UNIVERSIDADE ESTADUAL DE CAMPINAS
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BRUNA RODRIGUES SUSSAI

EXPERIMENTAL DESIGN OF ACID-ALKALINE PRETREATMENTS APPLIED TO RICE HUSKS AND STRAW FOR THE BIOETHANOL PRODUCTION

PLANEJAMENTO EXPERIMENTAL DE PRÉ-TRATAMENTOS ÁCIDO-ALCALINOS APLICADOS A CASCAS E PALHA DE ARROZ VISANDO À PRODUÇÃO DE BIOETANOL

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RESUMO

A produção de etanol a partir de biomassas lignocelulósicas por hidrólise via enzimática é dificultada pela estruturação da parede celular vegetal, sendo que pré-tratamentos são necessários para viabilizar o processo. Além disso, uma otimização de pré-tratamentos deve ser feita de forma específica para cada biomassa, devido às diferenças de composição e morfologia que os substratos apresentam. Neste trabalho, pré-tratamentos sequenciais ácidos e básicos aplicados a cascas e palha de arroz foram avaliados utilizando estratégias de design experimental (DOE).

Para cascas de arroz, utilizou-se um Planejamento Fatorial Fracionário (FFD) do tipo 2^{5-1} para avaliar os efeitos: concentração de H_2SO_4, concentração de NaOH, temperatura, tempo e razão sólido/líquido. Em seguida, utilizou-se um Planejamento Composto Central (CCD) para atingir condições experimentais ótimas. A melhor condição alcançada foi em um processo de duas etapas, utilizando H_2SO_4 1.8% m/m na etapa 1 e NaOH 6% m/m a 85 °C por 100 min e 12,5% m/m de teor de sólidos na etapa 2. Essa condição resultou em 58,7 mg de glicose/g substrato (12 h de hidrólise enzimática), um aumento de oito vezes, em comparação à casca *in natura* (7,3 mg/g de substrato). Adicionalmente, a etapa alcalina removeu elevada quantidade de sílica das cascas, que foi isolada com alto rendimento (70%) e pureza (97%), contribuindo para tornar mais viável a produção de etanol a partir de cascas de arroz.

Para palha de arroz, a avaliação das mesmas variáveis por um FFD do tipo 2^{5-1} já permitiu a otimização do processo. Nas melhores condições de hidrólise (sem etapa ácida, utilizando NaOH 4,5% m/m a 125 °C por 100 minutos e com 5% m/m de teor de sólidos), 481,9 mg de glicose/ g de substrato foram liberados em apenas 12 h de hidrólise enzimática. Com hidrólises de 24 h, foi possível alcançar rendimentos de conversão de celulose superiores a 90%, significantemente maior que para a palha in natura (HY = 18%). A otimização mostrou que a etapa ácida não é essencial para alcançar elevados rendimentos de hidrólise na palha de arroz, mas pode ser útil para extrair hemicelulose destas biomassas, valorizando o processo com base em uma abordagem de biorrefinaria.

ABSTRACT

The production of ethanol from lignocellulosic biomasses via enzymatic hydrolysis is hampered by the structure of the plant cell wall, requiring pretreatments to make the process viable. Furthermore, pretreatment optimization must be done specifically for each biomass, due to the differences in composition and morphology of the substrates. In this work, sequential acid and alkali pretreatments applied to rice husk and straw were evaluated using design of experiments (DOE).

For rice husks, a 2^{5-1} Fractional Factorial Design (FFD) was used to evaluate the effects: H_2SO_4 concentration, NaOH concentration, temperature, time and solid/liquid ratio. Then, Central Composite Design (CCD) was used to achieve optimal experimental conditions. The best condition achieved was in a two-step process, using 1.8% w/w H_2SO_4 in step 1 and 6% w/w NaOH at 85 °C for 100 min and 12.5% w/w of solid content in step 2. This condition resulted in 58.7 mg glucose/g substrate (12 h of enzymatic hydrolysis), an eight-fold increase compared to in natura rusk (7.3 mg/g of substrate). Furthermore, the alkaline removed a large amount of silica from the husk, which was isolated with high yield (70%) and purity (97%), helping to enable the production of ethanol from rice husk.

For rice straw, the evaluation of variables by a 2^{5-1} FFD has already allowed process optimization. Under the best hydrolysis conditions (without acid step, using 4.5% w/w NaOH at 125 °C for 100 minutes and with a solid content of 5% w/w, 481.9 mg of glucose were released /g of substrate after only 12 hours of enzymatic hydrolysis. With the 24-hour hydrolysis cellulose conversion yields greater than 90% were obtained, significantly higher than that of fresh straw (HY = 18%). The acid is not essential to achieve high hydrolysis yields from rice straw but can be useful to extract hemicellulose from this biomass, improving the process based on a biorefinery approach.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
CCD	Central composite design
DOE	Design of experiments
DSB	Dumbbell silica bodies
FESEM	Field-emission scanning electron microscopy
FFD	Fractional factorial design
FPU	Filter paper units.
HPLC	High performance liquid chromatography
HY	Hydrolysis yield
MS	Mean square
RA (1-19)	Rice husk sample pretreated using FFD
RB (1-13)	Rice husk sample pretreated using CCD
RG	Glucose released in hydrolysis
RH1	Rice husk sample pretreated with 1.8% w/w H_2SO_4
RH2	Rice husk sample pretreated with 3.6% w/w H_2SO_4
RIN	In natura rice husks
S (1-21)	Rice straw samples pretreated using FFD
S/L	Solid to liquid ratio
SEM	Scanning electron microscopy
SH1	Rice straw sample pretreated with 1.8% w/w H_2SO_4
SH2	Rice straw sample pretreated with 3.6% w/w H_2SO_4
SIN	In natura rice straw
XRF	X-ray fluorescence spectroscopy

CONTENTS

I. GENERAL INTRODUCTION

Rice figures as the third most produced agricultural commodity in the world, after sugarcane and corn (Abidea *et al.*, 2019). According to statistics of the Food and Agriculture Organization of the United Nations (FAO), the 2021/2022 annual global rice production (processed basis) surpassed 500 million tons (FAO, 2023). In Brazil, data from the National Supply Company (CONAB) show that the 2021/2022 rice harvest totaled approximately 10.8 million tons (Conab, 2023).

Rice production is accompanied by the generation of a great quantity of agricultural waste, resulting in 1.7 kg of husks and straws per each kg of milled rice (Binod *et al.*, 2010; Ebrahimi *et al.,* 2017). Although commonly underutilized, these residues have the potential to be transformed into various products, including chemicals, enzymes, textiles, bioplastics, pharmaceuticals, and biofuels (such as bioethanol, bio-oil, synthesis gas, and biogas) (Reddy and Yang, 2005; ElMekawy *et al.,* 2013). Other interesting alternative for rice waste recycling is the production of biochar via controlled burning, with possible applications in the adsorption of organic and inorganic contaminants, soil amendment, and carbon sequestration (Huang and Lo, 2019).

Rice-derived lignocellulosic residues have been reported in literature as source material for the production of biofuels, due to the following advantages: (1) origin in a renewable resource, (2) positive environmental impact, resulting in no net release of carbon dioxide and very low sulfur content, and (3) the expectation of significant economic potential yield, considering that fossil fuel prices will increase in the future (Manas *et al.,* 2019).

The production of ethanol from lignocellulosic biomass involves four main steps: pretreatments, enzymatic hydrolysis, fermentation, and distillation (Kothari *et al.*, 2022). Enzymatic hydrolysis is a central step of the process because it is responsible for converting cellulose into glucose. As the name suggests, the conversion occurs through the action of hydrolytic enzymes, named cellulases, which act synergistically to produce monosaccharides. However, the enzymatic hydrolysis is considered a challenging step of the biomass to ethanol conversion process since the action of these enzymes is influenced by the high crystallinity of the cellulose and by the presence of hemicellulose (19 to 27%) and lignin (5 to 24%) in the biomass, which hinder the access to the active sites of the cellulosic substrate. Therefore, the pretreatment step is necessary to reduce cellulose crystallinity and separate it from the other components of plant cell wall (Ogeda and Petri, 2010).

In addition to lignin and hemicellulose, rice straw and rice husks are rich in ash (10 to 20%). The ash fraction is composed mainly of silica (SiO_2), which can represent circa 75%–95% of the biomass inorganic fraction in a hydrated amorphous form (Mirmohamadsadeghi and Karimi, 2020). SiO_2 must be removed before processing because it interferes with hydrolytic processes and can form insoluble incrustations in reactors and filtration systems (Le *et al*., 2015).

There are several pretreatment methods applicable to lignocellulosic biomasses, which can be classified as physical (particle size reduction, irradiation, heating, etc.), chemical (using bases, acids, solvents, and others), biological (with enzymes or fungi) or their combination (Mankar *et al*., 2021; Haldar and Purkait, 2021). Due to the variable chemical composition, heterogeneity, and complexity of different lignocellulosic biomasses, no pretreatment is effective and suitable for all biomasses (Jin *et al*., 2020). Therefore, the study and optimization of specific pretreatments for different potential biomasses is essential.

Considering the importance of selecting an adequate and optimized pretreatment to enable the production of ethanol from rice husks and straw, the focus of this thesis was to evaluate the effect of a two-step pretreatment approach to each of these agricultural residues. Pretreatments comprise an initial treatment with dilute acid (known to hydrolyze mainly the hemicellulose fractions of lignocellulosic substrates), followed by a second step with dilute base (to remove both lignin and silica) (Das *et al.,* 2021; Rezende *et al.,* 2011). The alkali method was considered especially suitable for rice residues due to its very particular ability to remove both lignin and silica. Additionally, the acid step was included since previous results of our research group and others showed the potential of combining acid-alkali steps for cellulose isolation (Rezende *et al.,* 2011; Rezende *et al.,* 2018; Huong *et al*., 2022). Figure I.1 represents the improvement of enzymatic hydrolysis of rice residues using acid-alkaline pretreatment, due to the generation of a cellulose-rich solid, while removing hemicellulose, lignin, and silica.

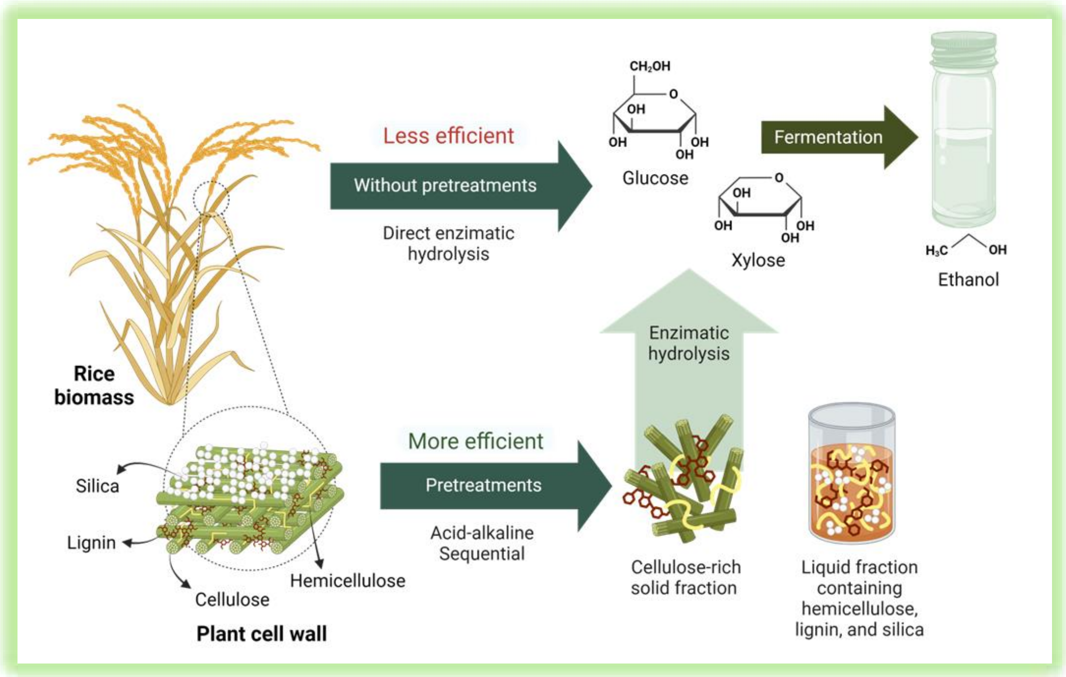

Figure I.1 Schematics of acid-alkaline pretreatment to increase enzymatic digestibility of rice waste.

The optimization of sequential pretreatments was performed using Experimental Design (DOE) strategies, allowing for simultaneous evaluation of several variables and identification of interactions between the different experimental test conditions. DOE refers to the process of planning, designing, and analyzing an experiment using statistical data to improve the functional performance of products, reduce quantity of residues, reagents, and process time, thus increasing its efficiency. Fractional Factorial Design is often used in industry for screening, since it allows studying main effects and their interactions using a minimum number of experimental runs (Antony, 2003).

In addition to optimizing production processes by reducing chemical inputs, time, and energy, another way to increase the viability of bioethanol production is the use of byproducts generated in the fractionation of biomass in a biorefinery (Alonso *et al.,* 2017). The biorefinery concept preconizes the use of the same facilities, equipment, and processes of biomass conversion to produce fuels, energy, chemicals, and biomaterials in an integrated way (Ragauskas, *et al.,* 2006), as illustrated in Figure I.2.

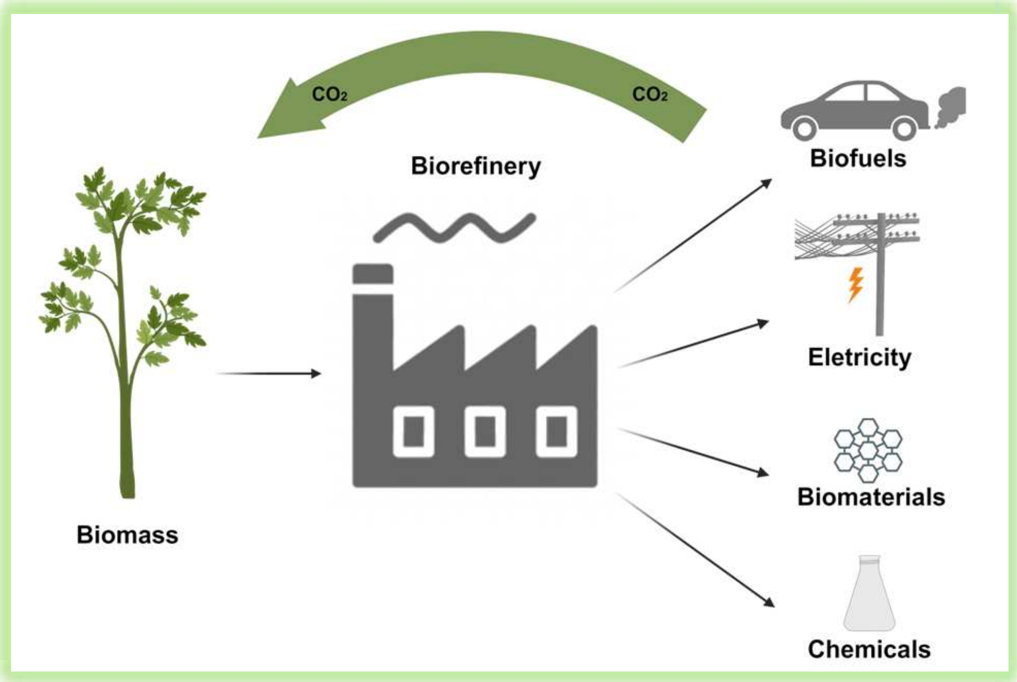

Figure I.2 Biorefinery schematics with integrated production of biofuels, bioenergy, and biomaterials. Adapted from Ragauskas *et al.* (2006).

The cellulose-rich fraction recovered from the pretreatment of rice waste, in addition to biofuel production, can result in value-added products, such as monomeric sugars, and nanocellulose (Rabelo *et al.*, 2023). Hemicellulose can be used in the production of furfural (used in the manufacture of paints, plastics, adhesives, nematicides, fertilizers, fungicides, and flavoring compounds (Raman and Gnansounou, 2015)), as well as for the generation of other products such as biogas, organic acids, lipids, and enzymes, through biochemical processing. (Rabelo *et al.*, 2023). Lignin has been used in scientific research as bulk lignin, or to produce lignin nanoparticles to be used in hydrogels (Chen *et al.*, 2019) and sunscreens (Wang *et al.*, 2019), due to the antifungal, antibacterial and UV protection properties in the nanometric scale. Finally, silica is a material with the potential for wide industrial applications, such as catalysis (Le *et al.*, 2015), anti-sticking agents, raw material to produce silicon (Todkar *et al.,* 2016), and as an adsorbent for heavy metals and organic contaminants in soil amendment and wastewater treatment plants (Shen, 2017). Figure I.3 illustrates the components that can be obtained from the fractionation of rice waste and their main applications.

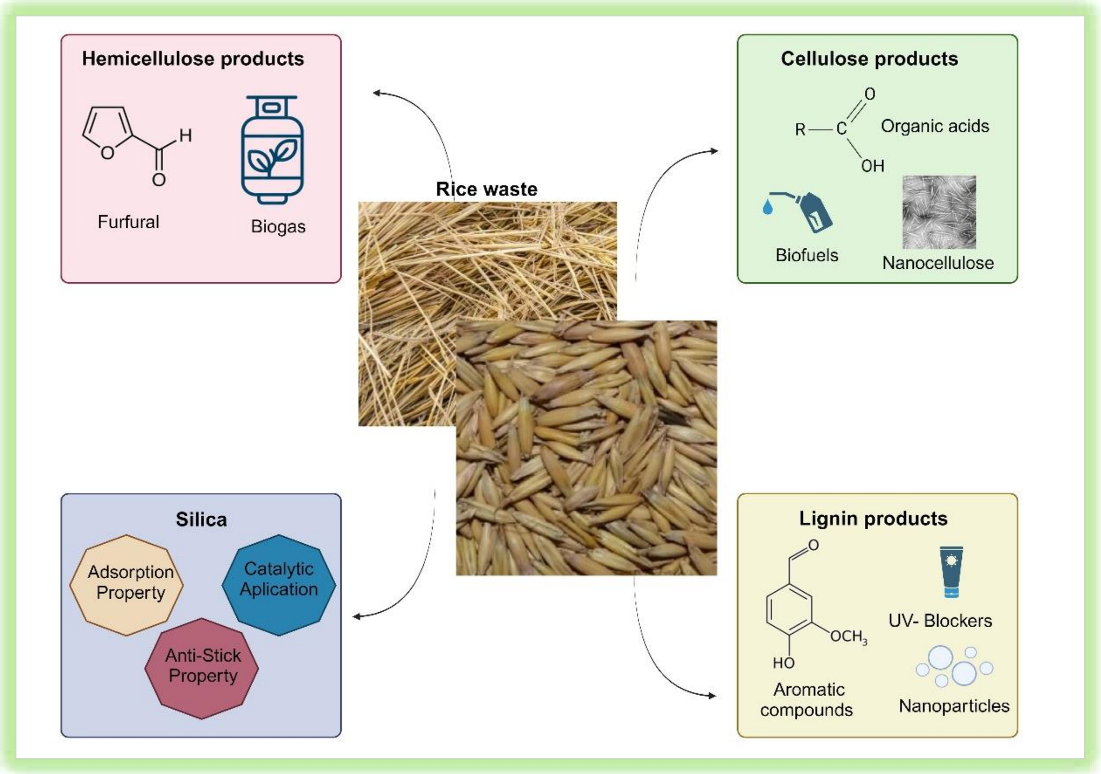

Figure I.3 Diagram showing examples of the various components that can be obtained from the fractionation of rice waste.

The simultaneous production and use of bioenergy and high-purity silica, from rice husks and straws, can significantly reduce the total cost of the process and release its full potential value (Umeda and Kondoh, 2010; Mirmohamadsadeghi and Karimi, 2018). However, most researches considering straw and rice husks as source materials concentrate on one of the applications: silica production, or bioenergy obtention, and therefore, integrated approaches are not reported (Mirmohamadsadeghi and Karimi, 2020). The present study focuses on both approaches: selection and combination of pretreatments aiming proper biomass fractionation, and the recovery of silica removed in the pretreatment of rice husks.

This thesis is structured in three chapters. The first contains a literature overview of the fractionation and conversion of rice residues into fermentable sugars and ethanol, introducing the following aspects: the main types of pretreatments applied to lignocellulosic materials, as well as their advantages and disadvantages; pretreatments that have been studied for rice straw and husks to enable and optimize bioethanol production; and trends and perspectives for future studies. The second chapter presents the part of this study regarding optimization of acid-base pretreatments of rice husks aiming ethanol production and the recovery of silica from the husks. The third chapter describes the optimization of acid-base pretreatments applied to rice straw, following the same experimental conditions used for the husks. By studying similar experimental conditions for both straws and husks, we seek to evaluate the possibility of treating these two different parts of the same plant in a single processing step.

II. OBJECTIVE

The objective of this study is to assess glucose release and enzymatic hydrolysis yield obtained from multiple rice husks and straw acid-alkaline pre-treatments conditions by using experimental design tools to improve the extraction of sugars used in the production of bioethanol from these biomasses.

CHAPTER 1 – LITERATURE REVIEW

1.1 Pretreatments

The hydrolytic processes required for ethanol production from lignocellulosic substrates are hampered by factors such as the complex interactions between cellulose, hemicellulose, and lignin; the crystalline nature of cellulose; and the physical barrier formed by lignin around cellulosic fibers. Therefore, it is necessary to pretreat the biomass, which involves breaking the cellulose-hemicellulose-lignin complex, reducing the crystallinity of the cellulose and, consequently, facilitating the action of cellulolytic enzymes.

Different substrates have different compositions and distributions of components in their cell walls, and consequently there is not a single universal pretreatment process that can be ideally applied to all substrates. Existing pretreatments can be classified into four categories: Physical, Chemical, Physicochemical, and Biological (Kothari *et al.*, 2022; Senatore *et al.*, 2021). Table 1.1 shows the four main classifications for pretreatments applied to lignocellulosic substrates, the most known processes in each category, their effects on the lignocellulosic substrate, and their main advantages and disadvantages. References where these pretreatments were applied to rice residues were included.

Table 1.1 Examples of pretreatments applied to lignocellulosic materials, their action mechanism, advantages, disadvantages, and references exemplifying the use of some of the pretreatments on rice residues.

