in this research area should focus on the optimization of different pretreatments for specific biomasses and also on using the biomass in a more integral way. This is paramount to allow an effective use of these raw materials, minimizing time, cost and the production of residues, while maximizing the amount of fermentable sugars produced. In this work, DOE was used to optimize and acid-alkaline pretreatment in rice husks and the following optimal conditions were obtained: 1.8 % w/w H2SO4 in the acid step and 6 % w/w NaOH at 85 °C for 100 min and 12.5% w/w of solids in the alkaline step. This resulted in 58.7 mg of glucose/g substrate, an 8-fold increase as compared to sample *in natura* (7 mg/g). A simple method was also used to isolate high purity silica (97% pure at a 70% yield) from the alkaline liquor, enabling the use of this important co-product for different applications. This may contribute to a more cost-effective production of cellulosic ethanol from this abundant but relatively recalcitrant agricultural residue.

**Funding**

This work was supported by the RSC mobility grant; Fapesp [grant numbers 2016/13602-7 and 2018/23769-1] and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001. Research at the CNAP was funded by The European Commission’s Seventh Framework Programme (FP7) [project SUNLIBB 211982] and by BBSRC [projects BB/G016178 and BB/G016194].

**Competing interests**

The authors have no competing interests to declare.

**Figure captions**

**Figure 1.** Half-normal plot of the standardized effects of the 25-1 fractional factorial design.

**Figure 2.** A) Graph of internally studentized residuals (residuals/standard deviation of regression) and B) graph of predicted *vs.* actual (experimental) responses of sugar release (mg/g), for the 25-1 fractional factorial design.

**Figure 3.** Response surface of the most important factors (H2SO4 and NaOH concentrations) based on the 25-1 fractional factorial design. The other factors were kept at their centre points.

**Figure 4.** Response surface of the model shown in Equation 2.

**Figure 5.** A) Graph of internally studentized residuals (residuals/standard deviation of regression) and B) graph of predicted *vs.* actual (experimental) responses of sugar release (mg/g) for the CCD.

**Figure 6.** Reducing sugars (mg/g substrate) released from rice husks after 12h enzymatic hydrolysis (bars in the left axis) and their percentage of lignin (black squares in the right axis), crystalline cellulose (grey squares) and ash (white circles) before and after pretreatments. Error bars are standard deviation values from replicates. RIN = rice husks *in natura*; RH1 = sample pretreated with 1.8% w/w H2SO4; RH2 = sample pretreated with 3.6% w/w H2SO4; RA 1 to RA 19 = samples of FFD with experimental conditions detailed in Table 2.

**Figure 7.** Scanning electron microscopy images of rice husk surfaces: A) *in natura*; B) after the acid treatment with 1.8% w/w H2SO4 (RH1) and C and D) after the acid and the alkali treatment with 6% w/w NaOH (RB2). Scale bars: 100 μm in C and 50 μm in A, B and D.

**Figure 8.** Reducing sugars (mg/g substrate) released from rice husks after 12 h enzymatic hydrolysis (bars in the left axis) and their percentage of lignin (black squares in the right axis), cellulose (grey squares) and silica (white circles). Error bars are standard deviation values from replicates. RB 1 to RB 13 = samples of CCD with experimental conditions detailed in Table 5.

**Figure 9.** Chemical composition of the material obtained in silica recovery from the liquor of alkali pretreatment (Sample RB2) and a photograph showing the recovered material after drying.