Pretreatment classification	**Type of pretreatment**	**Approaches (principle or reagent)**	**Advantages**	**Disadvantages**	**References related to rice residues**
Physical Pretreatments	Milling (hammer milling, disk milling, ball milling)	Mechanical reduction of the particle size and amorphization (reduction of crystallinity).	Reduction of cellulose crystallinity and increase of available surface area.	High-energy consumption.	Mustafa *et al.* (2017)
	Extrusion	Application of high shear forces and temperature to reduce the particle size and mechanically alter fiber structure.	Continuous process with high processing capacity that do not form fermentation inhibitors.	Necessity of an additive to improve the material fluidity.	Zhang *et al.* (2020)
	Ultrasonication	Cavitation effect caused by ultrasonic energy (an acoustic wave that oscillates at frequencies above 16 kHz).	Reduction of particle size and improvement in lignin removal.	High energy demand and consequent possibility of plant structure degradation.	Zhang *et al.* (2020) Wu *et al.* (2017)
	Microwave radiation	Heating via incidence of microwaves (non-ionizing radiation with wavelengths in the range of 0.01-1 m and frequencies ranging from 300 to 300,000 MHz).	Reduction of cellulose crystallinity and increase of available surface area.	Requirement of special equipment and possible degradation of some bioactive compounds.	Kumari *et al.* (2021)
Chemical pretreatments	Acidic pretreatment	Use of acids such as HCl, H_3PO_4, HNO_3, and H_2SO_4 in diluted solutions.	Hemicellulose hydrolysis into simple sugars and modification of lignin structure.	Corrosion of equipment due to high acid concentrations.	Kapoor *et al.* (2017) Lamb *et al*. (2018) Wu *et al.* (2017) Zahoor *et al.* (2021) Anu *et al.* (2020b)
	Alkaline pretreatment	Use of alkalis such as NaOH, KOH, and $Ca(OH)_2$.	Efficient removal of lignin and silica.	Formation of residual salts in	Anu *et al.* (2020 a) Wu *et al.* (2017)

				Biomass.	Shahabazuddin *et al.* (2018)
	Organosolv	Use of solvents such as ethanol, methanol, acetone, and organic acids.	Hydrolysis of lignin and hemicelluloses. Process requires low temperature, low pressure, and neutral pH conditions.	High costs due to the recovery of solvents.	Trinh *et al.* (2016) Tsegaye *et al.* (2020) Ebrahimi *et al.* (2017)
	Ionic liquids	Use of ionic liquids (composed of organic salt ions), such as imidazolium and pyrrolidinium salts.	Selective removal of hemicellulose and lignin. Recyclability. Process free of toxic and odorous emissions.	High costs of ionic liquids.	Mohammadi *et al.* (2019) Abdolmaleki *et al.* (2021) Gao *et al.* (2019) Sorn *et al.* (2019)
Physicochemical pretreatments	Supercritical fluid	Use of supercritical CO_2 due to its moderate critical temperature and pressure.	Effective lignin removal and low sugar degradation.	Expensive equipment for large-scale production.	Serna *et al.* (2016) Gao *et al.* (2010)
	Steam explosion	Autohydrolysis and explosion of the material, due to a fast pressure release.	Effective hemicellulose degradation. Low environmental impacts. Possible in the presence or absence of an acid as a catalyst.	Sugar loss during post hydrolysis process. Inhibitor formation.	Montipó *et al.* (2021) Anu *et al.* (2020 b)
	Ammonia fiber explosion (AFEX)	Exposure of biomass to liquid ammonia at high temperature and pressure, followed by a fast pressure release.	Increase of the internal area, breaking and separation of hemicelluloses and lignin. Very effective for biomasses containing low amounts of lignin.	High costs for ammonia and equipment.	Blümmel *et al.* (2018)
Biological pretreatments	Pretreatments based on microorganisms or enzymes	Use of microorganisms or enzymes to modify the lignocellulosic structure.	Delignification of biomass. Structural modification of cellulose and hemicellulose. Low-cost and eco-friendly (no release of toxic compounds; no effluent generation). No inhibitor formation.	Long incubation time. Slow rate of delignification. Carbohydrate loss.	Sreemahadevan *et al.* (2018) Chang *et al.* (2021) Cruz *et al.* (2019) Baramee *et al.* (2020)

According to Table 1, there is a wide range of pretreatments that can be applied to lignocellulosic materials, and the selection of the technology to be used in each case must consider the type of biomass, the capacity of improving the cost efficiency of the enzymatic hydrolysis, and the processing route itself. For rice waste, recent studies on pretreatments are based on ionic liquids, organosolv, and physical pretreatments (including milling, steam explosion, microwave, ultrasound, and extrusion), acids, alkalis, and also biological methods using fungi or bacteria, as indicated in the references on the right column of Table 1.

Biological biomass treatments use microorganisms or enzymes to modify the lignocellulosic structure. They are considered environmentally friendly, efficient, energy and cost effective, but require at least two weeks to perform delignification (Sreemahadevan *et al.*,2018).

Physical pretreatments, based on milling, steam explosion, microwave, ultrasound, and extrusion, for instance, generally disrupt the lignocellulosic structure, enhancing subsequent enzymatic hydrolysis (Jiang *et al.,* 2019). Pretreatments based on particle size reduction (milling and extrusion) can modify and depolymerize the biomass structure, thereby increasing enzymatic accessibility to the biomass (Harun *et al.,* 2011). Steam explosion, in turn, is an autocatalytic process that uses highly compressed water (200–2000 psi) and high temperature (180–230 °C) to form a superheated steam that penetrates the solid and is followed by sudden decompression. It modifies the biomass structure and results in a filtrate rich in hemicellulosic sugars (Chung and Washburn, 2016; Wood *et al.*, 2016). Microwave pretreatment involves rapid heating and low energy consumption, leading to crystalline cellulose relocation and increase of enzyme accessibility (Keshwani and Cheng, 2009; Fia and Amorin, 2023). Finally, ultrasonic pretreatment attacks and cleaves the hemicellulose-lignin bond, improving the enzymatic accessibility of cellulose with the removal of hemicellulose (Hassan *et al.*, 2018).

Among chemical pretreatment methods, ionic liquids (IL) have been studied due to their low toxicity, low chemical corrosion, considerable thermal stability, and non-flammable properties. Due to their polarity and ability to form hydrogen bonds, IL can dissolve cellulose and other carbohydrates, besides facilitating the action of cellulases by reducing the interactions among cellulosic structures and increasing the free volume (Abdolmaleki *et al.,*2021). Although IL pretreatment is considered highly efficient, its main disadvantage is its high cost.

Organosolv pretreatment uses organic solvents (ethanol, methanol, glycerol, ethers, ketone, and others), generally combined to water, to remove hemicellulose and lignin from plant biomasses. Its advantages are the lower production of fermentation inhibitors and the fact that it is considered an environmentally friendly method (Trinh *et al.,* 2016).

Acid pretreatments increase enzymatic accessibility by effectively hydrolyzing hemicelluloses (mainly xylans) into monomeric sugars, producing a xylose-rich liquor (as exemplified by the mechanism in Figure 1.1). Thus, it causes structural transformations of the biomass**,** such as separation of fibers from the medulla, loosening of the fibrous network and changes in the crystallinity (Chen *et al*., 2016, Kapoor *et al*., 2015).

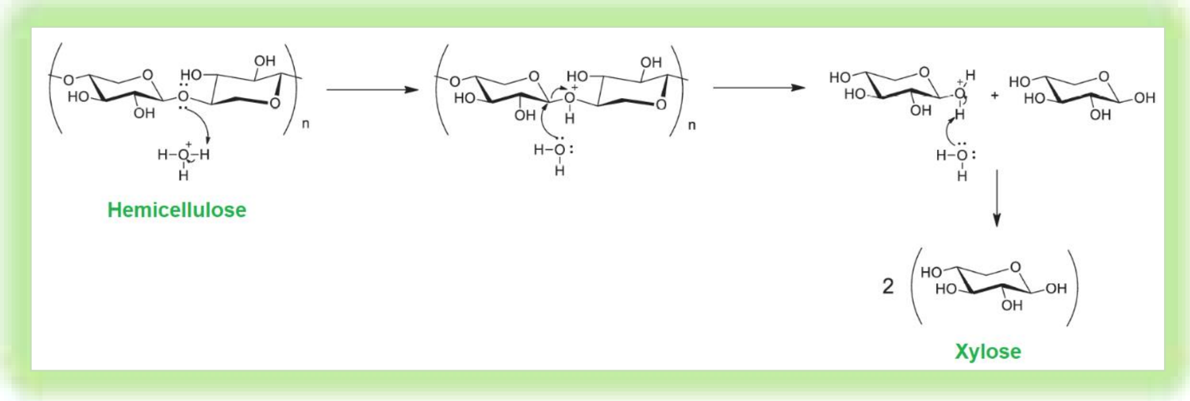

Figure 1.1 Expected mechanism of acid hydrolysis of hemicellulose. Extracted from Scopel, E. (2019).

Alkaline pretreatment has been widely studied and recognized as a highly effective and low-cost method for the solubilization of lignin present in different biomasses (Zahoor *et al.,* 2021). A mechanism that is known to help the dissolution and extraction of lignin from the plant cell wall in an alkaline medium is the disruption of the aryl bonds present in the lignin structure (McDougall *et al.,* 1993), as depicted in Figure 1.2.

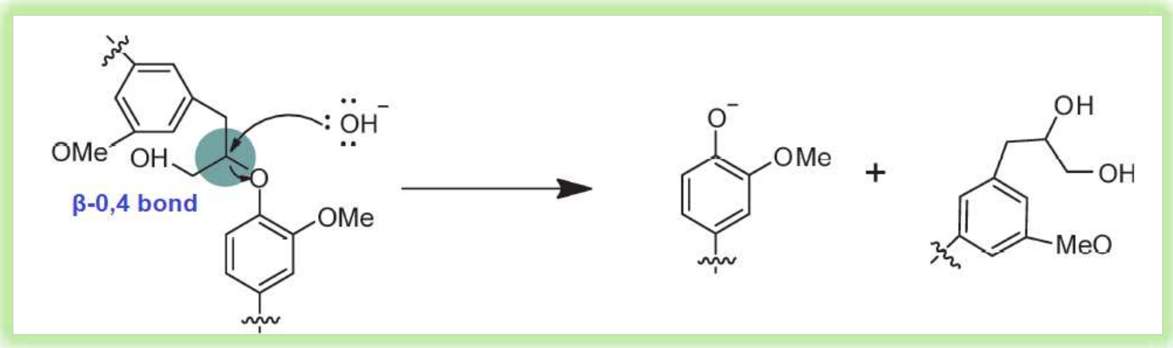

Figure 1.2 Mechanism of lignin fragmentation by alkaline hydrolysis of aryl bonds.

Besides, alkali methods are particularly suitable for rice residues due to their capacity of solubilizing silica. Although commonly represented by a monomeric structure, silica is found in the form of a polymer in plants and can be solubilized through the reversible reaction described in Equation 1.

$$SiO_{2\,(s)} + 2\,H_2O \leftrightarrows Si(OH)_{4\,(aq)} \qquad \text{Eq.1}$$

Figure 1.3 presents a proposed mechanism for the depolymerization of silica and its solubilization in alkaline media (Le *et al.*, 2015).

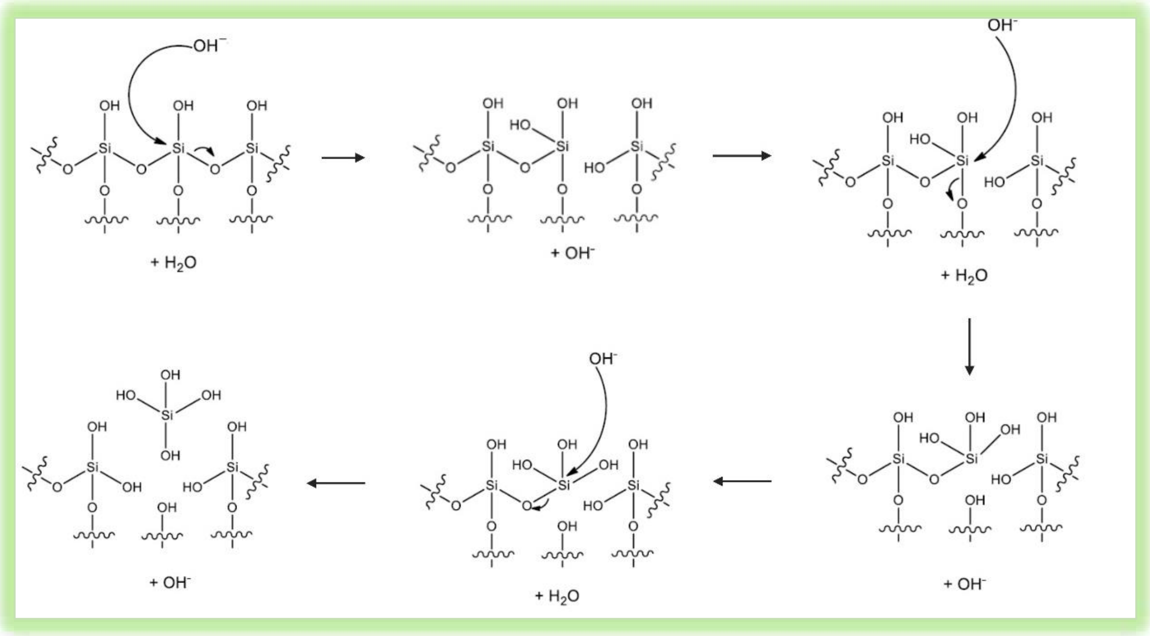

Figure 1.3 Proposed mechanism for the solubilization of silica in an alkaline medium initiated by the nucleophilic attack of the hydroxyl on Si, and subsequent breaking of Si-O bonds. Adapted from Le *et al.* (2015).

1.2 Pretreatments applied to rice straw and husks aiming at bioethanol production

Several types of pretreatments have been applied to rice residues. This section of the thesis is intended to report the primary studies and results obtained in recent years, focusing on the production of biofuels from rice straws and husks.

1.2.1 Biological pretreatments

Sreemahadevan *et al.* (2018) studied the effectiveness of rice straw delignification using a fungus (the alkalophilic dimorphic dimer encoded MVI.2011) known to synthesize large amounts of ligninases, which are key enzymes in the biological delignification of lignocelluloses. These authors studied pretreatments with MVI.2011 at 27 °C in solid and submerged states, which are two methods commonly used in culture of microorganisms. The solid-state culture of raw rice straw with MVI.2011 for 2 weeks improved the saccharification yield percentage by 54% after enzymatic hydrolysis (Celuclast Cellic Cetec2, 15 FPU/g glucan, S/L (solid/liquid ratio) = 2% w/v, 8h), while the one-week submerged culture showed a 74.2% improvement, as compared to untreated rice straw. According to these authors, the percentage of saccharification can be further improved if the submerged culture conditions are optimized to minimize the cellulolytic and hemicellulolytic activities of the fungus, specifically for rice straw. It is important to notice that the conditions of the biological pretreatment used in this study were those optimized for the action of the fungus on commercial lignin.

Chang *et al.* (2021) studied a pretreatment to increase the yields of delignification and saccharification of rice straw using laccase at 2U/g substrate (*T. versicolor*, a multicopper oxidase that oxidizes substituted phenols present in lignin structure) in combination with 100 - 1000 mg/L of ionic liquid or surfactant ([AMIM]Cl or TritonX-100). Results showed that the pretreatment using laccase in combination with 750 mg/L [AMIM]Cl and 500 mg/L TritonX-100 (24 h of incubation, at 50 °C) increased lignin removal to 18.49% and 31.79%, respectively, while the pretreatment performed with laccase alone removed 11.97% of lignin. Cellulose conversion values of 40.96%, 38.24%, and 37.91% were obtained in rice straw pretreated with laccase + TritonX-100, laccase + [AMIM]Cl, and laccase alone, respectively, *via* enzymatic saccharification (commercial glucosidase - Novozyme NS221118 - with 40 CBU/g rice straw and a cellulase complex - Novozyme NS220086- with 50 FPU/g rice straw, S/L = 2.5%, 72 h). These values were higher than the cellulose conversion obtained for the untreated straw (approximately 20%). Despite the positive results, these authors emphasize the need for a better optimization of experiments, focused mainly on increasing solid loading and reducing laccase doses, to achieve industrial viability.

Cruz *et al.* (2019) used *P. otreatus* (white-hot fungi) in the biological pretreatment of rice husk using a semi-solid culture (26.5 g of milled rice husk and 87 mL basal medium, S/L ≅ 30%) at 25° C for 25 days. On the 23rd day, maximum delignification (9%) was achieved, promoting a 57% increase in the enzymatic digestibility of rice husks (buffered cellulase from *Trichoderma reesi*, 1300 U/L; S/L = 1% w/v, 72 h of hydrolysis).

In addition to promoting delignification, biological pretreatments can be used to degrade the hemicellulose fractions (e.g. xylan). These fractions hinder the hydrolysis of cellulose, because they act as a physical barrier to cellulases. Baramee *et al.* (2020) proposed a bioprospecting pretreatment strategy using *B. firmus* K-1 (a xylanolytic bacterium that produces extracellular xylan enzymes, including xylanases, β-glycosidases, α-L-arabinofuranosidases, and acetyl esterases) to enhance cellulose degradation in rice. The proposed pretreatment (3 days of incubation, 37 °C) removed 30% of xylan and achieved a glucan conversion of approximately 74% (3.2 times higher than the value for untreated rice straw) after enzymatic hydrolysis (Accellerase 1500, 1.0 CU/mL, S/L = 100 g/L, 48 h). The results indicate that xylan removal can increase the accessibility of cellulose to cellulases, although the lignin content was not reduced.

1.2.2 Physical/Physicochemical pretreatments

Anu *et al.* (2020b) evaluated different physical pretreatments of rice straw, namely size reduction, steam explosion, microwave, and ultrasonic pretreatments; the authors showed that reducing the particle size of rice straw improved the amount of released reducing sugars after enzymatic hydrolysis (Cellulase, 20 U/g, S/L = 5% w/v, 6 h). The optimal result (47.53 ± 2.37 mg reducing sugars per 1 g of substrate) was achieved for ground rice straw (0.4 x 0.1 cm), when compared to a result of 32.48 ± 1.62 mg/g substrate for rice straw as larger particles (2.4 x 0.3 cm). The steam pretreatment of rice straw (S/L = 5 - 30% w/v) was carried out at 121° C and 15 psi for 60 minutes, and the maximum amount of reducing sugars (64.44 mg/g substrate) was released in the straw pretreated with a S/L of 30% w/v.

For microwave pretreatment, rice straw (S/L = 10% w/v) was immersed in distilled water and exposed to microwave (1200 W, 50 Hz, 220 V) radiations under low, medium, and high power for 5 min. Medium microwave power yielded the sample with maximum amount of released sugars (26.85 ± 1.34 mg/g substrate), but this result was lower when compared to the saccharification of untreated rice straw (32.48 ± 1.62 mg/g substrate), which is due to biomass carbonization associated to the degradation of some sugars, according to the authors. Finally, the pretreatment with ultrasonication was performed by immersing the rice straw (S/L = 10% w/v) in distilled water and exposing the slurry to ultrasonic radiation for 5 to 15 min. The sample pretreated for 15 min released the maximum content of sugars (34.50 ± 2.82 mg/g substrate), when compared to the other samples.

Montipó *et al.* (2021) studied the catalyzed steam pretreatment applied to rice husks (S/L = 5% w/v) employing 2.5% of SO_2 as a catalyst (amount based on the water content of wet RH). The pretreatment was optimized using Central Composite Rotational Design (CCRD), based on the response surface method (RSM), to evaluate the effect of temperature (183 – 227 °C) and time (1.1 – 8.9 min) on sugar content production. The optimized pretreatment condition was 218 °C for 2.3 min, capable of releasing 90.6 g/L (86.4% yield) of glucose, after enzymatic hydrolysis (Cellic® CTec2, 20 FPU/g, S/L = 22%, 48 h).

Extrusion is a mechanical processing technology which integrates mixing, stirring, shearing, and heating, promoting physical and chemical modifications in lignocellulosic biomass. It can disrupt the lignin structure and the crystalline structure of cellulose, besides increasing cellulose specific surface area and porosity (Duque *et al.*, 2013; Negro *et al.,* 2015; Moro *et al.*, 2017). Zhang *et al.* (2020) reported an optimization of the rice straw extrusion conditions (material size - previously ground and sieved rice husk-, material moisture, extrusion temperature, and screw speed) using RSM and then an optimization of ultrasound application time on the pretreated sample, aiming for maximum efficiency of enzymatic hydrolysis. Results showed that the optimal extrusion parameters were 143 °C at a screw rate of 350 rpm, with 29% of moisture, and material size particles that passed through the 60 mesh, for ultrasound pretreatment, optimal conditions were 40 kHz/500W for 1.5 h. The enzymatic hydrolysis (with cellulase at 240 U/g sample; β-glucosidase at 180 U/g, and hemicellulase at 500 U/g for 72 h) of the pretreated sample at optimum conditions reached 77.5% of conversion of total cellulose and hemicellulose, which is more than 4 times that of unpretreated rice husks (17.61%).

1.2.3 Chemical pretreatments

1.2.3.1 Ionic liquid pretreatment

Imidazolium-based ionic liquids, such as 1-ethyl-3-methylimidazolium acetate ([EMIM][Ac]), are known to be the most efficient for the pretreatment of lignocellulosic materials (Wang *et al.*, 2017; Aung *et al.*, 2018). In an attempt to reduce costs, Mohammadi *et al.* (2019) proposed a pretreatment on rice straw using morpholinium-based ionic liquids (1-H-3-methylmorpholinium), which have lower cost and higher stability than imidazolium IL. These authors evaluated the effect of temperature (90, 105, and 120 °C), time (2, 3, and 5 h), solid loading (5, 6, 7, and 10% w/w chloride ([HMMorph][Cl]), and straw particle size (< 0.177 mm, 0.177-0.841 mm, and 0.841-2 mm), as well as the use of ionic liquid with water (30, 40, and 50% w/w), and dimethyl sulfoxide (DMSO) (10 and 30% w/w) as co-solvents, aiming to further reduce the cost of the process. The conclusion showed that the optimized pretreatment set of conditions was a mixture of the IL with 50% water, at 120 °C and 5% w/w solid loading for 5 h with straw particle size of 0.177 - 0.841 mm. Under this set of conditions, the hydrolysis yield (72 h) was increased from 33.2% (untreated rice straw) to 70.1%, while the ethanol production yield was improved from 21.9% to 64.0% of the theoretical maximum. These optimal pretreatment conditions were also evaluated for rice straw pretreated with [EMIM][Ac], showing similar hydrolysis (77%) and ethanol (66.3%) yields, and confirming the potential of [HMMorph][Cl] under diluted conditions as a lower-cost IL pretreatment for rice straw.

Abdolmaleki *et al.* (2021) proposed a rice straw pretreatment using an acidic ionic liquid (1-(carboxymethyl)pyridinium chloride), which has been studied for other reactions in the past, but only in recent years was proposed as an alternative for the dissolution of biopolymers, such as chitosan, chitin and cellulose (Teheri *et al.,* 2018; Teheri *et al*., 2019). Using an aqueous solution of [CMPy]Cl, rice straw pretreatment was performed under different conditions of time (2, 3, and 5 h), temperature (25, 90, and 120° C), solid loading (5, 6, and 15% w/w) and the use of water as a co-solvent, seeking maximum ethanol production. The best ethanol conversion (62.2%) was obtained with fermentation of previously hydrolyzed (Celuclast, 20 FPU/g substrate, S/L = 5 g/L, 72 h) pretreated samples (35% water, at room temperature, and 6% w/w solid loading for 3 h), in contrast to a 21.9% conversion for the untreated straw. The authors also showed that, by using pretreatment with ([CMPy]Cl), the porosity and surface area significantly increased, while the crystallinity index decreased. Silica removal was also reported leading to a more accessible surface for enzymatic action.

The use of high water contents as co-solvent is a strategy to reduce the cost of IL pretreatments. However, molecular dynamic simulations (Hegde *et al*., 2016) have shown that the dissolution capacity of ILs is notably reduced when the content of water exceeds the limit of 25% m/m, due to the breakdown of IL ordering structures.

Inorganic salts can be used as additives to improve the tolerance of ionic liquids in the presence of water. A study conducted by Gao *et al.* (2019) considered inorganic salts (K_3PO_4 and K_2CO_3) as additives to pretreat rice straw using imidazolium-based ionic liquid (1-ethyl-3-methylimidazolium chloride)/water systems. The best reducing sugar yield was 82.45%, for the rice straw pretreated using 50% [C2mim]Cl + 49.5% water + 0.5% K_2CO_3 (w/w) at 95°C and S/L = 5% for 3 h. This value, obtained after enzymatic hydrolysis (Cellulase from Trichoderma reesei (Celluclast 1.5L) and β-glucosidase (Cellobiase from Aspergillus niger) (1:1), 20 FPU/g of cellulose, S/L = 0,67 g/L, 72 h), was considerably higher than that for rice straw *in natura* (14%), and was associated with a 69.32% lignin removal. These results showed that the salt addition strategy is valid to reduce IL pretreatment costs. To achieve further cost reduction, the authors suggested studies related to recycling of the used solutions, as well as optimization of the pretreatment parameters, such as ionic liquid concentration, salt concentration, time, and temperature.

Another way to reduce the costs of ionic liquid pretreatments was proposed by Sorn *et al.* (2019), combining the use of microwaves, which are capable of inducing rapid heating and reducing the process duration. These authors performed pretreatment of rice straw using 1-butyl-3-methylimidazolium chloride ([Bmim]Cl) and 1-butyl-3-methylimidazolium hydrogen sulfate ([Bmim]HSO_4) as an acidic-IL and under microwave irradiation, and recycling the ionic liquid. The pretreated rice straw samples showed highest lignin removal using microwave and [Bmim]Cl (57.02 ± 1.24%), followed by [Bmim]Cl alone (41.01 ± 2.67%), microwave with [Bmim]Cl, [Bmim]HSO_4 alone (20.77 ± 1.79%), and [Bmim]HSO_4 alone (16.88 ± 1.14%)]. The highest glucan conversion (61.14 ± 2.32%) was achieved through enzymatic saccharification (using the cellulase complex Novozyme NS220086 at 50 FPU/g substrate, and the β-glucosidase Novozyme NS221118, with an enzyme load of 40 CBU/g substrate at S/L = 1% w/v for 48 h) on the [Bmim]Cl + microwave sample, as compared to a glucan conversion of 16.53 ± 0.54% for the untreated straw.

1.2.3.2 Organosolv pretreatment

A study of organosolv pretreatment of rice straw was conducted by Trinh *et al.* (2016), using glycerol as a solvent. Initially, a pretreatment with glycerol was evaluated at temperatures ranging from 130 to 210° C for reaction times of 1 to 24 h, with a 5% solid loading. Then, the best pretreatment condition was applied with addition of hydrochloric acid (0.1 - 1%) as a catalyst. The best reducing sugar yield (56.6%, almost five times higher than the value obtained for the unprocessed sample) was obtained for the sample pretreated at 190° C for 10 h with S/L = 5% w/w and 0.5% HCl, after enzymatic hydrolysis (using an enzyme cocktail containing cellulase at 17.5 FPU/g, β-glucosidase at 6.25 CBU/g, and xylanase at 25 FXU/g, S/L = 5% w/w for 72 h).

According to these authors, in addition to being non-toxic, this solvent has a high boiling point, allowing the pretreatment to be performed at the high temperatures typically used in organosolv procedures, but under atmospheric conditions. However, it is important to highlight that although higher temperatures allow for higher pretreatment rates, they also increase the energy input and thus the cost of the process, and cause the process to be more expensive.

Tsegaye *et al.* (2020) proposed a pretreatment of rice straw using a mixture of formic and acetic acids as organic solvents, to solubilize lignin at temperatures lower than 100°

C. The authors used a central composite design and response surface analysis to optimize the pretreatment parameters (temperature: 60 to 100° C; time: 10 to 50 min, and acid concentration: 50 to 90%) aiming for maximum lignin solubilization. The statistical analyses showed that all the evaluated factors are significant and cause a positive effect on the depolymerization of rice straw. The optimum condition (73.17% of lignin removal) was achieved with an acid concentration of 69.85%, at 75.41°C for 29.68 min. The maximum hydrolysis efficiency was 62.09% (515 mg/g rice straw), although only after 9 days of hydrolysis as, in this study, the whole cell bacteria *Bacillus* sp. BMP01 was used to replace the more expensive commercial enzymes.

Ebrahimi *et al.* (2017) investigated pretreatments of rice husks with glycerol carbonate (at 130° C) and acidified aqueous glycerol (at 90° C) for different reaction times (15, 30, 60, and 120 min). Rice husks pretreated for 60 min with both pretreatments had the maximum glucan digestibility yields: 78.2% (with glycerol carbonate) and 69.7% (with acidified aqueous glycerol) after enzymatic hydrolysis (commercial cellulase from *Trichoderma reesi*, 15 FPU, S/L = 2% w/w, 72 h). Furthermore, the pretreatment with acidified glycerol carbonate was able to remove 55.8% of lignin from the biomass, while the pretreatment with acidified aqueous glycerol removed only 21.21%.

1.2.3.3 Acid pretreatment

Kapoor *et al.* (2017) researched dilute sulfuric acid pretreatment of rice straw at high S/L (25 to 35 % w/w) and pilot scale (250 kg/day). A sequence of pretreatment experiments on rice straw was carried out for 10 minutes, combining different temperatures (152, 157, 160, 162, and 166 °C) with different concentrations of sulfuric acid (0.25, 0.35, 0.65, and 0.70% w/w). Maximum glucan hydrolysis (72%) was achieved for the sample pretreated with 0.35% w/w acid concentration, at 162 °C for 10 min, after enzymatic hydrolysis (Cellulase, 10 FPU/g substrate, S/L = 20% w/w, 48h).

Anu *et al.* (2020 b) compared the enzymatic hydrolysis yield in rice straw samples pretreated with different acids (sulfuric, lactic, and citric acid) at 121° C and 15 psi for 60 min, obtaining superior performance for sulfuric acid. Within the various concentrations of sulfuric acid tested (between 0.5 and 3% v/v), 1% v/v was found to release the maximum amount of reducing sugars (about 185 mg/g substrate) after enzymatic hydrolysis (Cellulase, 20 U/g, S/L = 5% w/v, 6 h).

Lamb *et al.* (2018) studied a sequence of acid pretreatments of rice husk using different sulfuric acid concentrations (0.5, 0.1, and 2.5%) for 30, 60, and 90 min, at 121°C and

S/L = 10%; the best enzymatic hydrolysis result (63.4% of glucose) was achieved for the sample pretreated with 1% H_2SO_4 for 60 min, after 48 h of enzymatic hydrolysis (Cellic® CTEC2, 40 FPU, S/L = 10%, 48 h).

1.2.3.4 Alkaline pretreatment

Wu *et al.* (2017) performed rice straw pretreatment using ultrasound-assisted sodium hydroxide (NaOH) solution to obtain improved lignin removal under shorter reaction times. These authors showed that the pretreatment of rice straw, using a 1% w/w NaOH solution and S/L = 5% w/v, was able to remove 46% of hemicellulose and 57% of lignin from the biomass, with negligible degradation of cellulose. The sample pretreated under these conditions was able to release an amount of reducing sugar of 2.91 g/L after enzymatic saccharification (Cellulase at 10,000 U/g, S/L = 1% w/v, 48 h), which is about 3.5 times higher than the untreated sample (0.85 g/L).

Shahabazuddin *et al.* used Box-Behnken Design (BBD) based on RSM to optimize an alkaline pretreatment on rice husks, evaluating the influence of biomass loading (10-25% w/w), particle size (0.25-1 mm), NaOH loading (0.5-2% w/w), and reaction duration (20-60 min) on reducing sugar yields after enzymatic hydrolysis (cellulase, 20 FPU/g of substrate, S/L = 10% w/v, 48 h). Maximum sugar release (371 mg/g of biomass) was obtained with 10% biomass loading, 0.25-0.625 mm particle size, 2% w/w NaOH and a 40 min reaction, by removing 54% w/w of the native lignin and increasing the cellulose content from 32.65 to 51.65% w/w. Within the experimental range, the reducing sugar yields ranged from 26.1 to 371 mg/g.

Potassium hydroxide (KOH) is another alkali which received much attention in recent research due to its ability to remove lignin from biomass (Sharma *et al.*, 2013: Jaffar *et al.,* 2016; Xie *et al.,* 2018; Veerangouda *et al.*, 2019, Zahoor *et al.* (2021)).

Zahoor *et al.* (2021) performed a study to optimize a rice straw pretreatment using KOH/urea (KU) at low temperatures (< 100 °C). The optimization used Box-Behnken Design (BBD), based on RSM, to evaluate the effects of pretreatment conditions (reaction time ranging from 0.5 to 3 h, temperature varying from 50 to 90 ◦C, KU concentration from 1 to 3%, and solid-liquid ratio from 5 to 20%) on the efficiency of enzymatic hydrolysis. The optimal conditions (3% KU, S/L = 1:15, for 3 h at 70 °C) resulted in 32.47% lignin and 21.82% hemicellulose removals. The rice straw sample pretreated under these optimized conditions showed a reducing sugar yield of 98.38 % (five times higher than that of straw *in natura*, after enzymatic hydrolysis (20 FPU cellulase from *Trichoderma longibrachiatum*/g glucan, S/L =

5% w/v, 72 h), and an ethanol yield of 87.13% (six times higher than that of the straw *in natura*). In addition, this work showed that this type of pretreatment generates a residue rich in potassium and nitrogen, with the potential to be used as agricultural fertilizers.

Pretreatment with sodium carbonate (Na_2CO_3) has also shown effective delignification of rice waste (Salehi *et al.*, 2012; Khaleghian *et al.*, 2015; Shen *et al.*, 2018). Anu *et al.* (2020a) described an optimization of sodium carbonate pretreatment of rice straw using Central Composite Design (CCD), based on RSM, to evaluate the effects of sodium carbonate concentration (0.25 to 2.25% w/v), substrate concentration (5 to 35% w/v), and autoclaving (at 121 °C) time (10 to 50 min) on the liberation of reducing sugars. Statistical optimization resulted in a higher release of reducing sugars (531.2 mg/g substrate after enzymatic hydrolysis (cellulase 20 U/g, S/L = 2.5% w/v, 6 h)) for the sample obtained with pretreatment using 5% rice straw, 1.25% sodium carbonate, and an autoclaving period of 30 min. Moreover, the maximum release of reducing sugars reached the value of 728.45 mg/g substrate after 48 h of hydrolysis. The first author of the cited study applied the same optimization method in another research (Anu *et al.*, 2020b), using ammonia as alkali in the pretreatment and the optimized condition (5% rice straw, 12% ammonia and autoclaving for 30 min). After enzymatic hydrolysis for 6 h (cellulase 20 U/g, S/L = 2.5% w/v), the saccharification rate showed an increase of 1.9-fold, releasing 451.96 mg/g substrate, and after 48 h of hydrolysis the maximum release of reducing sugars reached 635.37 mg/g of substrate.

1.2.4 Comparison between the pretreatments studied for rice residues and justification of this work

Comparisons among saccharification results, obtained in different pretreatments applied to a specific biomass, should be done with caution since the conditions for enzymatic hydrolysis are crucial for the sugar release and vary significantly in different studies. Table 2 summarizes the pretreatments reported in recent years, which achieved the best enzymatic hydrolysis results for rice straw and rice husks.

Table 1.2 Summary of the three best pretreatments applied to rice straws and husks in recent studies, and their respective enzymatic hydrolysis conditions and sugar yields.

Rice waste	**Pretreatment**	**Enzymatic hydrolysis conditions**	**Sugar Yield**	**Reference**
Rice straw	Alkaline using KOH/urea	Biomass loading: 5% w/v Cellulase from *Trichoderma longibrachiatum* (20 FPU/g glucan) at 50 °C, 150 rpm, pH 4.8 for 72 h	98.4%	Zahoor *et al.* (2021)
	Biological pretreatment of rice straw with cellulase-free xylanolytic enzyme-producing *Bacillus firmus* K-1.	Biomass loading: from 10 to 200 g/L Accellerase 1500, Cellulase from *Trichoderma reesei (*Celluclast 1.5L) (1.0 CU/mL), at 50 °C, 130 rpm, pH 5 for 48 h	74%	Baramee *et al.* (2020)
	Ionic liquid (IL/inorganic salt/water), using inorganic salts (K_3PO_4 and K_2CO_3) as additives for imidazolium-based IL/water systems	Biomass loading: 0.67 g/L Cellulase from *Trichoderma reesei* (Celluclast 1.5L) and β-glucosidase (Cellobiase from Aspergillus niger) 1: 1 (20 FPU/g of cellulose) at 50 °C, 120 rpm, pH 4.8 for 72 h	82.4%	Gao *et al.* (2019).
Rice husk	Steam pretreatment	Biomass loading: 22% Cellic® CTec2 (20 FPU/g substrate) at 50° C, pH 4.8, 150 rpm for 48 h	86.4%	Montipó *et al.* (2021)
	Diluted acid sulfuric pretreatment	Biomass loading: 10% Cellic® CTEC2 (40 FPU) at 50° C, pH 5, 210 rpm for 48 h	79.8%	Lamb *et al*. (2018)
	Acidified aqueous glycerol and glycerol carbonate pretreatment	Biomass loading: 2% w/w Cellulase from *Trichoderma reesei* (10 FPU/g glucan) at 50 °C, pH 4.8, 150 rpm for 72 h	78.2%	Ebrahimi *et al.* (2017)

Different types of pretreatments are promising for rice straw and husk in terms of enzymatic hydrolysis yields. However, in addition to the efficiency of enzymatic hydrolysis, it is important to choose a process that minimizes the generation of polluting byproducts and maximizes the economic benefit of cellulose conversion technology. Important points that also need to be considered are the prediction of these pretreatments based on the cost-benefit assessment of these processes, and the possibility of recovery and use of the other components of the plant wall of straw and rice husk, as in a lignocellulosic biorefinery.

Acid and alkaline pretreatments were among the most promising for rice waste and it is important to emphasize that they are not especially expensive when compared to other pretreatment methods. A comprehensive work by Tao and collaborators performed a techno-economic analysis of six pretreatment processes to convert switchgrass into fermentable sugars and cellulosic ethanol. They compared ammonia fiber expansion (AFEX), dilute acid (DA), alkaline pretreatment, liquid hot water (LHW), moisture in wet ammonia (SAA), and sulfur dioxide impregnated steam explosion in terms of overall ethanol production, total capital investment, and minimum ethanol selling price (Tao *et al.*, 2011). The authors concluded that there is a limited differentiation between economic performances comparing these pretreatment options. Another work by Eggeman and Erlander (2005) compared five pretreatment processes (DA, LHW, AFEX, alkaline, and ammonia recycling percolation (ARP)) for releasing sugars from corn stover, concluding that the cost differences of these pretreatments are also insignificant. The authors attribute the low variation in pretreatment costs to a balance between the low cost of pretreatment reactors, in some pretreatments, and the higher costs associated with the recovery of pretreatment catalysts and of ethanol product in others (Eggeman and Elander, 2005).

Considering these examples and all the factors involved in the economic viability of a pretreatment - for example, the price of biomass and reactors, the cost of energy, the possibility of reusing chemicals and water, residence time and temperature, ethanol purification, etc.-, the techno-economic analysis of pretreatments has shown that acid and alkaline pretreatments are not particularly expensive when compared to other common pretreatments (Baral and Shah, 2017; Tao *et al.*, 2011; Eggeman and Elander, 2005).

In this work, acid pretreatments using H_2SO_4 and alkaline treatments using NaOH were studied. Regarding their polluting characteristics, it is difficult to find studies in literature comparing different pretreatments in terms of the environmental impact of their involved reagents. But it is important to highlight that in both the acid and the alkaline pretreatments proposed here, chemicals are used in dilute conditions. NaOH and H_2SO_4 are reagents widely applied to obtain a variety of products in the chemical industry, and the treatment options of waste streams are routine and well-established processes. NaOH and H_2SO_4 do not contain heavy metals nor use organic or chlorinated solvents, which are much more harmful to the environment and difficult to dispose of. A sodium hydroxide solution can be neutralized to pH = 7.0 with a few drops of concentrated hydrochloric acid (HCl), forming a salt solution (NaCl) that can be discharged directly into the wastewater system. In turn, a H_2SO_4 solution can be neutralized with CaO or $Ca(OH)_2$, forming a salt ($CaSO_4$) and water. Furthermore, it is possible that future studies will focus on strategies to allow the reuse of pretreatment water (by salt isolation, for example), contributing to reduce the environmental impact of the process.

The combined use of the acid and alkaline pretreatments chosen in this work (evaluated using experimental design strategies (DOE)) allows the fractional extraction of the main byproducts of bioethanol production - hemicellulose (extracted in the acid stage), and lignin and silica (extracted in the alkaline stage), in an aqueous solution and without the use of toxic solvents, adding value to the bioethanol production process from rice waste.

1.2.5 Design of Experiments (DOE)

DOE consists of carrying out successive experiments with controlled changes (Silva *et al.,* 2023), in which all experimental variables can be varied simultaneously, allowing the maximum information to be extracted from a minimum number of tests. This approach reduces the amount of time and resources needed, and allows the identification of synergistic and/or antagonistic effects between factors (interactions) on a given response of interest, thus facilitating the understanding of the final result of an experiment (Barros Neto *et al.,* 2010; Breitkreitz *et al.,* 2014)

Factorial designs are used to study statistically independent variables, that is, those that can have their values changed and controlled independently of each other. The selection of the type of factorial design to be used depends on the researcher's objective and the stage at which the project is (Breitkreitz *et al.,* 2014).

In full factorial designs, the responses of all main effects and interactions are evaluated, however these types of design are rarely used when the number of factors or levels is relatively large (Number of levels > 3, number of factors > 5) (Román -Ramírez and Marco, 2022). The number of trials in the full factorial design (2^k) increases proportionally with the number of variables (also defined as factors, k) necessary to carry out an experimental design. For a number of variables k greater than or equal to 5, there is a considerable increase in the number of experiments, in addition to a longer time to perform all tests and a higher cost of reagents and samples. However, a portion of interactions between variables (i.e., higher order interactions) have a high probability of being considered non-significant for the outcome, which makes it possible to obtain the results needed for statistical analysis with only a fraction of the total assays (Silva *et al.*, 2023).

The planning method that uses only a fraction of the tests is called in the literature as fractional factorial (2^{k-b}), with k being the number of variables studied and b being the size of the applied fraction. This planning is used mainly when the variables studied are many and there is little information about the system. In this way, fractional factorial planning works as a screening of variables that are truly significant, which can later be used in optimization planning (response surface). The fractional factorial design strategy consists of considering the effects (contrasts) of interactions of higher orders (i.e., interactions between two or more variables) as little or no significant. In this way, the effects of these higher-order interactions (with low significance) mix with the effects of significant variables or interactions, generating a contrast value for these mixed variables or interactions. This mixture of the effects of non-significant interactions with the effects of significant variables or interactions allows only a fraction of the experiments to be carried out without significant losses to the result (Barros Neto *et al.*, 2010).

To exemplify, when evaluating the influence of five different factors (variables) at two levels, one high and the other low, on the response of a complete factorial design (2^5), it is necessary to carry out 32 experiments. This large number of experiments can make planning execution difficult. This way, it is possible to reduce this number of tests by half (obtaining similar results) by using a fractional design (2^{5-1}), totaling 16 experiments. In this planning (2^{5-1}), the strategy adopted is to mix the main effects with those of interactions of four factors, while the interactions of degree two are mixed with those of degree three. The result obtained is a planning with 16 experiments with the same capacity to carry out the variable screening study. (Barros Neto *et al.,* 2010).

Therefore, for an initial study with the objective of screening variables and identifying those that have the greatest influence on the response, fractional planning is a quite useful approach. Factor planning allows the construction of linear models in factors using the Least Squares method, which describe flat response surfaces. However, when the response surface has a curvature, it is necessary to construct quadratic models. To achieve this, new levels must be added to the factorial planning (Breitkreitz *et al.,* 2014).

Central Composite planning is an interesting option for generating quadratic models because it is a natural extension of factorial planning with a central point, which can be carried out in a first screening stage. To this initial planning, axial points are added (star planning) that provide additional levels for calculating the coefficients of the quadratic model. The distance from the axial points to the planning center is ± α, where α can vary from 1 to √k, where k is the number of factors. The value of α depends on the desirable properties for planning and the possibility of carrying out experiments in the experimental domain (Myers and Montgomery, 2002).

After carrying out the experiments designed by a DOE, it is extremely important to evaluate the constructed model and its adequacy to the experimentally obtained responses. This diagnosis can be carried out based on the Analysis of Variance (ANOVA), the evaluation of the residual graph (differences between the values obtained experimentally and those predicted by the model), and the graph of experimental values *vs.* values predicted by the model. The residual graph must present a random pattern and the graph of experimental values *vs*. predicted values must contain points distributed close to a straight line with a slope of 45° and an intercept close to zero. For linear models, a simple way to assess whether there is a lack of fit, that is, whether the curvature on the response surface is significant, is to add a point at the center of the factorial design (center point), measure the response at this point and compare with the value predicted by the linear model. If curvature exists, the predicted value will be statistically different from that measured experimentally (Breitkreitz *et al.,* 2014).

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CHAPTER 2 – 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 2^{5-1} 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: H_2SO_4 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 H_2SO_4 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, an 8-fold increase compared to the sample *in natura* (7 mg/g). In rice husks, in contrast to the results commonly found in literature, NaOH extracts mainly silica instead of lignin, while H_2SO_4 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 making ethanol production from this biomass economically viable.

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

2.0 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 valorization mitigates 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 in 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 can be used in different applications after extraction, 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 allow 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 making ethanol production more sustainable and economically feasible.

Unfortunately, in practice, there is no such perfect pretreatment attending to all the aspects mentioned, 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 many of the biomass components as possible, 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 2^{5-1} fractional factorial design (FFD) was applied to screen the sugar release as a function of the variables: H_2SO_4 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, $\alpha = \sqrt{2}$ and five replicates at the central 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 while hemicellulose, lignin and silica contents were extracted into 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.

2.1 MATERIALS AND METHODS

2.1.1 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.

2.1.2 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 H_2SO_4, 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 (Tables 2.1 and 2.2). 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. An autoclave (Phoenix AV-75, Araraquara-SP, Brazil) was used when the pretreatment temperature was above 100 °C, while the water bath (Fisaton model 550, São Paulo-SP, Brazil) was used when the temperature was below 100 °C. 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 2^{5-1} FFD was first applied as a screening to evaluate the sugar release as a function of the variables: H_2SO_4 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 2.1 and 2.2 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 ($\alpha = \sqrt{2}$ and five replicates at the central 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 (Tables 2.4 and 2.5). The combination of the levels of the CCD is graphically shown in Figure S2, using the coded variables.

2.1.3 Compositional and morphological analysis

2.1.3.1 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.

2.1.3.2 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 1 g of solid biomass samples in muffle oven (EDG F-1800 10P, São Carlos, Brazil) at 600 °C for 24 h.

2.1.3.3 Scanning electron microscopy

Rice husk surface morphologies before and after pretreatments were 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) and a current of 11.3 mA for 120 s.

2.1.4 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) with 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 H_2SO_4 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 *et al.*, 2017b).

$$HY(\%) = \frac{RG(mg/g)}{C(mg/g)\times 1.1} \times 100 \qquad \text{(Equation 1)}$$

2.1.5 Silica recovery

Silica was recovered from the liquor of the alkaline pretreatment of rice husks (condition RB2 in Table 2.5 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 H_2SO_4 (Minu *et al.,* 2012), and kept for 12 h at room temperature for silica precipitation. Subsequently, the liquor was vacuum filtered and the 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).

2.2 RESULTS AND DISCUSSION

2.2.1 Design of experiments

Two DOE approaches were carried out in this work to optimize the sugar release of rice husk samples. A 2^{5-1} 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.

2.2.1.1 2^{5-1} Fractional factorial design

Table 2.2 shows the sugar release for the samples prepared following FFD and Figure 2.1 shows the half normal plot of the factors, where significant effects are those that deviate from the straight line centred in zero. Concentration of H_2SO_4 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 2.3 The calculated F value of $MS_{Regression}/MS_{residual}$ is equal to 3.55, whereas the tabulated F value (8, 10, 95% confidence level) is 3.07. The calculated F value of $MS_{lack\ of\ fit}/MS_{pure\ error}$ 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 2.2 Because the $MS_{lack\ of\ fit}$ is high, it inflates the $MS_{residual}$ causing the calculated F value for $MS_{Regression}/MS_{residual}$ 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 2.2A and 2.2B.