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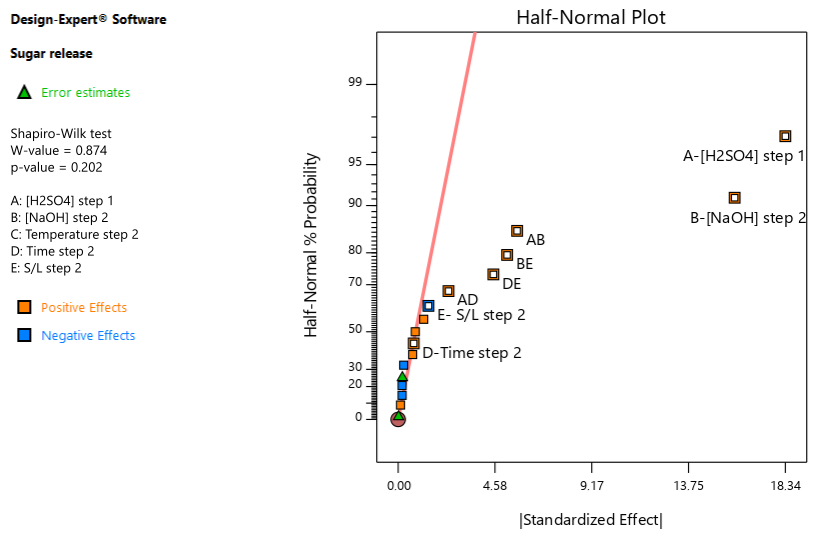
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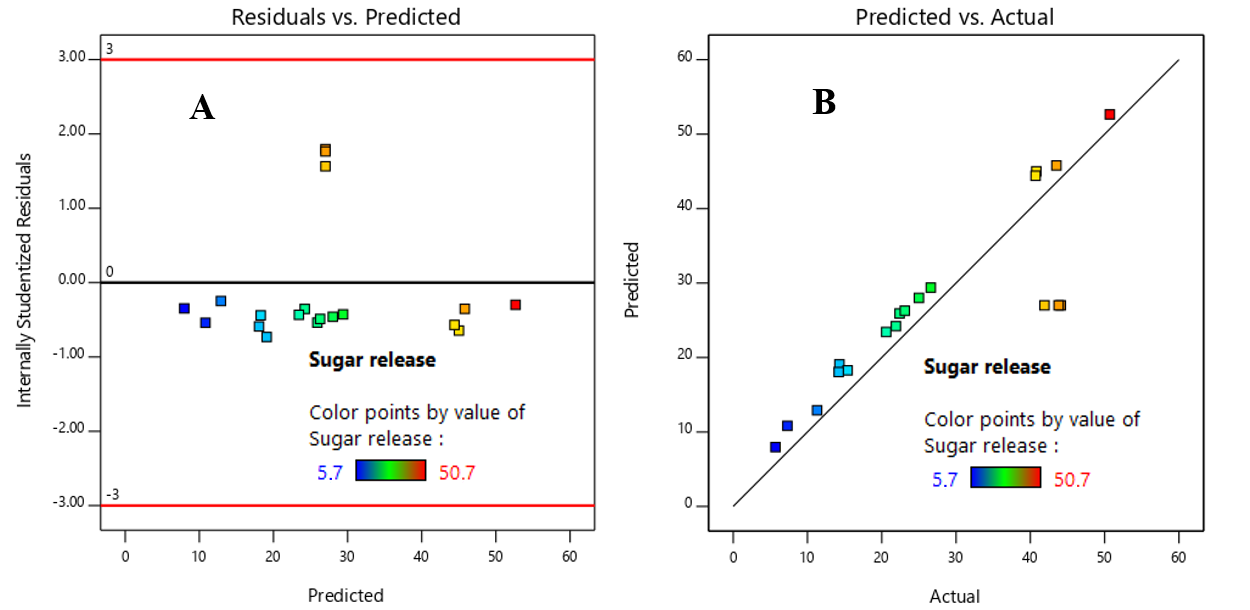
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**Figure 1.** Half-normal plot of the standardized effects of the 25-1 fractional factorial design.



**Figure 2.** A) Graph of internally studentized residuals (residuals/standard deviation of regression) and B) graph of predicted *vs.* actual (experimental) responses of sugar release (mg/g), for the 25-1 fractional factorial design.