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 2.2). On the other hand, residuals are not random, and the central points are clearly far from the rest of the points (Figures 2.2A and 2.2B). The response surface shown in Figure 2.3 also indicates that there is a curvature in the middle of the experimental domain, since the experimental central points are above the surface.

Table 2.1. Levels of the factors in the 2^{5-1} fractional factorial design.

	Factors		
	Low level (-1)	**High level (+1)**	**Central (0)**
A- [H_2SO_4] (% 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 2.2 Sample identification with the corresponding experimental conditions and the response of sugar release in the 2^{5-1} fractional factorial design with 3 replicates at the central point.

Sample RA	**[H_2SO_4] 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	25.0
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.

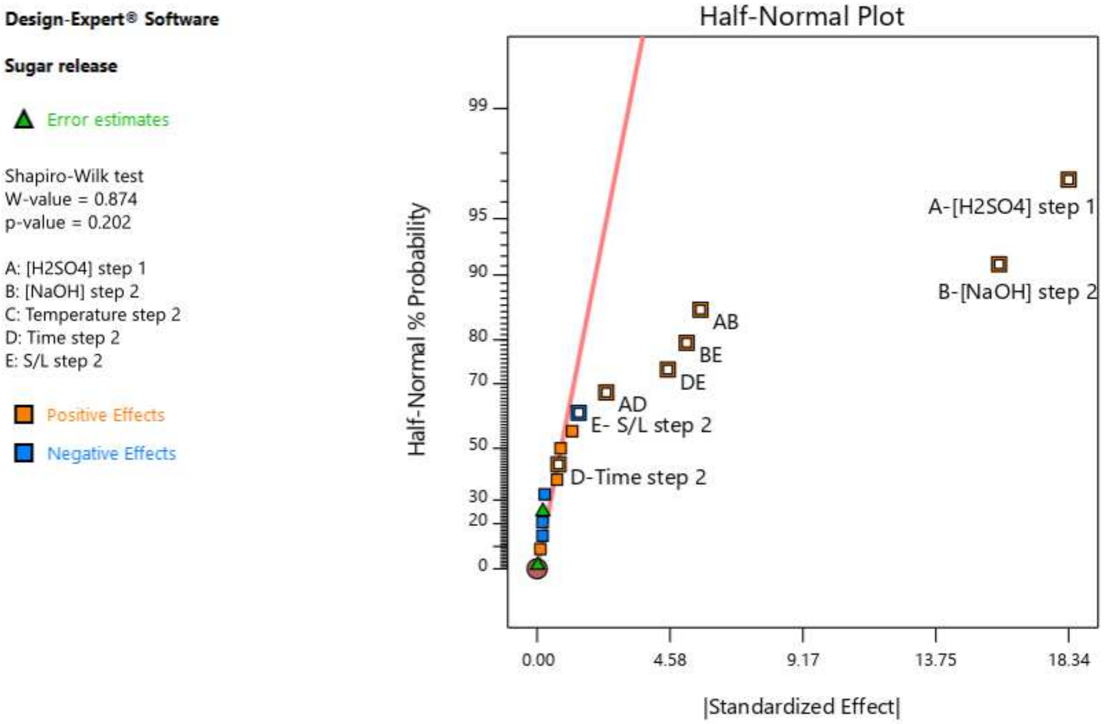

Figure 2.1 Half-normal plot of the standardized effects of the 2^{5-1} fractional factorial design

Table 2.3 ANOVA table of the model describing the sugar release as a linear function of the selected coefficients, based on 2^{5-1} 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-[H_2SO_4] 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			

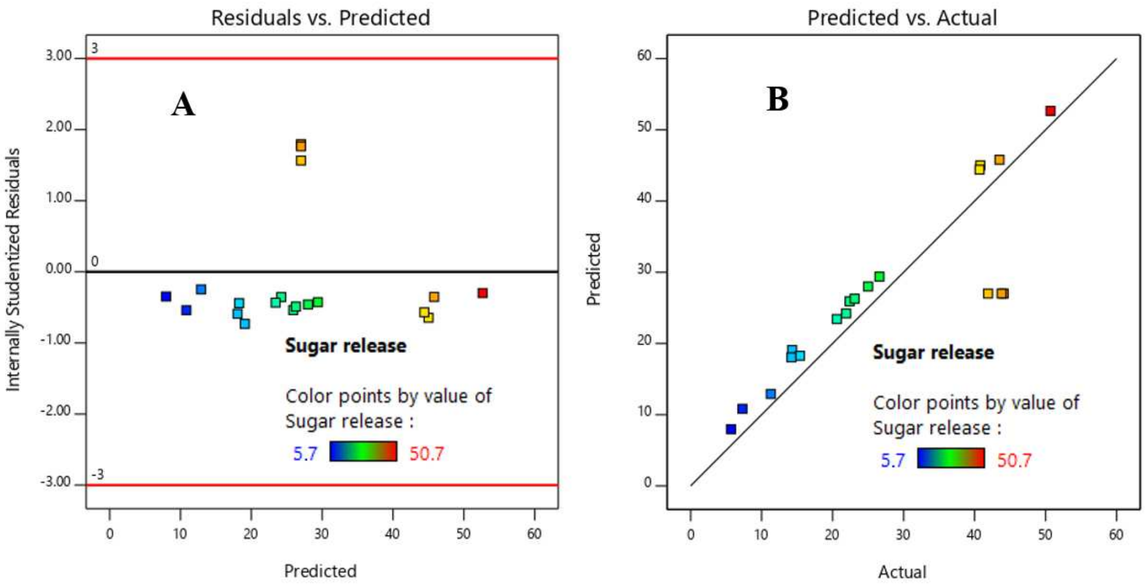

Figure 2.2 A) Graph of internally studentized residuals (residuals/standard deviation of regression) and B) graph of predicted *vs.* actual (experimental) responses of sugar release (mg/g), for the FFD.

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: [H_2SO_4] = 1.8 to 5.4% w/w and [NaOH] = 2 to 6% w/w, since the results of FFD indicated this could be a promising region. The response surface in Figure 2.3 shows that the higher responses (close to 50 mg/g) are obtained towards higher values of H_2SO_4 and NaOH concentrations.

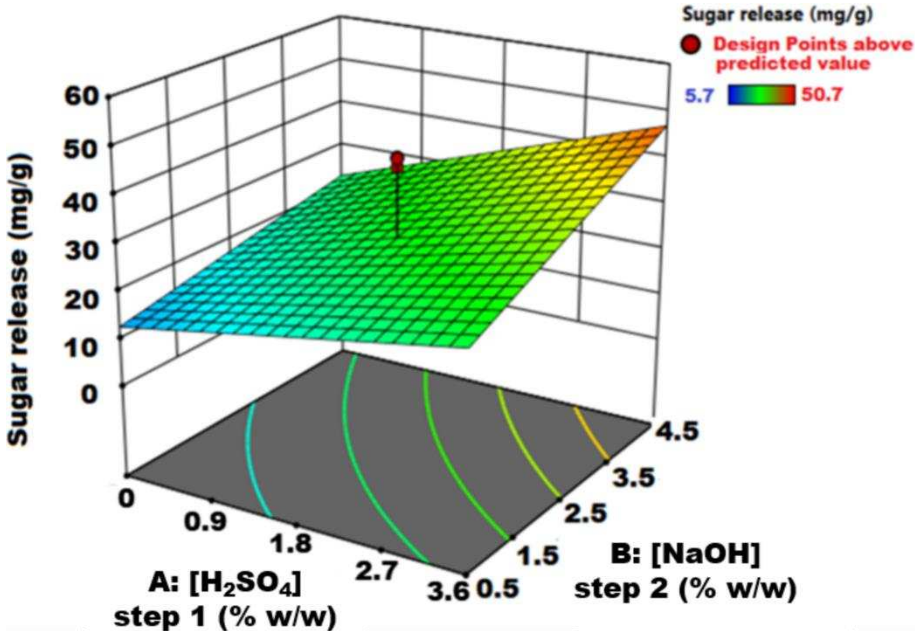

Figure 2.3 Response surface of the most important factors (H_2SO_4 and NaOH concentrations) based on the 2^{5-1} fractional factorial design. The other factors were kept at their central points.

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, [H_2SO_4] x Time (AD). If all the optimal conditions are kept as follows: ([NaOH] = 4.5% w/w, [H_2SO_4] = 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.

2.2.1.2 *Central composite design*

Table 5 shows the sugar released from the samples prepared according to the central composite design ($\alpha = \sqrt{2}$ with 5 replicates at the central 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 central point (authentic replicates) was 43.7 – 49.2.

The selected model is presented in Equation 2:

$$Sugar\ release\ (y) = 45.54 + 5.93\,[NaOH] + 2.65\,[H_2SO_4] - 1.95\,[NaOH]^2 + 2.35\,[H_2SO_4]^2 - 8.03\,[NaOH]^2*[H_2SO_4]. \qquad \text{(Equation 2)}$$

Table 2.4 Levels of the factors in the central composite design ($\alpha = \sqrt{2}$).

	Factors				
	Low level (-1)	**High level (+1)**	**-α**	**Central (0)**	**+α**
A- [H_2SO_4] (% w/w)	1.8	5.4	1.1	3.6	6.1
B- [NaOH] (% w/w)	2	6	1.2	4	6.8

Table 2.5 Sample identification with the corresponding experimental conditions and the responses of sugar release according to the central composite design ($\alpha = \sqrt{2}$) with 5 replicates at the central point.

Sample RB	**[H_2SO_4] 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 quintuplicated measurements in the central point.

The ANOVA data for this model is shown in Table 2.6, where NaOH shows the most significant influence on the response ($p = 0.0004$), followed by the term $[NaOH]^2*[H_2SO_4]$ ($p = 0.0035$). The remaining coefficients were kept for hierarchical purposes. The model indicates a complex response surface (Figure 2.4). Nevertheless, fit was observed with $MS_{\text{lack of fit}}/MS_{\text{pure error}}$ = 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 $MS_{\text{Regression}}/MS_{\text{residual}}$ 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 2.5A) and the predicted values are in good agreement with the actual values (Figure 2.5B).

Table 2.6 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-H_2SO_4	28.13	1	28.13	4.05	0.0839
A^2	26.49	1	26.49	3.82	0.0916
B^2	38.38	1	38.38	5.53	0.0509
A^2B	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			

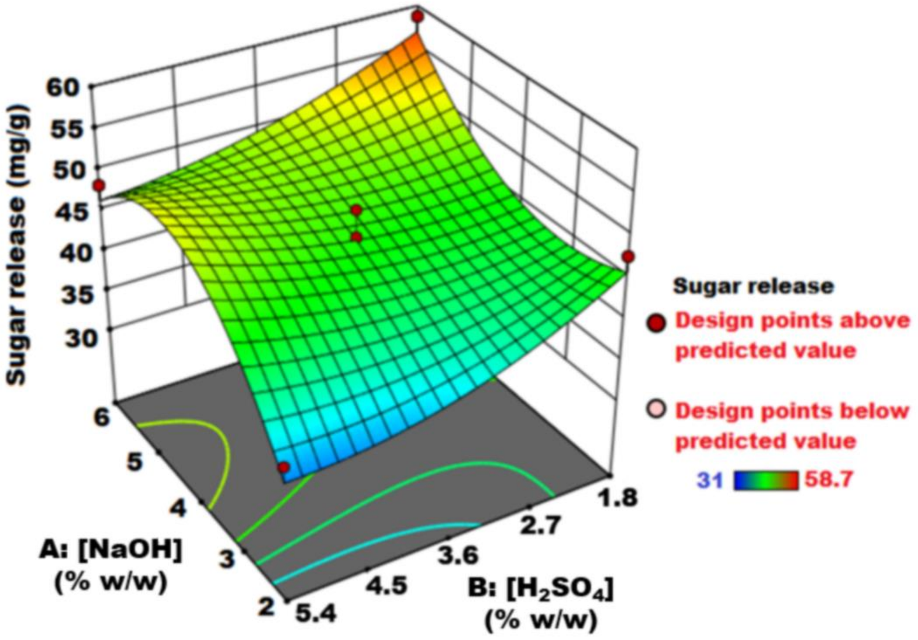

Figure 2.4 Response surface of the model shown in Equation 2.

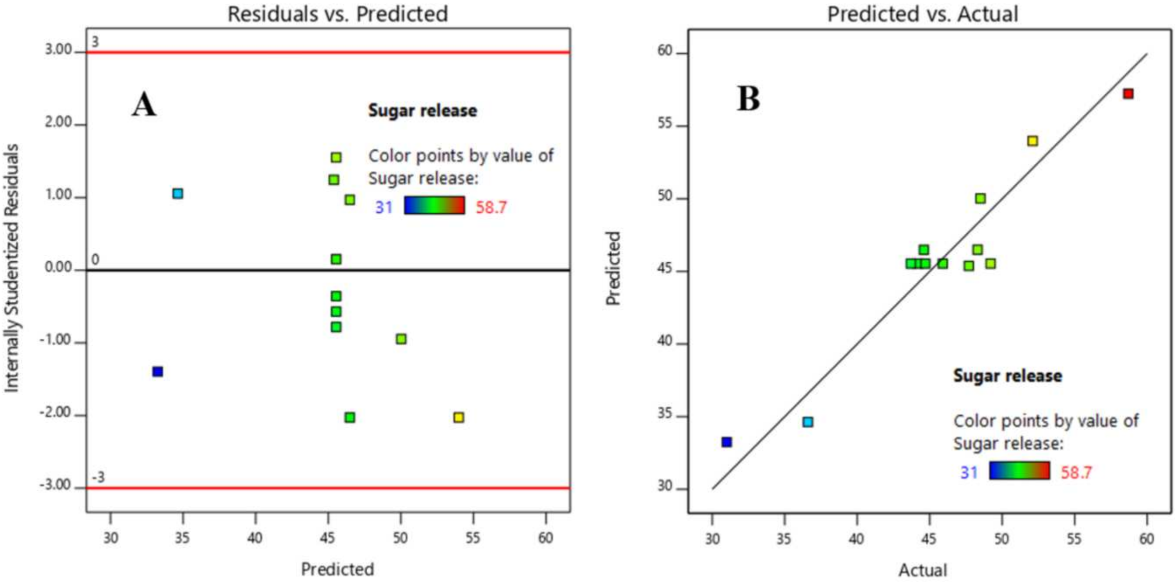

Figure 2.5 A) Graph of internally studentized residuals (residuals/standard deviation of regression) and B) graph of predicted *vs.* actual (experimental) responses of sugar release (mg/g) for CCD.

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 72 h 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 2.4 All the other experimental conditions were fixed (85 °C; 100 min and S/L = 12.5%) and the results after 12 h of hydrolysis were:

- [H_2SO_4] = 1.8% w/w; [NaOH] = 8% w/w: sugar release = 47.9 mg/g
- [H_2SO_4] = 0.9% w/w; [NaOH] = 6% w/w: sugar release = 47. 9 mg/g
- [H_2SO_4] = 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: [H_2SO_4] = 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* (7 mg/g).

2.2.2 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 2.6 shows the reducing sugars obtained after enzymatic saccharification (12 h 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% w/w H_2SO_4 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 H_2SO_4; 4.5% w/w NaOH; 125 °C; 100 min; 12.5% w/w S/L in Table 2.2).

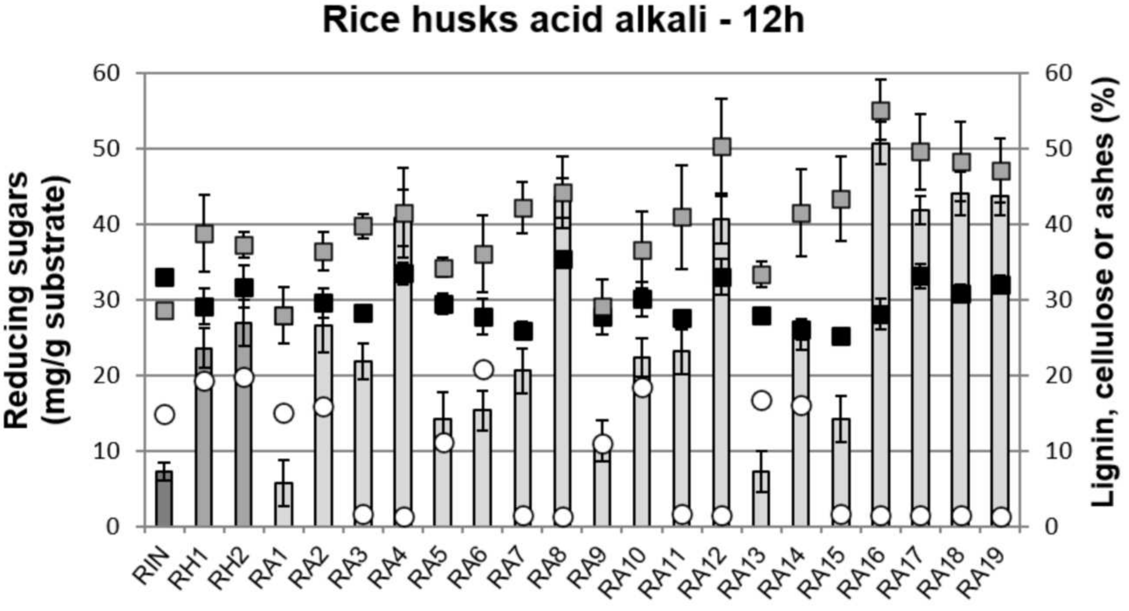

Figure 2.6 Reducing sugars (mg/g substrate) released from rice husks after 12 h enzymatic hydrolysis (bars in the left axis) and their percentage of lignin (black squares in the right axis), crystalline cellulose (grey squares) and ash (white circles) before and after pretreatments. Error bars are standard deviation values from replicates. RIN = rice husks *in natura*; RH1 = sample pretreated with 1.8% w/w H_2SO_4; RH2 = sample pretreated with 3.6% w/w H_2SO_4; RA 1 to RA 19 = samples of FFD with experimental conditions detailed in Table 2.2.

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 2.6). 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 2.7. Figure 2.7A 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 2.7B, which shows the sample morphology after the acid treatment (sample RH1 in Table 2.2). 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. In contrast, after the alkaline treatment with 6% w/w NaOH (sample RB2 in Figure 2.7C and 2.7D), the 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 2.7C and 2.7D are perfectly in accordance with the removal of these silica grains and with the decrease in the ash content in these samples (from ca. 15 to 1.4% w/w). Inversely, ash contents in the post-acid samples remain high (ca. 15-20% w/w) in line with the surface unmodified morphology.

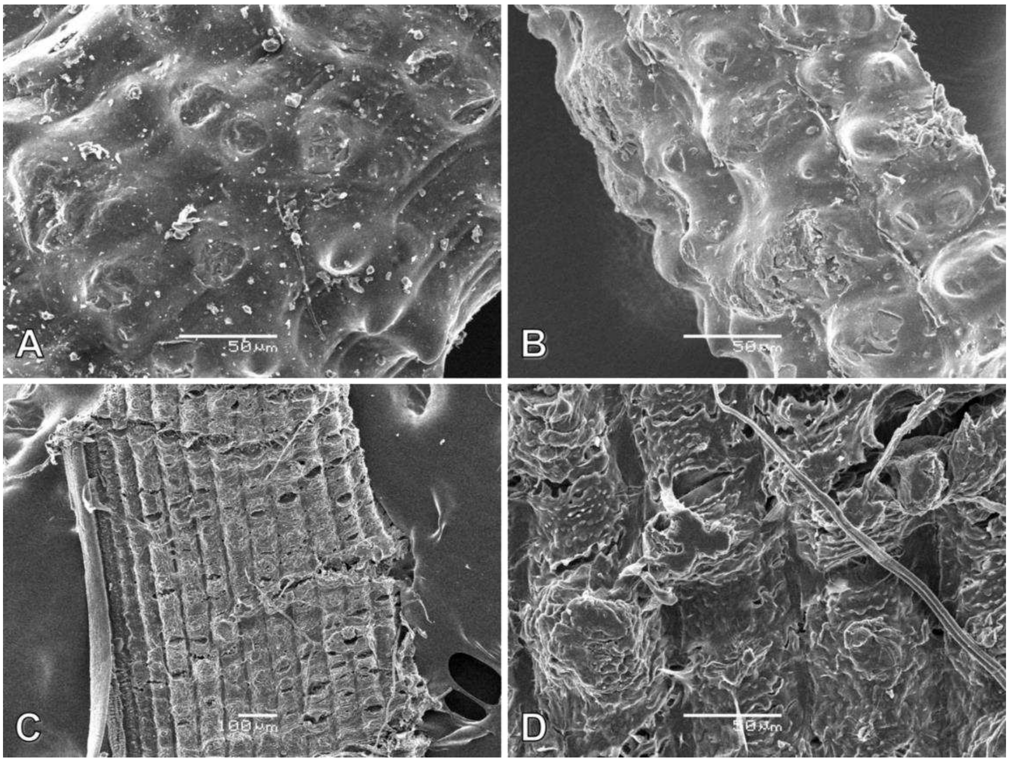

Figure 2.7 Scanning electron microscopy images of rice husk surfaces: A) *in natura*; B) after the acid treatment with 1.8% w/w H_2SO_4 (RH1) and C and D) after the acid and the alkali treatment with 6% w/w NaOH (RB2). Scale bars: 100 µm in C and 50 µm in A, B and D.

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 content 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 content. 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 (H_2SO_4 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 H_2SO_4 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 (H_2SO_4 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 H_2SO_4 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 2.8). 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%).

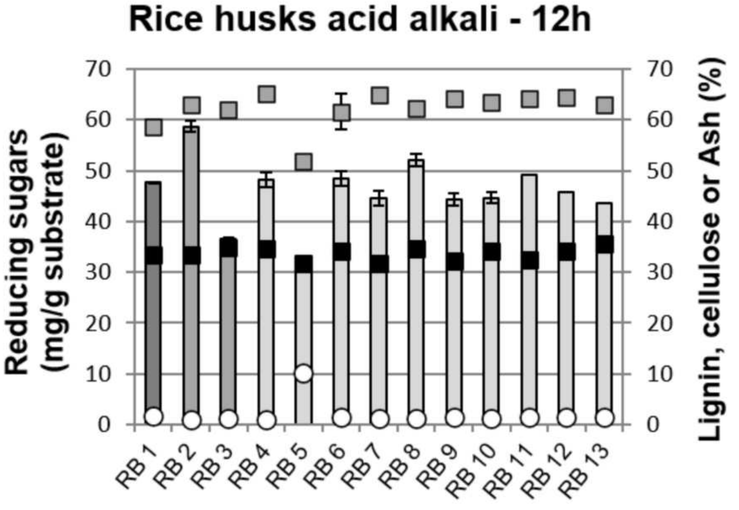

Figure 2.8 Reducing sugars (mg/g substrate) released from pretreated rice husks after 12 h enzymatic hydrolysis (bars in the left axis) and their percentage of lignin (black squares in the right axis), cellulose (grey squares) and silica (white circles). Error bars are standard deviation values from replicates. RB1 to RB13 = samples of CCD with experimental conditions detailed in Table 2.5.

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 (H_2SO_4 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.

2.2.3 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 2.9).

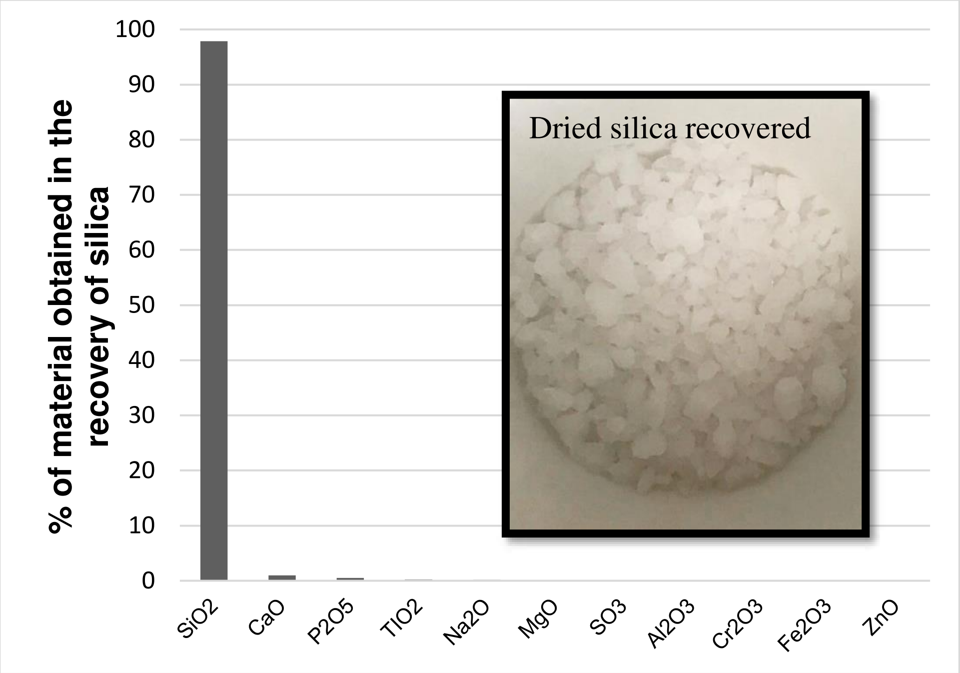

Figure 2.9 Chemical composition of the material obtained in silica recovery from the liquor of alkali pretreatment (Sample RB2) and a photograph showing the recovered material after drying.

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 requires previous lignin isolation, centrifugation steps and high temperature (Barana *et al.,* 2016).

2.3 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 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 an acid-alkaline pretreatment in rice husks and the following optimal conditions were obtained: 1.8% w/w H_2SO_4 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.

ACKNOWLEDGEMENTS

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APPENDIX A - Supplementary Material for the paper

IMPROVED HYDROLYSIS YIELDS AND SILICA RECOVERY BY DESIGN OF EXPERIMENTS APPLIED TO ACID-ALKALI PRETREATMENT IN RICE HUSKS

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Temperature profile in pretreatments

The times to reach the pretreatment temperature (15 min) and to cool down (80 min) to room temperature were controlled to be the same in all the temperatures tested (85, 105, 120 and 125 °C) to ensure a comparable contact time between the biomass and the pretreatment liquids. Figure S1 exemplifies the temperature protocol used in the samples pretreated at 125 °C (in autoclave, blue curve) and at 85 °C (in water bath, orange curve).

For the pretreatment at 125 °C, the autoclave was initially heated to 100 °C (boiling water), then the samples were allocated, the equipment was locked, and it took 15 min to reach the pretreatment temperature. The pretreatment time (20, 60 or 100 min) started to be recorded at this point and when the time was over, the autoclave was turned off. The equipment took 40 min to depressurize and to cool down to *ca.* 100 °C, so that it could be opened, and the samples removed. Samples were then placed on the bench and after 40 min a safe temperature for filtration was reached (around 40 °C).

For the pretreatment at 85 °C, samples were placed in the water bath at 40 °C and the temperature was gradually increased to 85 °C in 15 min. Then, the pretreatment time at constant temperature was counted and subsequently the bath was turned off. Samples were removed from the water bath and let to cool down on the bench for 80 min, keeping similar heating and cooling times as the pretreatments in autoclave.

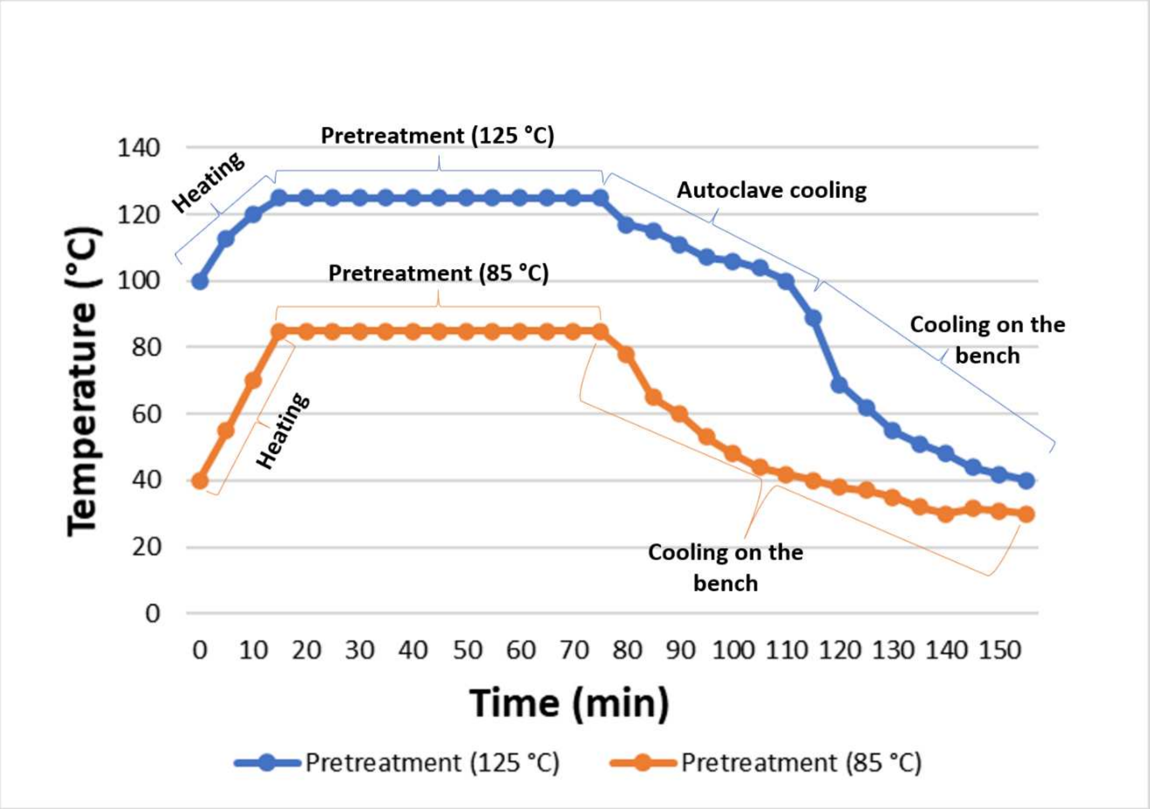


Figure S1. Temperature profile for heating, pretreating and cooling down the samples pretreated at 125 °C (in autoclave, blue curve) and at 85 °C (in water bath, orange curve), as measured directly in the samples pretreated in each temperature.

Design of experiments

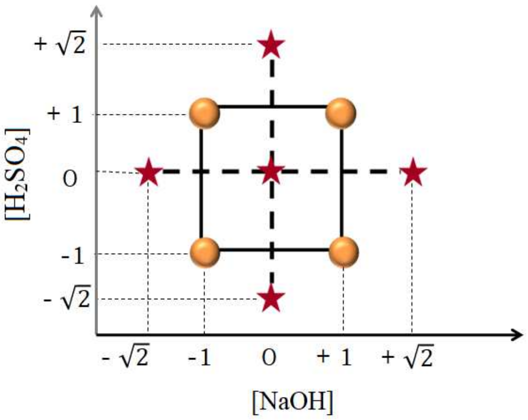


Figure S2. Combination of the levels of the central composite design (CCD) using the coded variables.

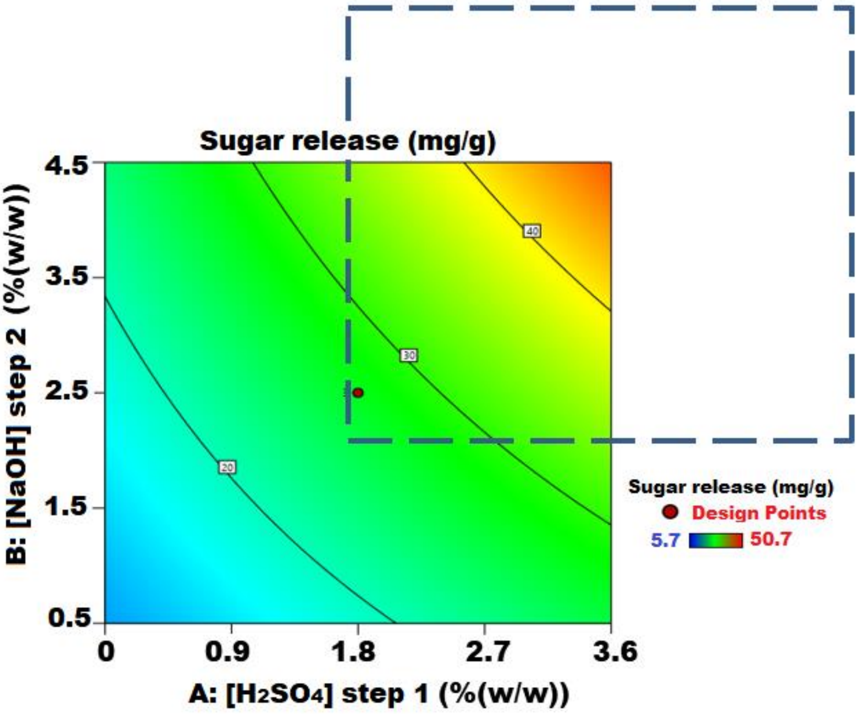


Figure S3. Shifted region in relation to the original experimental domain used to plan the central composite design.

Tables of compositional analysis and pretreatment yields

Table S1. Pretreatment yield* (%) and the quantities of cellulose, lignin, hemicellulose and ash for samples *in natura* (RIN), after the acid treatment (RH1 and RH2) and RA1 to RA19 (FFD).

Sample	Pretreatment yield (%)	Cellulose (%)	Lignin (%)	Hemicellulose (%)	Ash (%)
RIN	-	28.6 ± 0.1	33.1 ± 0.3	16.6 ± 0.0	14.8 ± 0.2
RH1	72.5	38.8 ± 5.0	29.2 ± 2.4	4.4 ± 0.4	19.3 ± 0.2
RH2	71.3	37.3 ± 1.7	31.8 ± 2.8	3.7 ± 0.6	19.8 ± 0.1
RA1	80.8	27.9 ± 3.7	28.0 ± 0.6	7.8 ± 0.6	15.1 ± 0.2
RA2	80.8	36.4 ± 2.6	29.7 ± 1.9	3.2 ± 0.8	15.9 ± 0.0
RA3	65.8	39.8 ± 1.6	28.2 ± 0.6	8.4 ± 0.6	1.6 ± 0.0
RA4	68.7	41.6 ± 5.9	33.5 ± 1.5	4.0 ± 0.3	1.3 ± 0.1
RA5	73.2	34.3 ± 1.3	29.5 ± 1.3	8.3 ± 0.6	11.2 ± 0.5
RA6	70.9	36.1 ± 5.1	27.8 ± 2.4	3.0 ± 0.5	20.8 ± 0.0
RA7	47.1	42.3 ± 3.4	25.9 ± 1.2	8.9 ± 0.7	1.5 ± 0.3
RA8	48.8	44.3 ± 4.8	35.4 ± 0.4	2.6 ± 0.2	1.4 ± 0.2
RA9	77.3	29.1 ± 3.7	27.7 ± 0.8	8.4 ± 0.6	11.0 ± 0.2
RA10	78.1	36.6 ± 5.1	30.1 ± 2.3	2.9 ± 0.3	18.5 ± 0.3
RA11	57.0	41.0 ± 6.8	27.6 ± 0.3	8.5 ± 0.6	1.7 ± 0.0
RA12	61.6	50.4 ± 6.3	33.1 ± 2.4	2.7 ± 0.2	1.5 ± 0.1
RA13	76.9	33.4 ± 1.8	27.9 ± 1.0	7.7 ± 0.6	16.8 ± 0.2
RA14	70.6	41.6 ± 5.8	26.1 ± 1.4	1.9 ± 0.2	16.1 ± 0.3
RA15	44.6	43.5 ± 5.6	25.2 ± 0.1	6.8 ± 0.5	1.6 ± 0.3
RA16	47.3	55.2 ± 0.2	28.2 ± 2.0	2.6 ± 0.3	1.6 ± 0.4
RA17	61.6	49.6 ± 5.0	33.2 ± 1.6	2.7 ± 0.2	1.5 ± 0.1
RA18	62.8	48.3 ±5.2	30.9 ± 1.1	2.9 ± 0.4	1.5 ± 0.3
RA19	62.5	47.1 ± 4.3	32.1 ± 1.2	3.0 ± 0.1	1.4 ± 0.2

* Pretreatment yield is the total of dried pretreated solid remaining after pretreatment in comparison to the initial solid.

Table S2. Pretreatment yield* (%) and the quantities of cellulose, lignin, hemicellulose and ash for samples RB1 to RB13 (CCD).

Sample	**Pretreatment yield (%)**	**Cellulose (%)**	**Lignin (%)**	**Hemicellulose (%)**	**Ash (%)**
RB1	66.5	58.6 ± 0.3	33.5 ± 0.1	4.7 ± 0.3	1.7 ± 0.1
RB2	67.4	62.9 ± 0.2	33.5 ± 0.2	3.6 ± 0.5	1.1 ± 0.1
RB3	61.3	61.9 ± 0.1	34.8 ± 0.0	2.6 ± 0.1	1.3 ± 0.0
RB4	65.5	65.2 ± 0.5	34.5 ± 0.1	1.0 ± 0.7	1.1 ± 0.3
RB5	60.2	51.7 ± 1.2	31.7 ± 0.0	3.6 ± 0.9	10.1 ± 0.6
RB6	78.4	61.6 ± 3.5	34.1 ±0.1	2.5 ± 0.3	1.4 ± 0.5
RB7	62.6	64.8 ± 0.4	31.7 ± 0.1	4.2 ± 0.1	1.2 ± 0.1
RB8	60.9	62.3 ± 0.6	34.6 ± 0.3	2.1 ± 0.3	1.2 ± 0.0
RB9	60.1	64.3 ± 0.1	32.3 ± 0.5	2.8 ± 0.3	1.4 ± 0.1
RB10	65.1	63.5 ± 1.0	34.2 ± 0.1	2.6 ± 0.3	1.3 ± 0.1
RB11	64.7	64.2 ± 0.1	32.5 ± 0.2	2.9 ± 0.3	1.4 ± 0.1
RB12	63.9	64.4 ± 0.2	34.1 ± 0.1	2.9 ± 0.6	1.4 ± 0.1
RB13	65.5	63.0 ± 0.2	35.6 ± 0.2	2.8 ± 0.2	1.5 ± 0.1

* Pretreatment yield is the total of dried pretreated solid remaining after pretreatment in comparison to the initial solid.

Quantification of silica and total ash in rice samples

Silica was determined by x-ray fluorescence spectroscopy (XRF), in duplicate, following a method previously described in detail (Rezende *et al.*, 2018).

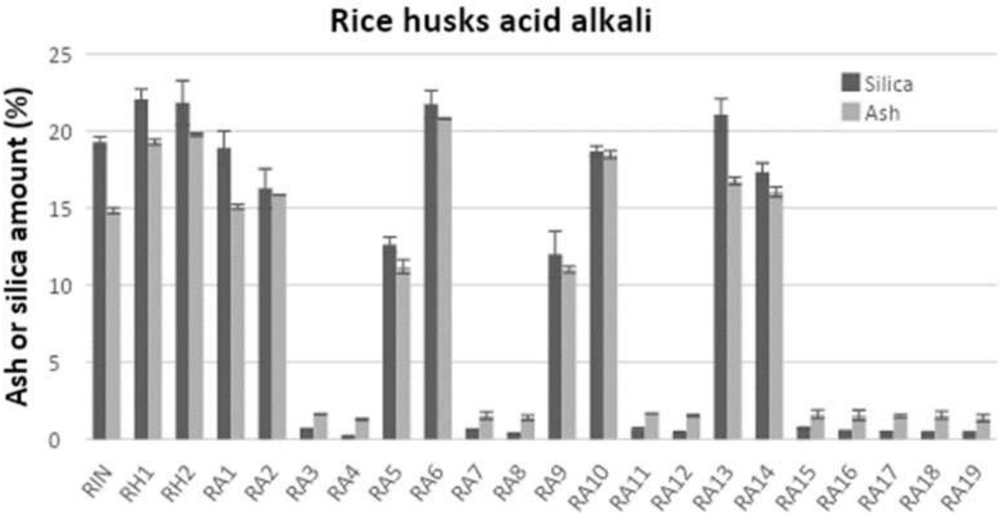


Figure S4. Comparison of silica and total ash amounts before (RIN) and after pretreatments in RH and RA samples.

Correlation between saccharification results (reducing sugar release – mg/g) and cellulose, lignin and ash contents (w/w%) in FFD samples (RA)

Correlations between reducing sugar release and the contents of ash, cellulose, and lignin are shown in Figures S5 to S7, respectively, for rice husks pretreated under acid-alkali conditions of the 2^{5-1} FFD (RA samples). In these samples, the correlation between sugars and ash is negative and moderate (R = -0.59 in Figure S5). The correlation between sugar release and cellulose is positive and relatively high (R = 0.87), indicating that higher sugar release is achieved in samples with increased cellulose contents (Figure S6). Finally, the correlation between the sugar released and the lignin content is positive and moderate (R = 0.66). This is an unexpected result since high lignin amounts are often associated with low yields of sugar released in enzymatic hydrolysis.

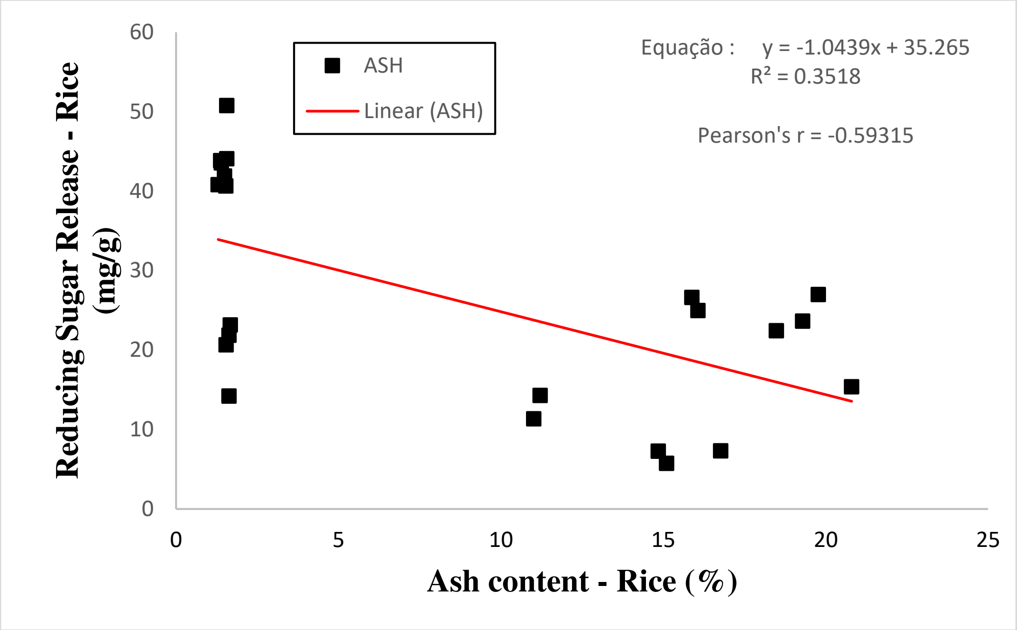


Figure S5. Sugar release as a function of ash content in samples RA1-19 (R = -0.59).

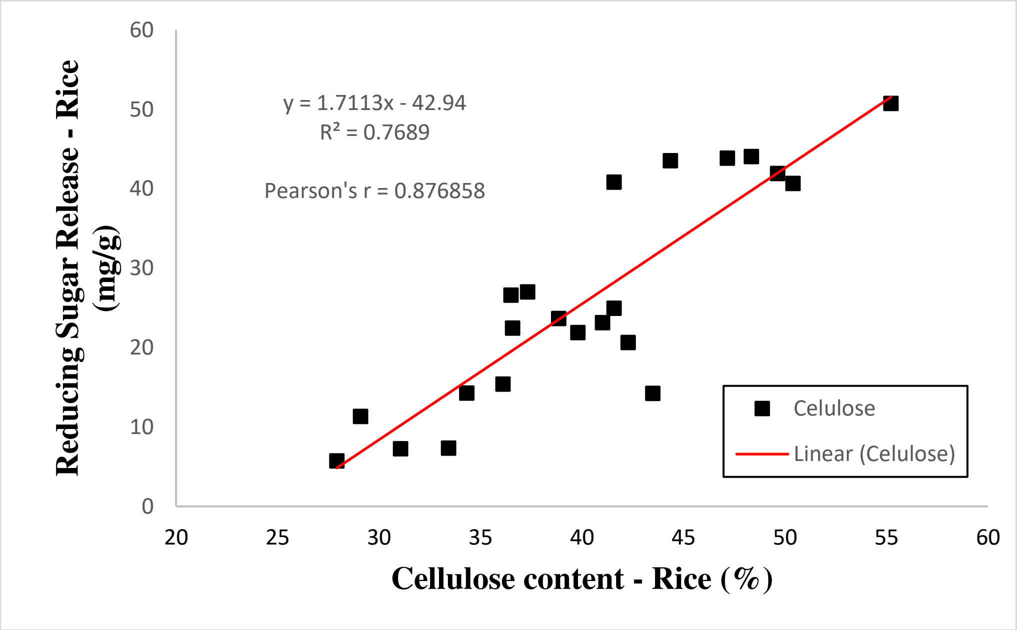


Figure S6. Sugar release as a function of cellulose content in samples RA1-19 (R = 0.87).

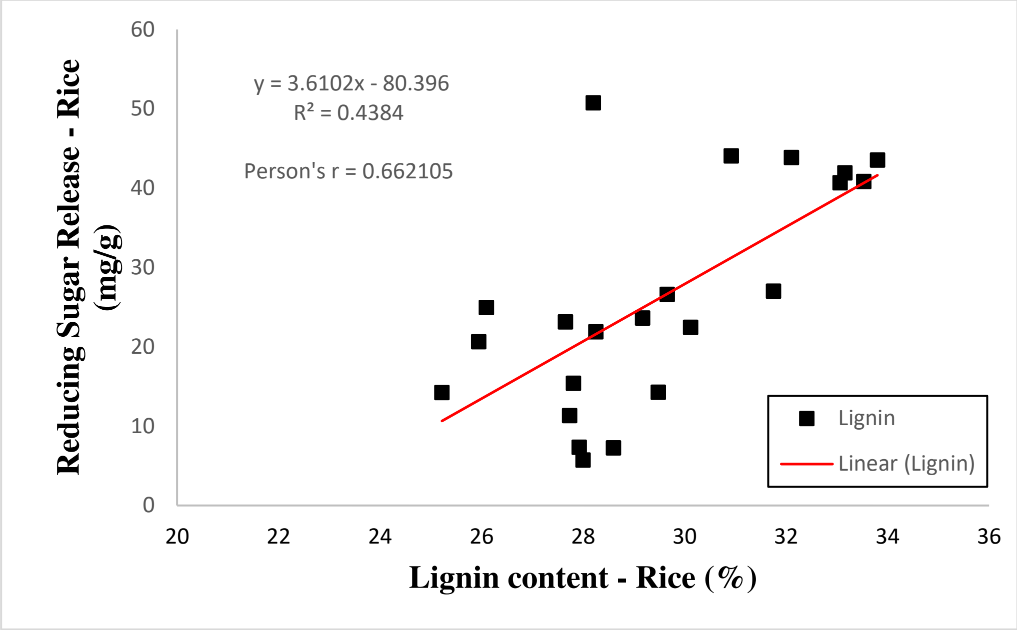
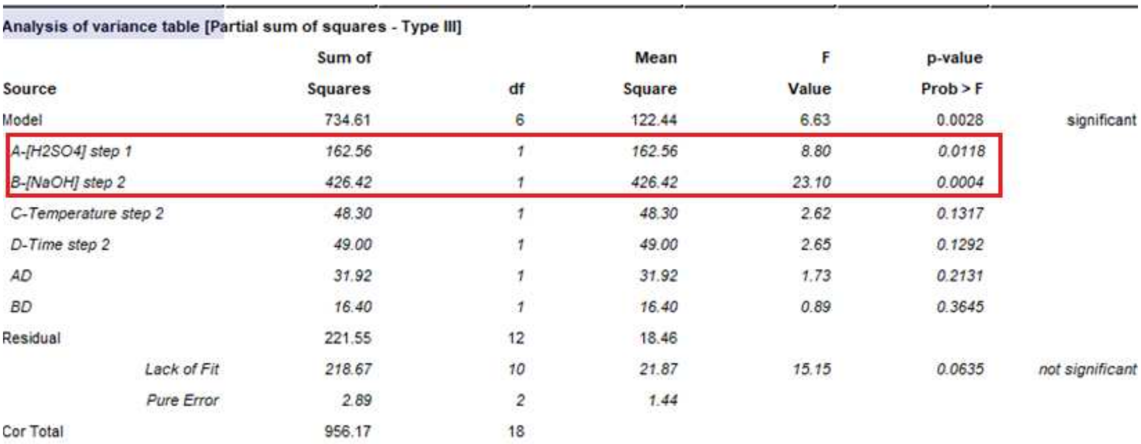


Figure S7. Sugar release as a function of lignin content in samples RA1-19 (R = 0.66).