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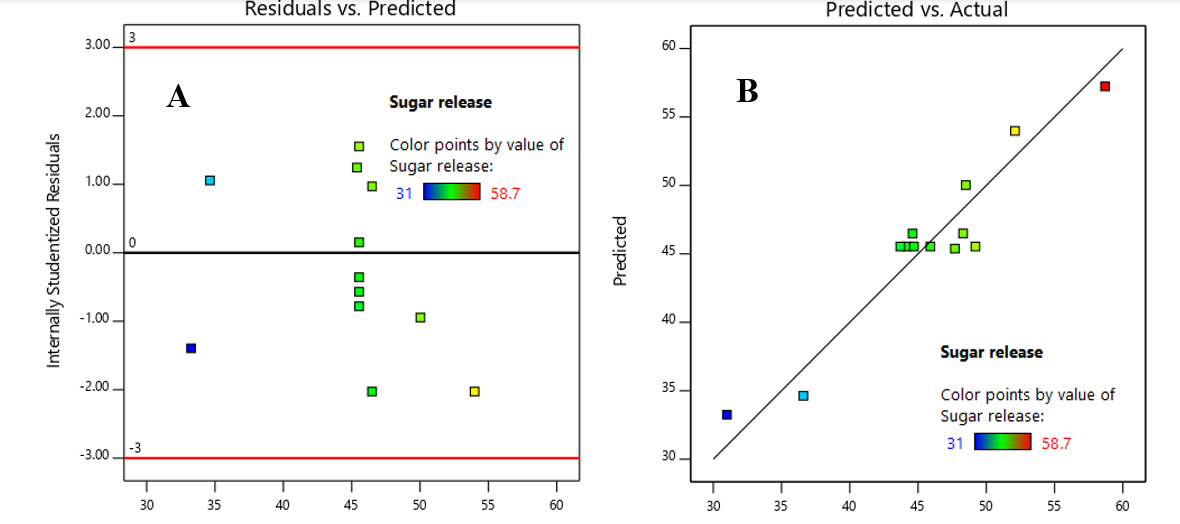
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**Gráfico, Gráfico de superfície

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**Figure 4.** Response surface of the model shown in Equation 2.



**Figure 5.** A) Graph of internally studentized residuals (residuals/standard deviation of regression) and B) graph of predicted *vs.* actual (experimental) responses of sugar release (mg/g) for CCD.

**Gráfico

Descrição gerada automaticamente**

**Figure 6.** Reducing sugars (mg/g substrate) released from rice husks after 12h enzymatic hydrolysis (bars in the left axis) and their percentage of lignin (black squares in the right axis), crystalline cellulose (grey squares) and ash (white circles) before and after pretreatments. Error bars are standard deviation values from replicates. RIN = rice husks *in natura*; RH1 = sample pretreated with 1.8% w/w H2SO4; RH2 = sample pretreated with 3.6% w/w H2SO4; RA 1 to RA 19 = samples of FFD with experimental conditions detailed in Table 2.

Mapa

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**Figure 7.** Scanning electron microscopy images of rice husk surfaces: A) *in natura*; B) after the acid treatment with 1.8% w/w H2SO4 (RH1) and C and D) after the acid and the alkali treatment with 6% w/w NaOH (RB2). Scale bars: 100 μm in C and 50 μm in A, B and D.

Gráfico

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**Figure 8.** Reducing sugars (mg/g substrate) released from rice husks after 12h enzymatic hydrolysis (bars in the left axis) and their percentage of lignin (black squares in the right axis), cellulose (grey squares) and silica (white circles). Error bars are standard deviation values from replicates. RB 1 to RB 13 = samples of CCD with experimental conditions detailed in Table 5.



Dried silica recovered

**Figure 9.** Chemical composition of the material obtained in silica recovery from the liquor of alkali pretreatment (Sample RB2) and a photograph showing the recovered material after drying.