Lignin, silica and cellulose amounts as responses in RA samples

For comparison, lignin, silica and cellulose quantities in RA samples were also used as responses in the FFD. Table S3 shows ANOVA of the model describing the cellulose amount as a linear function of the selected coefficients for RA samples. The significant factors for this response (p-value< 0.05) are the same also influencing the sugar release, named H_2SO_4 and NaOH concentration. In this case, the predictive capacity of the model is also bad.

Table S3. ANOVA table of the model describing the cellulose amount as a linear function of the selected coefficients for RA samples, as obtained from Design Expert software.

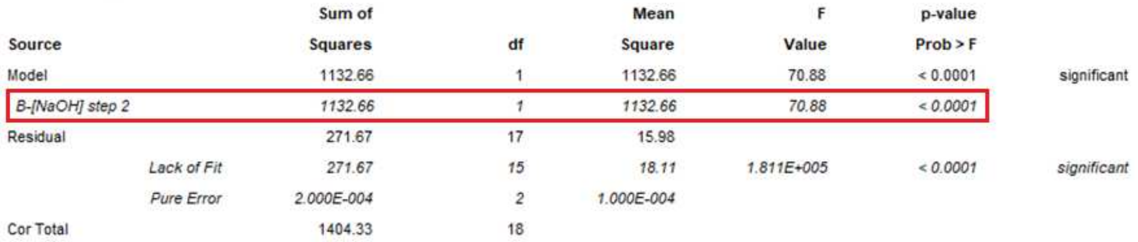


Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	734.61	6	122.44	6.63	0.0028	significant
A-[H2SO4] step 1	*162.56*	*1*	*162.56*	*8.80*	*0.0118*	
B-[NaOH] step 2	*426.42*	*1*	*426.42*	*23.10*	*0.0004*	
C-Temperature step 2	*48.30*	*1*	*48.30*	*2.62*	*0.1317*	
D-Time step 2	*49.00*	*1*	*49.00*	*2.65*	*0.1292*	
AD	*31.92*	*1*	*31.92*	*1.73*	*0.2131*	
BD	*16.40*	*1*	*16.40*	*0.89*	*0.3645*	
Residual	221.55	12	18.46			
Lack of Fit	*218.67*	*10*	*21.87*	*15.15*	*0.0635*	*not significant*
Pure Error	*2.89*	*2*	*1.44*			
Cor Total	956.17	18				

Table S4 shows ANOVA of the model describing the silica amount as a linear function of the selected coefficients for RA samples. The only significant factor for this response is the NaOH concentration and the model has a poor predictive capacity.

Table S4. ANOVA table of the model describing the silica amount as a linear function of the selected coefficients for RA samples, as obtained from Design Expert software.



Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	1132.66	1	1132.66	70.88	< 0.0001	significant
B-[NaOH] step 2	*1132.66*	*1*	*1132.66*	*70.88*	*< 0.0001*	
Residual	271.67	17	15.98			
Lack of Fit	*271.67*	*15*	*18.11*	*1.811E+005*	*< 0.0001*	*significant*
Pure Error	*2.000E-004*	*2*	*1.000E-004*			
Cor Total	1404.33	18				

In Table S5, ANOVA of the model describing the lignin amount as a linear function of the selected coefficients for RA samples is shown. Significant factors for this response are H_2SO_4 concentration and its interaction with NaOH concentration, as highlighted.

Table S5. ANOVA table of the model describing the lignin amount as a linear function of the selected coefficients for RA samples, as obtained from Design Expert software.

Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	62.78	3	20.93	4.64	0.0173	significant
A-[H2SO4] step 1	*30.53*	*1*	*30.53*	*6.77*	*0.0200*	
B-[NaOH] step 2	*4.95*	*1*	*4.95*	*1.10*	*0.3113*	
AB	*27.30*	*1*	*27.30*	*6.05*	*0.0265*	
Residual	67.63	15	4.51			
Lack of Fit	*64.99*	*13*	*5.00*	*3.78*	*0.2285*	*not significant*
Pure Error	*2.65*	*2*	*1.32*			
Cor Total	130.41	18				

Effect of pretreatments on the removal of hemicellulose from rice husks

Detailed quantification of all monosaccharides of hemicellulose present in FFD samples (RA1 to RA19) was carried out following the method described in [Rezende, 2018] (Figure S7). The total maximum amounts of hemicellulose sugars quantified in these samples were *ca.* 90 mg/g in sample *in natura* (RIN) and in samples odd-numbered from RA1 to RA15, which are the ones without a first acid step. A sharp decrease in the total hemicellulose fraction is noticed in samples that underwent the acid step (RH1-2, sample even numbered from RA2 to 16 and the centre points RA17 to 19). Acid-treated samples show a hemicellulose amount of *ca.* 1/3 as compared to the sample *in natura*, while samples treated with the alkali step only (RA1, RA3, RA5, RA7, RA9, RA11, RA13, RA15) have total hemicellulose content similar to RIN. This is consistent with the acid pretreatment efficiency to remove hemicellulose fractions in different biomasses (Rezende *et al.,* 2018; Ang *et al*., 2013).

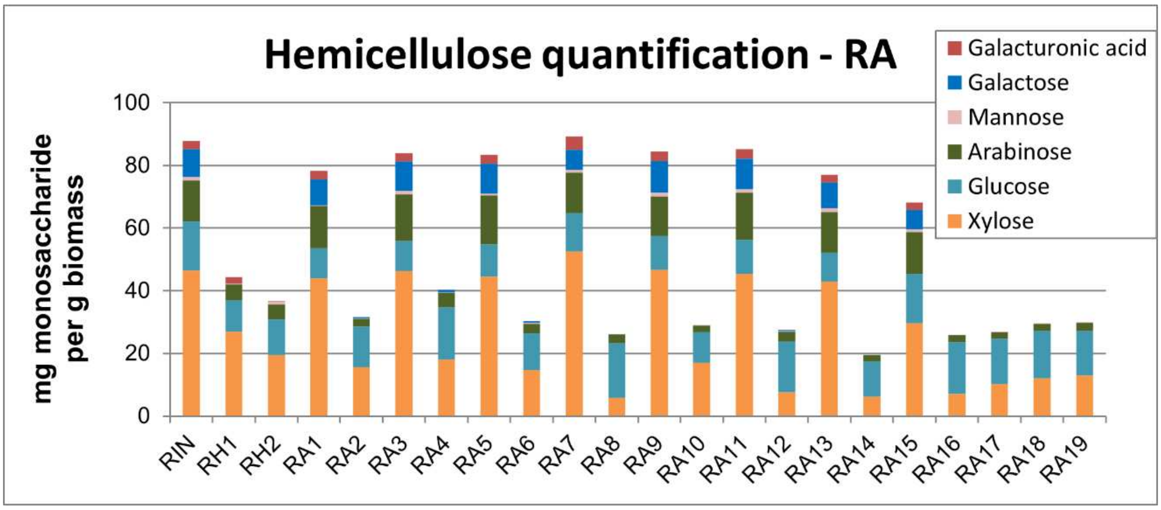


Figure S8. Quantification of hemicellulose monosaccharides (in mg/g substrate) in rice husks before and after pretreatments (FFD samples).

Correlation between saccharification results (reducing sugar release – mg/g) and cellulose, lignin and ash contents (w/w%) in CCD samples (RB)

Correlations between reducing sugar release and the contents of ash, cellulose, and lignin are shown in Figures S9 to S11, respectively, for rice husks samples of the CCD pretreated under acid-alkali conditions (RB samples). In these samples, the correlation between sugars and ash is negative and moderate (R = -0.67 in Figure S9). The correlation between sugar release and cellulose is positive but also moderate (R = 0.56, Figure S10), while the correlation between the sugar released and the lignin content is positive and low (R = 0.21, Figure S11).

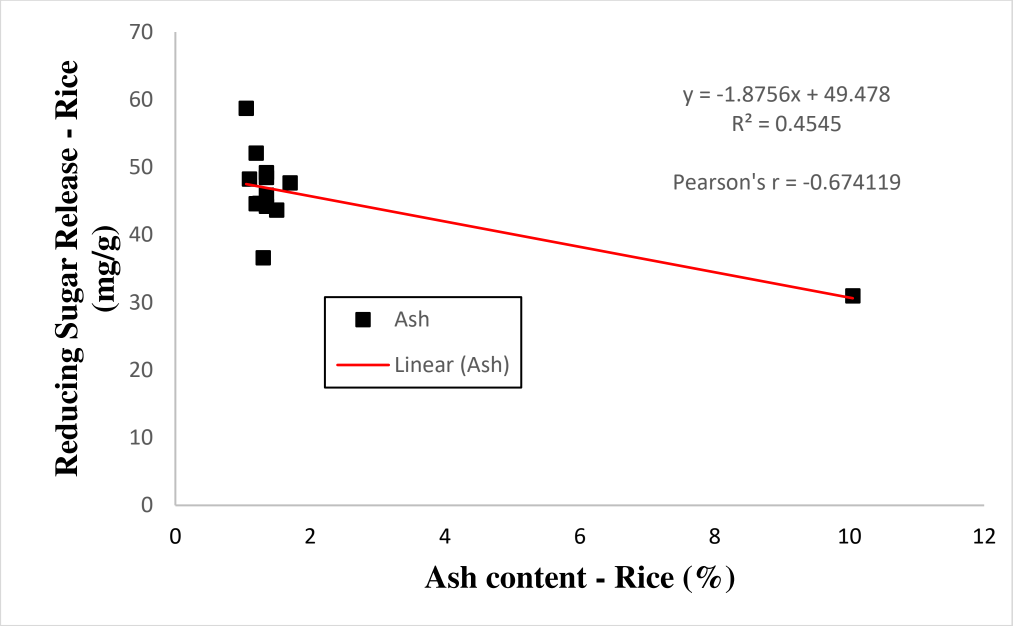


Figure S9. Sugar release as a function of ash content in samples RB1-13 (R = -0.67).

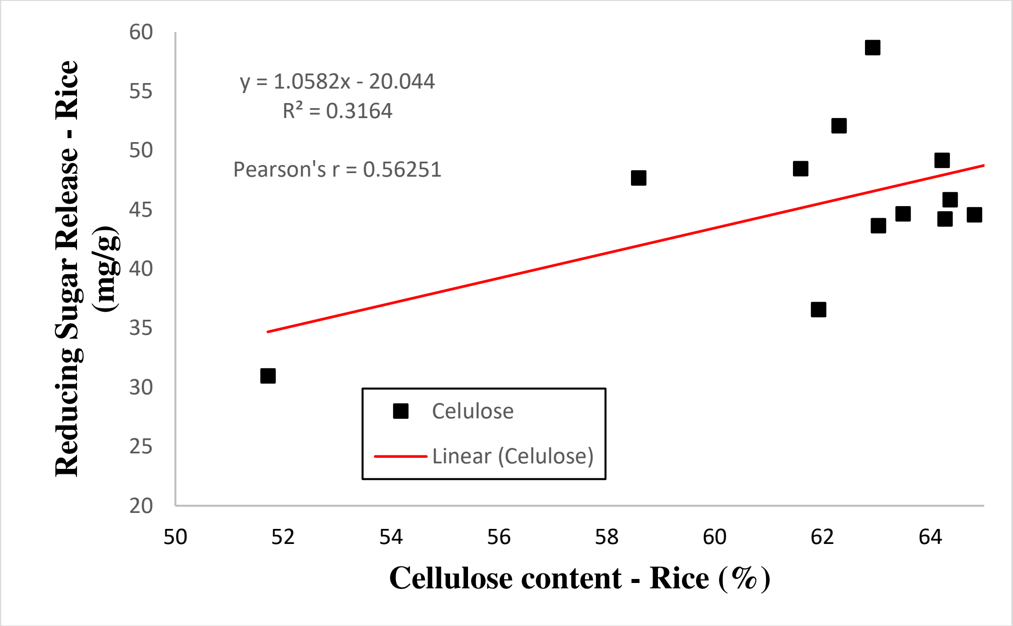


Figure S10. Sugar release as a function of cellulose content in samples RB1-13 (R = 0.56).

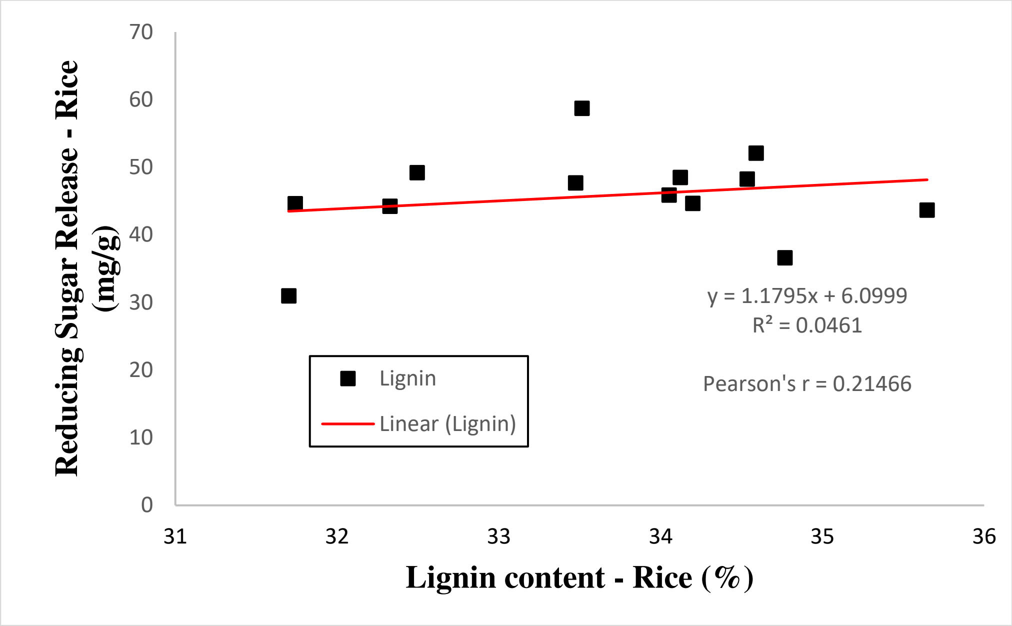
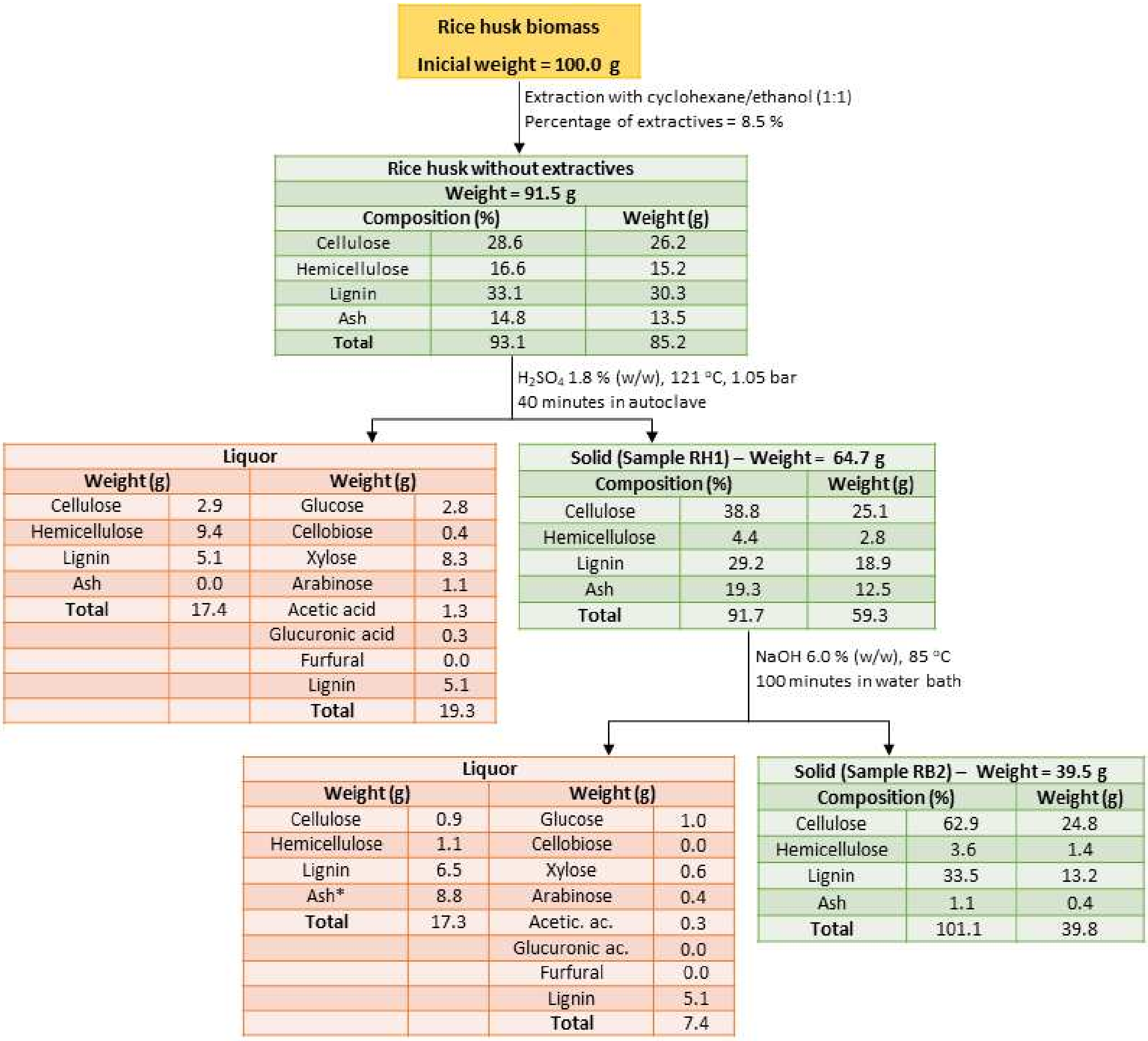


Figure S11. Sugar release as a function of lignin content in samples RB1-13 (R = 0.21).

Detailed composition analysis of the solid and liquid fractions obtained after the acid and alkaline treatments that resulted in the preparation of RB2.



Rice husk biomass
Inicial weight = 100.0 g

Extraction with cyclohexane/ethanol (1:1)
Percentage of extractives = 8.5 %

Rice husk without extractives Weight = 91.5 g	Composition (%)	Weight (g)
Cellulose	28.6	26.2
Hemicellulose	16.6	15.2
Lignin	33.1	30.3
Ash	14.8	13.5
Total	93.1	85.2

H$_2$SO$_4$ 1.8 % (w/w), 121 °C, 1.05 bar
40 minutes in autoclave

Liquor			
Weight (g)		**Weight (g)**	
Cellulose	2.9	Glucose	2.8
Hemicellulose	9.4	Cellobiose	0.4
Lignin	5.1	Xylose	8.3
Ash	0.0	Arabinose	1.1
Total	17.4	Acetic acid	1.3
		Glucuronic acid	0.3
		Furfural	0.0
		Lignin	5.1
		Total	19.3

Solid (Sample RH1) – Weight = 64.7 g	Composition (%)	Weight (g)
Cellulose	38.8	25.1
Hemicellulose	4.4	2.8
Lignin	29.2	18.9
Ash	19.3	12.5
Total	91.7	59.3

NaOH 6.0 % (w/w), 85 °C
100 minutes in water bath

Liquor			
Weight (g)		**Weight (g)**	
Cellulose	0.9	Glucose	1.0
Hemicellulose	1.1	Cellobiose	0.0
Lignin	6.5	Xylose	0.6
Ash*	8.8	Arabinose	0.4
Total	17.3	Acetic. ac.	0.3
		Glucuronic ac.	0.0
		Furfural	0.0
		Lignin	5.1
		Total	7.4

Solid (Sample RB2) – Weight = 39.5 g	Composition (%)	Weight (g)
Cellulose	62.9	24.8
Hemicellulose	3.6	1.4
Lignin	33.5	13.2
Ash	1.1	0.4
Total	101.1	39.8

Figure S12: Detailed composition analysis of the solid and liquid fractions obtained after the acid and alkaline treatments that resulted in the preparation of RB2 sample from the *in natura* biomass. In liquors, individual components, and biomass fractions (cellulose, hemicellulose, lignin and ash) are expressed in weight, while in solids, fractions are expressed in percentage (% w/w) or weight (g). (*) ash content in the alkaline liquor is determined as the silica quantity that can be precipitated and is underestimated since part of the silica remains soluble.

Experimental methods used to obtain the compositional analysis of the liquor and the solid phases presented in Figure S12 followed the procedures described in (Nascimento *and Rezende,* 2018). The distribution of specific components can be followed from the sample *in natura* until the final RB2 sample in the different pretreatment fractions. From 100 g of *in natura* rice husks, 91.5 g remain after extractive removal, and the extracted sample contains 26.2 g of cellulose, 15.2 g of hemicellulose, 30.3 of lignin and 13.5 of ashes. Cellulose remains mostly in the solid fractions after the pretreatment with acid (25.1 g) or alkali (24.8 g) so that only a little quantity of this component is hydrolysed and transferred to the liquors. The ash fraction (mainly composed by silica) is also little changed in the solid after the first pretreatment step with H_2SO_4 1.8% w/w (13.5 g to 12.5 g after the acid step). On the other hand, its content is drastically reduced to 0.4 g in the solid after the treatment with NaOH 6% w/w, indicating that most ashes are hydrolyzed to the alkali liquor, where 8.8 g could be quantified. It is important to highlight that this weight (8.8 g) refers to the silica that could be precipitate from alkaline liquor, though we know that a significant amount remained in the soluble form, forming a gel.

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CHAPTER 3 – SIMULTANEOUS OPTIMIZATION OF ALKALINE AND ACID-ALKALINE PRETREATMENTS APPLIED TO RICE STRAW TO PRODUCE GLUCOSE CORRELATED WITH CHEMICAL AND MORPHOLOGICAL EFFECTS

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Abstract

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

Keywords: Experimental Design; acid pretreatment; alkali pretreatment; rice straw; biorefinery; silica.

3.0 INTRODUCTION

Rice is a widely available food source in several regions around the globe and ranks as the third most-produced agricultural commodity, having yielded an estimated 518.14 million metric tons in 2023, prominently in Asia, South America, and Africa (Abaide *et al.*, 2019; "Rice Production by Country | World Agricultural Production 2023/2024," n.d.). The production of rice results in a significant amount of straw (approximately 1.35–1.5 kg per 1 kg of rice), offering substantial biotechnological potential (Satlewal *et al*., 2018). However, nearly 50% of rice straw is currently disposed of through burning, landfilling, or used as fodder, increasing thus environmental issues (Satlewal *et al*., 2018).

The potential of rice straw extends to chemical and biotechnological pathways for biofuel and chemical production, showcasing its versatility. The high cellulose content (23–47% wt) in rice straw (Binod *et al*., 2010; Jin et al., 2020) makes it an attractive source for second-generation (2G) ethanol, a promising alternative to oil-based fuels. 2G ethanol can supplement the production of first-generation (1G) ethanol, produced by direct fermentation of the sucrose present in sugarcane juice or corn starch, and is widely used in some countries such as Brazil. This strategy is a clever alternative to enhance bioethanol production and respond to the increasing demand for more sustainable fuels. Also, it is environmentally beneficial because it allows the production of higher quantities of fuel without the need to increase the cultivated land area, which reduces competition with food production (Satlewal *et al.*, 2018).

2G ethanol production involves pretreating lignocellulosic biomass to enhance cellulose accessibility for enzymatic conversion into fermentable sugars like glucose. Pretreatments are a mandatory step in 2G ethanol production due to the intrinsic association of cellulose with hemicellulose (19–27% wt) and lignin (5–24% wt) in the plant cell wall (Baruah *et al.*, 2018), which hinders the polysaccharide conversion into valuable bioproducts *(Tan et al.*, 2023).

Effective pretreatment is crucial for optimizing 2G ethanol production, especially considering the elevated costs of these processes (Singh and Patel, 2022). Chemical pretreatments, such as those using diluted acids and alkalis, efficiently extract lignin and hemicellulose and increase the substrate surface area and porosity (Agbor *et al.*, 2011). They are particularly important for rice straw due to its high silica content (up to 20%) (Binod *et al.*, 2010), considering the detrimental effect of silica on enzymatic action.

Diluted acid and alkaline pretreatments stand out for their ability to remove hemicellulose, lignin, and silica from rice straw, allowing their recovery as byproducts, which is in accordance with the biorefinery concept (Barana *et al.*, 2016; Scopel and Rezende, 2021). Acid treatments extract mainly hemicellulose by hydrolyzing the polysaccharide glycosidic bonds and producing oligomers and monomers, which are solubilized into the liquid fraction (Cheah *et al.*, 2020). Conversely, alkaline treatments effectively hydrolyze ester and ether linkages between cellulose, hemicellulose, and lignin, solubilizing the aromatic molecule into the alkaline liquid fraction (Lorenci Woiciechowski *et al.*, 2020). In sequence, hydroxide ions can efficiently cleave the internal β-O-4 linkages of lignin macromolecules, contributing to its solubilization. In rice residues, alkaline pretreatments have a fundamental role in silica extraction and solubilization, which is not efficiently achieved by simple hydrothermal treatments based on hot water (Le *et al.*, 2015; Moreira *et al.*, 2021).

Design of Experiments (DOE) is a valuable tool for optimizing pretreatment conditions, allowing efficient screening of an extensive experimental range by simultaneously changing the variables using fewer experiments *(*Han*s et al., 2022;* Mota *et al., 2021;* Valles *et al.*, 2021). In other words, DOE allows the detection of interactions between the experimental variables, which is not possible using experiments that vary only one condition at a time (Bruns *et al.*, 2006; Rezende *et al.*, 2018).

DOE is a strategy already adopted for optimizing pretreatments applied to lignocellulosic biomasses, including rice straw. However, while previous studies have separately optimized only acids (Hans *et al.*, 2022; Hsu *et al.*, 2010; Molaverdi *et al.*, 2022), only alkalis (Cheng *et al.*, 2010; Kim and Han, 2012; Valles *et al.*, 2021), or sequential acid-alkaline treatments (Sun *et al.*, 2016; Weerasai *et al.*, 2014), ours aims to simultaneously optimize alkaline and acid-alkaline pretreatments in the same DOE set of experiments. Simultaneous alkaline and acid-alkaline optimization takes advantage of the DOE ability in predicting interactions between acid treatment and the alkaline variables. This approach allows for a comprehensive exploration of diverse scenarios for biomass fractionation, considering the enzymatic hydrolysis outcomes.

The simultaneous optimization emerged as an interesting way to evaluate biomass processing, based on the fact that acid step was considered not strictly necessary in acid-alkaline treatments applied to some biomasses if glucose production is the focus of the process (Moreira *et al.*, 2021; Rezende *et al.*, 2018). In these cases, the biomass could be directly forwarded to a delignification step using alkaline methods, avoiding acid step, which is beneficial from an economic point-of-view for a single-product approach (in this case, in glucose). However, the use of an acid step before alkaline treatments is more suitable for an improved fractionation of the biomass components and a preferable alternative to valorize both hemicellulose and lignin fractions (Cheah *et al.*, 2020; Scopel and Rezende, 2021). Indeed, alkaline treatments applied directly to the *in natura* substrates hinder component fractionation because hemicellulose and lignin are extracted in the same liquid stream. To the best of our knowledge, no studies have simultaneously optimized alkaline and acid-alkaline pretreatments applied to rice straws.

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

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

3. 1 EXPERIMENTAL

3.1.1 Biomass and materials

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

3.1.2 Biomass pretreatments

In natura rice straw was treated using H_2SO_4 solutions similarly to previously reported procedures (Rezende *et al.*, 2011), following the concentrations indicated by the DOE (Table 3.1 and Table 3.2). These concentrations varied from 0 (when the acid step was not performed and *in natura* samples were directly forwarded to alkaline treatments) to 3.6 wt.%. All acid treatments were conducted in an autoclave (Phoenix AV-75) at 120 °C for 40 min using a solid:liquid ratio of 1:10 (g:mL). At the end of the pretreatment time, the system was cooled to room temperature, and the solid was separated from the liquid fraction by filtration and rinsed until neutral pH was obtained.

In the alkaline step, *in natura* or acid-treated substrates underwent an alkaline treatment with NaOH solutions following the conditions indicated in Table 3.1. The range of acid and alkali concentrations was determined based on previous studies for other biomasses, such as rice husks and elephant grass (Moreira *et al.*, 2021; Rezende *et al.*, 2018). Experiments above 100 °C were performed in an autoclave (Phoenix AV-75), similarly to the described for acid treatments, while pretreatments below 100 °C were performed in a water bath (Fisatom, model 550), ensuring similar temperature ramp conditions (Moreira *et al.*, 2021). The solids obtained after each pretreatment were filtered, rinsed until neutral pH and dried.

Experiments were carried out following a 2^{5-1} Fractional Design, in which 5 variables were evaluated at two levels (Table 3.1), and 5 replicates were used in the central points. The experimental factors considered were: 1) H_2SO_4 concentration in the acid step ([H_2SO_4]; 2) NaOH concentration [NaOH]; 3) Temperature; 4) Time; and 5) Solid-to-liquid ratio (S/L) in the alkaline step. Hydrolysis yields (HY) at 12 and 24 h (calculated following Equation 1) were evaluated as responses. The range of each factor was defined based on the previous evaluation carried out in rice husks (Moreira *et al.*, 2021).

Table 3.1 Factors and levels evaluated in the 2^{5-1} FFD

Factors			
	Low Level (-)	**High Level (+)**	**Central Point (0)**
A- [H_2SO_4] (% w/w)	0*	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

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

3.1.3 Enzymatic hydrolysis

Enzymatic hydrolysis was carried out using two types of enzymatic cocktails. Firstly, all samples from DOE were hydrolyzed using a mixture of Celluclast 1.5L and Novozyme 188 (Novozymes) (ratio 4:1) in a shaking incubator with a minimum of 4 replicates in each experimental condition. Following previous studies, the reactions were conducted with a biomass weight of 4.5 mg and a total volume of 850 µL at 50 °C, pH 4.5 (25 mM sodium acetate buffer) with an enzyme loading of 8 FPU/g biomass (Gomez *et al.*, 2010; Mota *et al.*, 2021). Hydrolysis residence times of 12 and 24 h were evaluated as DOE responses. Additional hydrolysis times (48 and 72 h) were carried out in a kinetic assay using samples with lower (S1) and higher (S8, S15, and S16) cellulose conversion yields at 12 and 24 h of enzymatic hydrolysis (Supplementary Information). This assay was carried out to ensure that 12 and 24 h were the most indicated hydrolysis times to be considered in DOE.

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

Before all enzymatic hydrolysis experiments, substrates underwent a hydration step for 2 h at room temperature. The glucose quantification was performed using High-Performance Liquid Chromatography (HPLC) equipment (Agilent 1200) (Scopel and Rezende, 2021). Hydrolysis yields (HY) were determined according to Equation 1, considering the total glucose released on hydrolysis (GL in mg/g substrate), the cellulose content (C, mg/g) in the hydrolyzed substrate, and a correction factor (1.1) due to polysaccharide hydrolysis (Ebrahimi *et al.*, 2017).

$$HY(\%) = \frac{GL(mg/g)}{C(mg/g)\times 1.1} \times 100 \qquad \text{(Equation 1)}$$

3.1.4 Statistical evaluation

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

3.1.5 Chemical composition

Cellulose, hemicellulose, lignin, ash, and extractive contents were quantified according to the National Renewable Energy Laboratory (NREL) procedure (Sluiter and Sluiter, 2011). Briefly, 0.3 g of substrate were hydrolyzed with H_2SO_4 72 wt.% (3mL) for 1 h at 30 °C. Next, 84 mL of deionized water were added to dilute H_2SO_4 to 4 wt.% and the system was allocated in autoclave at 121 °C for 1 h. Liquid fraction was separated from solids by filtration using a porous-bottom crucible. Carbohydrates and their degradation products were quantified in liquid fraction by high-performance liquid chromatography (HPLC) (Agilent 1200) using a BIORAD HPX87H column (45 °C, H_2SO_4 5 mmol/L as mobile phase). Acid-soluble lignin present in liquid fraction was quantified by UV–vis spectroscopy (Agilent, Cary 5000). The solid fraction present in the crucible was dried until constant weight (105 °C) and then calcinated to quantify acid-insoluble lignin. Ashes were quantified by calcinating the substrate (800 °C, 2 h). Soxhlet extraction (ethanol:cyclohexane, 8 h and water, 24 h) was carried out only in *in natura* samples to determine the amount of extractives

3.1.6 Morphological analysis

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

3.2 RESULTS AND DISCUSSION

3.2.1 Fractional factorial design (FFD) analysis

FFD was selected as the DOE tool in this study since it reduces the number of runs compared to a full factorial design. In the case of a DOE using five variables, the 2^{5-1} FFD presents resolution V, indicating that the main effects are aliased with fourth-order interactions (which are unlikely to be significant), and second-order interaction effects are aliased with the third-order interactions (also unlikely to be significant). Therefore, primary and second-order interactions can be estimated in 16 runs (central points not considered) against 32 runs needed in a full factorial design (Bruns *et al.*, 2006; Rezende *et al.*, 2018).

Table 3.2 describes the experiments conducted according to the 2^{5-1} FFD and the two evaluated responses (HY after 12 or 24 h of enzymatic hydrolysis using a mixture of Celluclast 1.5L and Novozyme 188 enzymatic cocktails). The results showed HY up to 6-fold higher than *in natura* straw (SIN), even after only 12 h of hydrolysis, indicating the efficiency of the pretreatment approach. At this hydrolysis time, the best performance was achieved under condition S15 (HY=57.6%), using no acid step and NaOH 4.5 wt.% at 125 °C for 100 min with S/L of 5 wt.% in the alkali step. Considering 24 h of enzymatic hydrolysis, the efficiency was even higher, achieving values higher than 90% for several experimental conditions (S8, S15, and S16).

Previous studies using acid or alkaline diluted solutions to pretreat rice straw typically used enzymatic hydrolysis times longer than 24 h and higher enzyme load to achieve similar results. The yields of enzymatic hydrolysis of rice straw treated using sequential acid-alkaline treatments resulted in 70-90% cellulose conversion to glucose after 72 h of enzymatic hydrolysis (Sun *et al.*, 2016; Weerasai *et al.*, 2014). Nevertheless, alkaline treatments used directly in rice straw also resulted in high cellulose conversion rates, closer to 60% (Kim and Han, 2012). For example, sequential treatment with 0.5% H_2SO_4 (130 °C for 2 h) and 1.5 % NaOH (80 °C for 3 h) resulted in HY of 92.7% using an enzymatic load of 17 FPU/g substrate after 72 h of enzymatic hydrolysis (Sun *et al.*, 2016). Considering other pretreatment approaches, such as micro-emulsions of eutectic solvents (Gao *et al.*, 2020) and ionic liquid systems (Gao *et al.*, 2019; Sorn *et al.*, 2019), HY range between 61 to 88% using an enzymatic content of around 20 FPU/g cellulose for 72 h.

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

Higher HY were achieved here using hydrolysis conditions typically milder than those reported in the literature (15–20 FPU, 48–72 h, respectively) (Abdolmaleki *et al.*, 2021; Mohammadi *et al.*, 2019; Scopel and Rezende, 2021). Two main causes that can be associated with our high HY are the high cellulose content in the substrates (which will be discussed in section 3.2.2) and the use of a reduced solid concentration (0.47%), which will be assessed in section 3.2.4. The high glucose release results, specifically using 24 h of enzymatic hydrolysis, are potentially linked to the chemical composition and morphology of the substrates, which will be assessed in section 3.2.2 and section 3.2.3, respectively.

Table 3.2 2^{5-1} FFD experiments and the responses of hydrolysis yields (HY) after 12 and 24 h of enzymatic hydrolysis.

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

* Reported values are an average of 5 replicates of enzymatic hydrolysis. Standard deviation calculated for the 5 replicates at the central point is ± 2.6% (12 h) e ± 0.6% (24 h)

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

The Half-Normal plots of the effects are shown in Figure 3.1, where the most important effects are those that deviate more from the straight line (centered in zero). In this case, NaOH concentration is the factor that most influences the response since it is far from the straight line, having a positive effect. It indicates that the increase of NaOH concentration is expected to increase the hydrolysis yields both for 12 and 24 h.

It is noticeable that the factors and their degree of influence on HY differ depending on the residence time in enzymatic hydrolysis. For 12 h of enzymatic hydrolysis, the most important factors after NaOH concentration are the S/L (negative effect), Time (positive effect), [H_2SO_4] (negative effect), and Temperature (positive effect). Also, the binary BC ([NaOH]-Temperature) interaction proved significant. On the other hand, if hydrolysis residence time is changed to 24 h, the main factors influencing HY after [NaOH] are Temperature (positive effect) and the BC interaction.

Comparing the two enzymatic hydrolysis times, Temperature became more relevant as enzymatic hydrolysis times increased. In contrast, the S/L ratio, Time, and [H_2SO_4] concentration, which were relevant for 12 h, were less expressive and virtually unimportant for HY obtained at 24 h. This difference indicates that HY are more sensitive to pretreatment conditions using shorter hydrolysis residence times. This can be explained by considering that at 12 h of enzymatic hydrolysis, enzymes have a more limited time to convert cellulose into glucose. Hence, the accessibility of the substrate becomes more critical (more accessible substrates present higher cellulose conversion). On the other hand, longer hydrolysis times allowed a slower kinetic for cellulose conversion, and the specific limitations of the substrate were thus less crucial (enzymes have time to convert higher amounts of cellulose even in less accessible substrates). Likewise, different pretreatment conditions allowed an almost total conversion of the available cellulose (Rezende *et al.*, 2011).

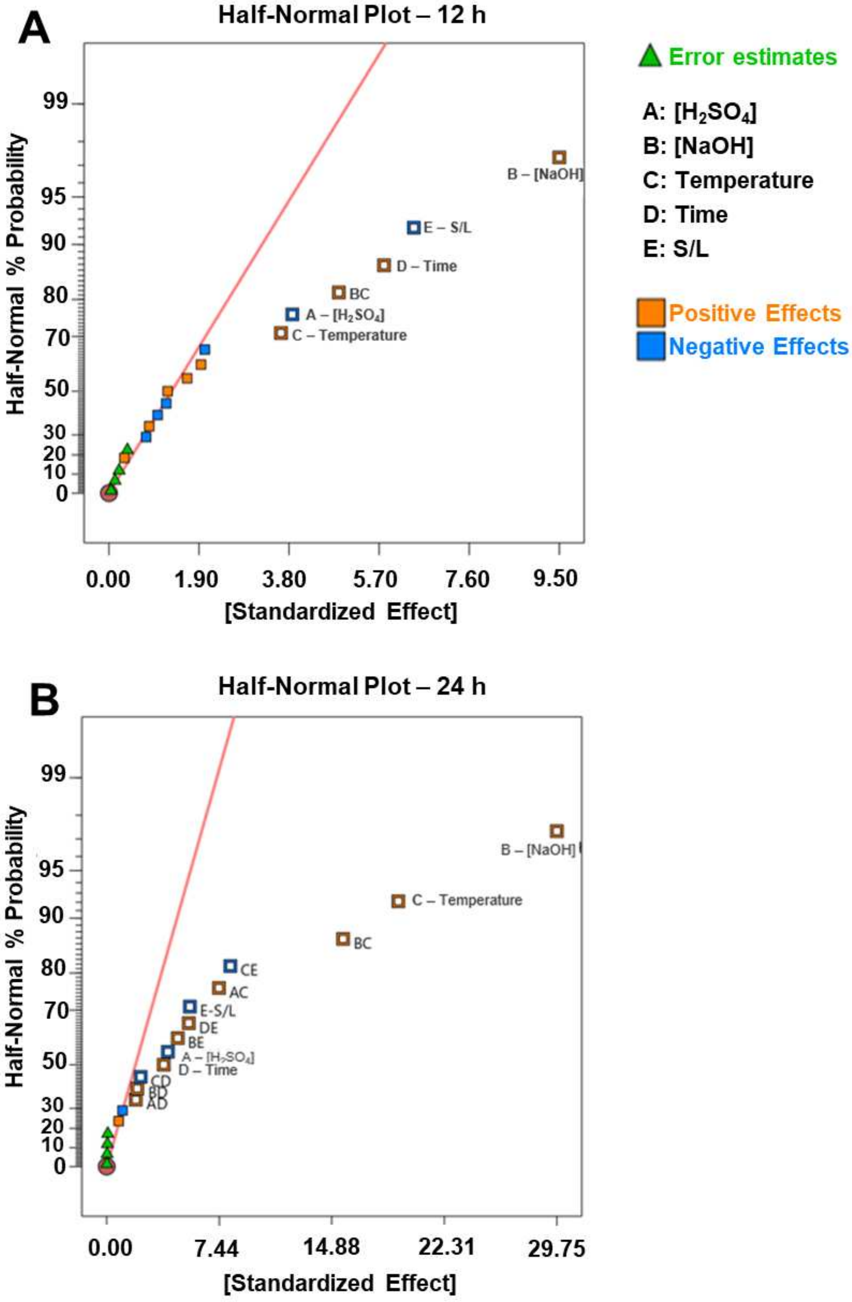

Figure 3.1 The Half-normal plot of the standardized effects of the 2^{5-1} fractional factorial design for hydrolysis residence times of A) 12 h; and B) 24 h.

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

The response surfaces using 12 h of enzymatic hydrolysis as a function of the most critical factors (NaOH concentration and S/L ratio in step 2) are shown in Figure 3.2. The surface curvature is not significant (p-value = 0.7804 in Table S1), indicating the linear model adequacy in describing experimental results within the studied domain. Therefore, based on the model, it is possible to predict that an optimal HY (12 h) could be reached at [NaOH] at the high level, keeping S/L at lower values. The highest conversion value (*ca.* 57%) was obtained without the acid step and using the following conditions in the alkaline pretreatment: [NaOH] = 4.5 wt.% at 125 °C and 100 min using an S/L = 5 wt.%, which coincides with the pretreatment conditions used to obtain S15.

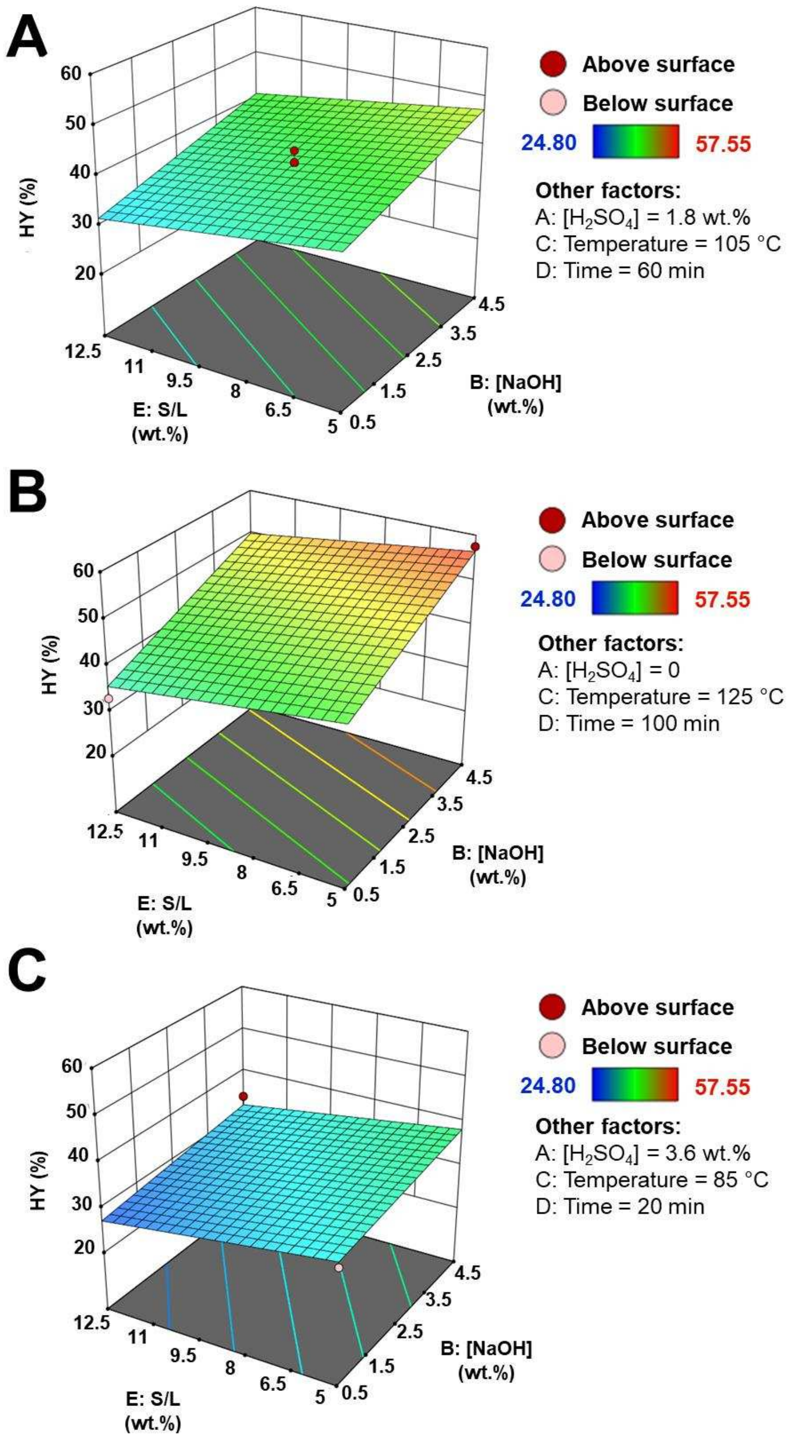

Figure 3.2 Response surface (HY after 12 h of enzymatic hydrolysis) of the most relevant factors for HY in rice straw samples ([NaOH] and S/L): A: with all the other factors kept at their central points ([H_2SO_4] = 1.8% w/w, Temperature = 105 °C, Time = 60 min); B: under the conditions that resulted in the highest conversion (without acid step, Temperature = 125 °C; Time = 100 min); and C: under the conditions that resulted in lowest conversion ([H_2SO_4] =

3.6% w/w, Temperature = 85 °C; Time = 20 min). Surface points above and below are shown to highlight the curvature analysis.

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

The response surfaces after 24 h of enzymatic hydrolysis as a function of the most important factors ([NaOH] and Temperature) are shown in Figure 3.3. The condition for the highest HY was [NaOH] = 4.5 % (w/w), Temperature = 125 °C, S/L = 5 % (w/w). [H_2SO_4] and Time are practically indifferent and have been shown at their lowest levels. It is noteworthy that between the conditions that led to the highest (Figure 3.3B) and the lowest results (Figure 3.3C), the maximum value is always when Temperature and [NaOH] are at their highest levels. This observation is also valid for response surfaces evaluating the HY after 12 h of enzymatic hydrolysis when [NaOH] and Temperature are varied (Figure S1). Still, the maximum values using 12 h of enzymatic hydrolysis reached up to 57.6%.

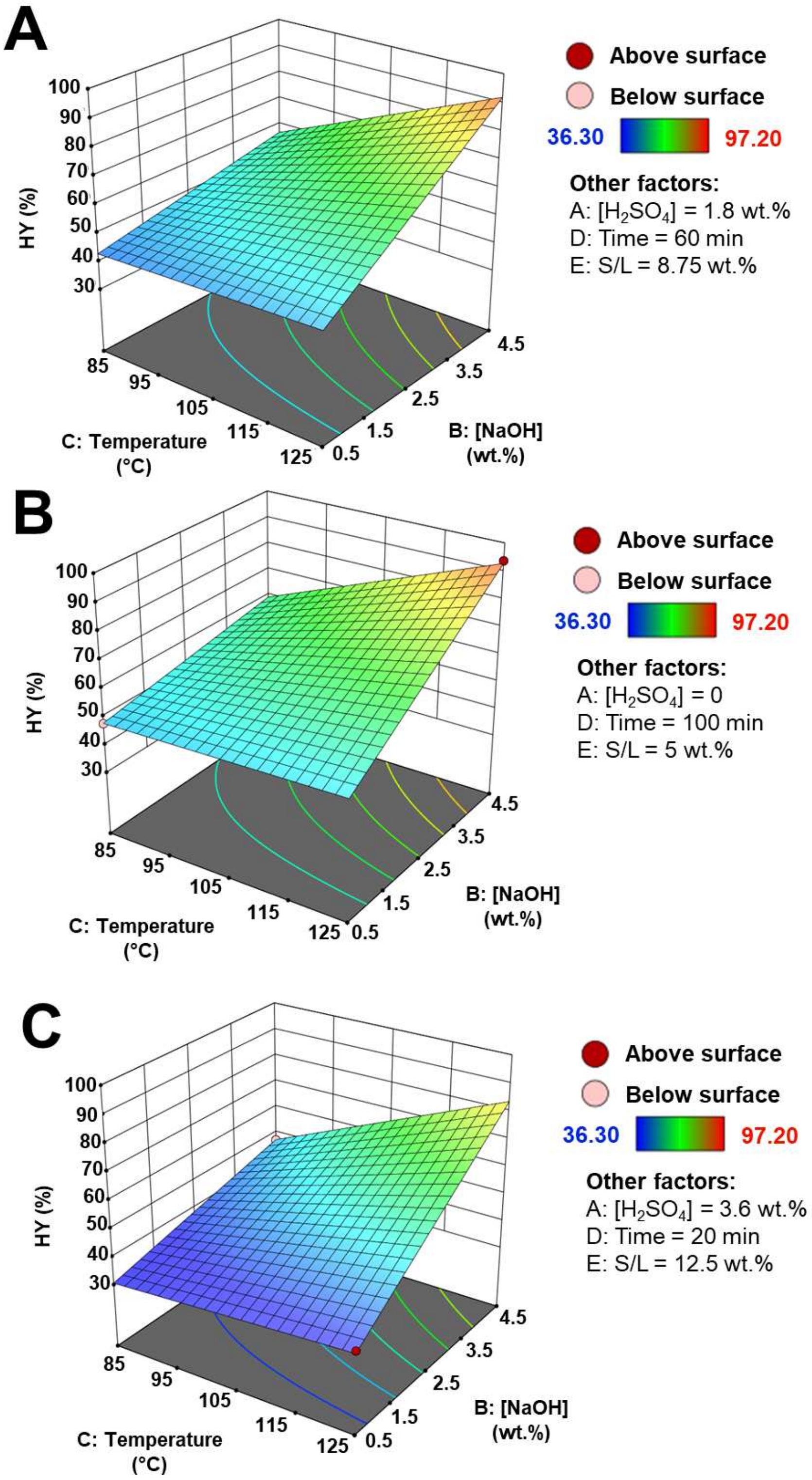

Figure 3.3 Response surface (HY after 24 h of enzymatic hydrolysis) of the most relevant factors for HY in rice straw samples ([NaOH] and Temperature): A: with all the other factors kept at their central points ([H_2SO_4] = 1.8% w/w, Time = 60 min, S/L = 8.75% w/w); B: under the conditions that resulted in the highest conversion (without acid step, Time = 100 min, S/L = 5% w/w); and C: under the conditions that resulted in lowest conversion ([H_2SO_4] = 3.6%

w/w, Time = 20 min; S/L = 12.5% w/w). Surface points above and below are shown to highlight the curvature analysis.

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

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

3.2.2 Chemical compositions and their relations with glucose production

Concomitant with evaluating the efficiency of the pretreatments in the enzymatic action, it is also essential to assess their effect on the chemical composition of the substrates. This evaluation is crucial because it explains the enzymatic hydrolysis results and defines the best conditions between similar HYs, considering a biorefinery scenario.

The chemical composition and the quantity of glucose released after enzymatic hydrolysis using the mixture of Celluclast 1.5L and Novozyme 188 in the substrates *in natura* and after pretreatments indicated by DOE (Table 2) are reported together in Figure 3.4 and also in Table S3. The compositional analysis showed that the rice straw *in natura* (SIN) comprises 36.9% cellulose, 25.0% hemicellulose, 12.9% lignin, 7.7% ash, and 17.4% extractives. The lignin content is typically lower than that reported for other grasses, such as elephant grass (Scopel and Rezende, 2021), sugarcane bagasse (Rezende *et al.*, 2011), and residues of corn (Camargos *et al.*, 2019), but similar to the values reported in the literature for other rice straw samples (Abaide *et al.*, 2019; Amnuaycheewa *et al.*, 2016). As lignin is a major factor hindering enzymatic action, these relatively lower contents highlight the applicability of rice straw for glucose production by enzymatic hydrolysis.

As expected, acid treatments extracted mainly hemicellulose, reducing its content by 65% and 75% as the *in natura* substrate is treated using [H_2SO_4] = 1.8 wt.% (SH1) and 3.6 wt.% (SH2). Alkaline treatments applied in acid-treated samples resulted in minimum hemicellulose contents (up to 2.2%). On the other hand, samples treated only with alkaline treatments had a maximum reduction of 43% in the hemicellulose content in the assay using the different conditions in high levels (S11). Indeed, alkaline treatments resulted mainly in lignin extraction (up to 77% in S8 using [H_2SO_4], [NaOH], and Temperature in high levels and Time and S/L in low levels), which follows the known effect of these treatments (Kim *et al.*, 2016). Alkaline treatments also effectively reduced the ash content to 0.7% in the most efficient conditions, essential for rice straw processing.

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

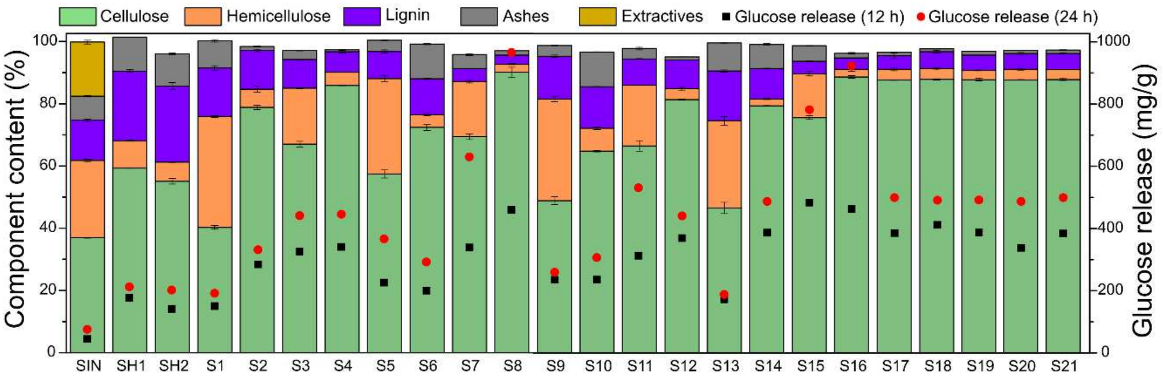

Figure 3.4 Chemical composition of solid substrates before and after pretreatments and glucose released (mg/g of substrate) after 12 and 24 h of enzymatic hydrolysis. Error bars are standard deviation values of replicates. SIN = raw rice straw; SH1 = sample pretreated only with [H_2SO_4] = 1.8% w/w; SH2 = sample pretreated only with [H_2SO_4] = 3.6% w/w; S1 to S21 = FFD samples with experimental conditions detailed in Table 3.1. Detailed values are described in Supplementary Information, Table S3.

In addition to the changes in chemical composition, the crystallinity of samples also changed because of the pretreatments, according to the determination of the Crystallinity Index (CrI, Table S5) based on the X-Ray diffraction (Figure S4, SI) (Park *et al.*, 2010). CrI increased from 46% to 52-54% in samples SH2 and S1, which still presented high lignin contents, and to 63-64% in samples with high cellulose content (S8, S15, and S16). Samples S8, S15, and S16 presented the highest cellulose conversion, which indicates that cellulose accessibility caused by hemicellulose and lignin removal played a more significant role than the increase in CrI. Indeed, the increase in CrI could be interpreted as a drawback for enzymatic hydrolysis since enzymes usually present better performance in amorphous substrates, which are less organized and easily converted into monosaccharides (Hall *et al.*, 2010). However, it is essential to highlight that the increase in CrI observed here is a consequence of the extraction of the amorphous components of lignocellulosic biomasses (hemicellulose and lignin) (Nascimento and Rezende, 2018; Scopel and Rezende, 2021). Since cellulose is a semicrystalline polymer (Klemm *et al.*, 2011), cellulose-rich substrates presented a higher CrI than *in natura* or less modified substrates.

Based on the chemical characterization of the solid substrates and HY, it is possible to suggest some approaches for rice straw processing (Figure S7, SI). Condition S15 is the most economical because it uses no acid step. However, the acid step plays a significant role in hemicellulose extraction (Figure 3.4). Therefore, acid steps can be conveniently applied in a cascade approach to separate hemicellulose and lignin in different streams. Amongst the conditions using acid-alkaline pretreatments, S8 and S16 showed the highest HY. However, S8 is more advantageous because it demands only 20 min of alkaline pretreatment, extracting the same quantity of lignin (87-88%, according to Table S4, Supplementary Information) and producing the same amount of glucose from each 100 g of *in natura* substrate (20.6 g, Figure S5, SI). A more detailed mass balance of the solid fractions and an estimate of component recovery from liquid fractions can be found in Supplementary Information (Figure S6).

The recovered hemicellulose can be further converted into bio-based chemicals, such as furfural (Gomes *et al*., 2023; Gómez Millán *et al.*, 2020) and xylitol (de Souza Queiroz *et al.*, 2021). If hemicellulose remains in the substrate, it can enzymatically be converted to pentoses along with the cellulose conversion into glucose. Still, the fermentation of pentoses into ethanol would demand microorganisms other than the usual *Saccharomyces cerevisiae* (Martins *et al.*, 2018), which should be considered. In addition, the partial removal of hemicellulose to the liquid fraction will require a proper separation from the lignin, also solubilized in the alkaline liquid fraction, to enable its application (Rabelo *et al.*, 2022).

Regardless of the acid step, alkaline treatments effectively removed lignin up to 90% (Table S4). Lignin can be precipitated from the alkaline liquid fraction by acid addition (Trevisan and Rezende, 2020) and used to produce lignin nanoparticles (Schneider *et al.*, 2021), polyols by depolymerization (Mahmood *et al.*, 2016), or carbon materials (Qu *et al.*, 2021). Rice straw also has a significant quantity of extractives, commonly composed of several high-value-added molecules, including sterols, fatty acids, and terpenes, which can be fractionated before acid or alkaline steps, enhancing biomass use and the revenue process, as was previously shown in similar biomasses, such as elephant grass leaves and stems and maize (Attard *et al.*, 2018; Scopel *et al.*, 2020).

3.2.3 Morphological characterization

Significant morphological changes in the solid substrates followed the chemical changes that increased the glucose release. Figure 3.5 shows FESEM images of the substrates before treatments and the silica mapping of amplified areas. *In natura* rice straw has a surface covered by silica structures, as shown in the secondary electron images (Figure 3.5A, Figure 3.5C, and Figure 3.5E) and on the silicon maps (Figure 3.5B, Figure 3.5D, and Figure 3.5F). The two main silica structures identified in rice straw were papillae (indicated as P, Figure

3.5B) and dumbbell silica bodies (DSB, Figure 3.5B) (Kaur *et al.*, 2019). DSB are solidly silicified cells (Figure 3.5B and Figure 3.5F). In contrast, papillae are tiny outgrowths of silica structures (Figure 3.5B and Figure 3.5D) (Markovich *et al.*, 2019). Indeed, 75-91% of the rice straw ashes are Si (Satlewal *et al.*, 2018), vital in improving rice growth, providing mechanical strength, and protecting the plant from pathogens. In rice, silica is presented preferentially in the epidermal cell wall, negatively affecting cellulase action. As determined by the silica quantification after pretreatment, the extent of silica removal from rice straw depends on the alkalinity of the pretreatment.

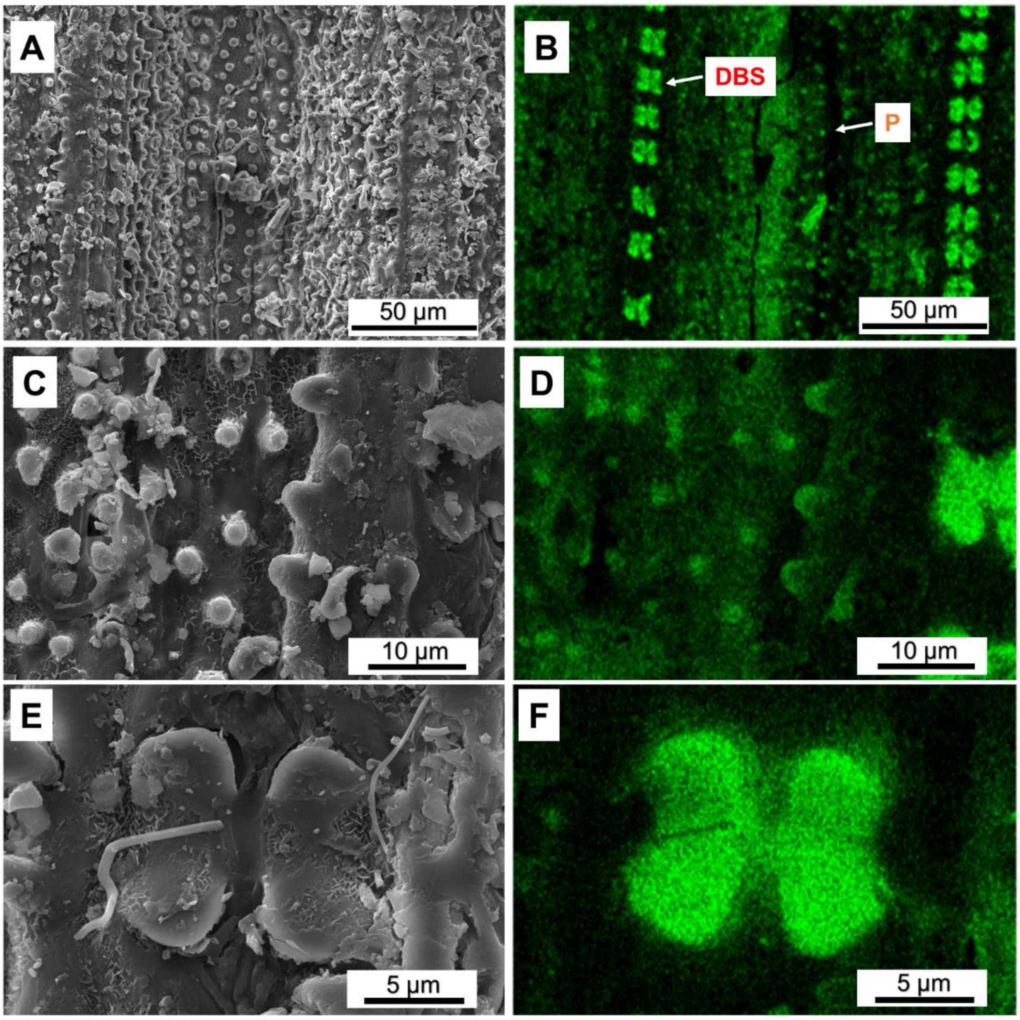

Figure 3.5 Scanning electron microscopy images of rice straw *in natura* (A, C, E) and Si mapping obtained by EDS (B, D, F) in the same areas of A, C, and E, respectively. Si is indicated as green in the images. DBS is the code for dumbbell silica bodies, and P is for papillae. The ash content in this sample is 7.7 ± 0.2%.

After the treatments, biomass morphology was changed by removing hemicellulose, lignin, and silica, resulting in more exposed and separated cellulose fibers. After the acid step, morphological changes were subtle, as shown for sample SH2, treated with 3.6% wt.% H_2SO_4 (Figure 3.6A and Figure 3.6B). The slight differences in chemical composition (Figure 3.4) and substrate morphology explain the slight increase in the HY only after acid treatments (Table 3.2). However, it is noticeable that the acid treatment caused a disorder in silica structures compared to *in natura* rice straw. DBS are less oriented and more rounded. In addition, unlike *in natura* substrate (Figure 3.5), silica is more distributed (lower contrast) on the fiber surface (Figure 3.6B), probably due to the partial silica removal and redeposition.

Regarding alkaline treatments, the mildest method used in this study (S1) did not significantly change the biomass structure (Figure 3.6C and Figure 3.6D), which is related to the slight change in the chemical composition (Figure 3.4). On the other hand, substrate S16 (acid-alkaline) (Figure 3.6E), which resulted in higher glucose releases, was significantly modified by the pretreatments, showing open bundles of cellulose fibers more exposed to enzymatic action. This morphological effect of bundle separation is similar to that obtained for other biomasses, such as sugarcane bagasse (Rezende *et al.*, 2011) and elephant grass (Scopel and Rezende, 2021), and it is assigned to lignin removal from the interfibrillar regions. In addition, no silica structures were observed in the EDS analysis of sample S16 (Figure 3.6E), showing the apparent effect of the alkali pretreatment on the silica domains of the plant cell wall.

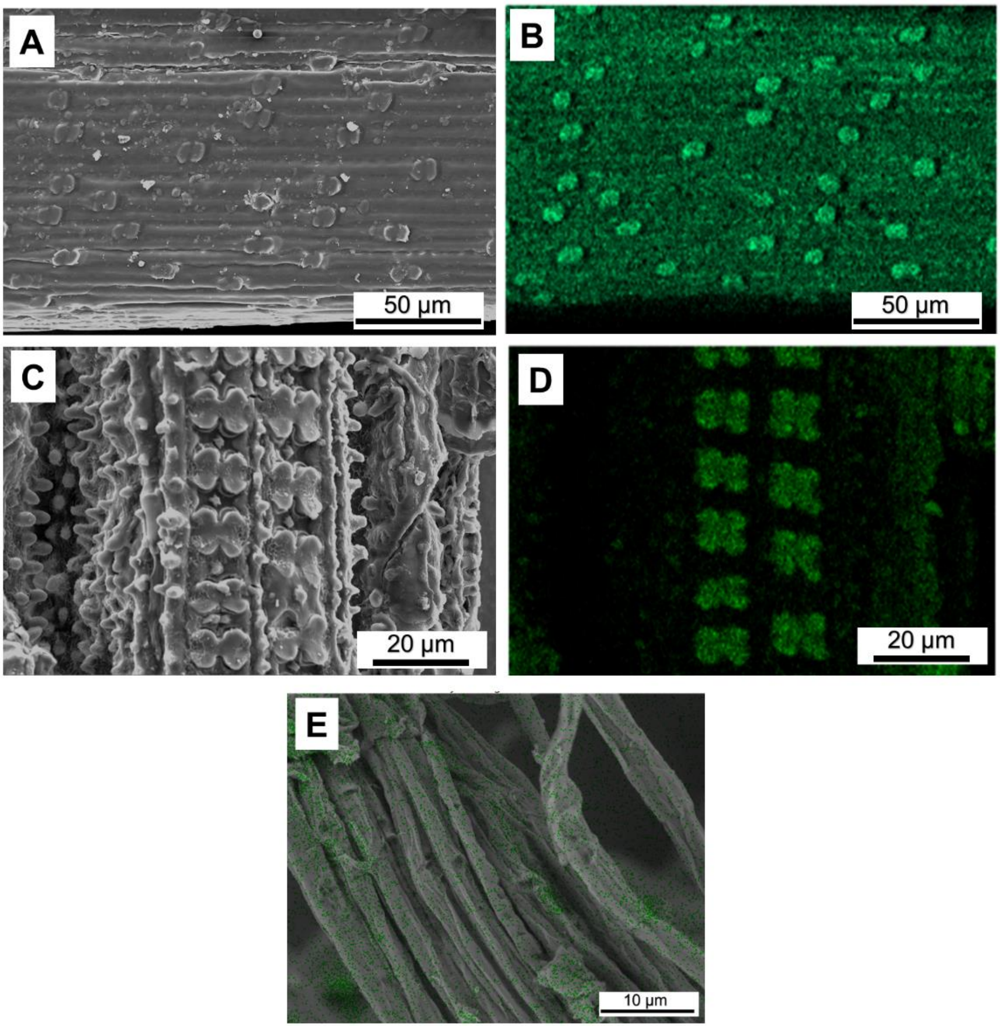

Figure 3.6. Scanning electron microscopy and Si map of samples SH2 (A and B), S1 (C and D), and S16 (E).

3.2.4 Considerations of enzymatic hydrolysis with different enzymes

Samples S8, S15, and S16, which resulted in the best results in DOE, were also hydrolyzed using the enzymatic cocktail Cellic CTec2 for 24 h. This assay was carried out with two main goals: compare two different sets of enzymes and provide a proof of concept using higher solid/ratio conditions in enzymatic hydrolysis aiming at increasing glucose concentration to enable fermentable conditions more economically viable. Firstly, we kept the enzymatic dosage in 8 FPU for assays at a solid content of 0.47% (same as the one used in DOE) and then evaluated higher solid contents (2.5 and 5%). Also, we evaluated higher enzyme dosages (20 FPU) for conditions using higher solid contents.

Figure 3.7 shows the HY and glucose concentration for S8, S15, and S16. Cellic CTec2 (8 FPU, 0.47% of solids) resulted in lower HY (between 67 to 85%) compared to the conditions using Celluclast 1.5L/Novozyme 188. When Cellic CTec2 was used, sample S15 presented a higher HY between the samples. The comparison indicates that Celluclast 1.5L/Novozyme 188 was more efficient for hydrolyzing the analyzed samples, probably because it employs a combination of two enzymes. Still, it is noteworthy that increasing residence time for Cellic CTec2 could increase yields closer to those achieved in DOE since previous use of this enzymatic cocktail for 72 h resulted in HY closer to 100%, when it was applied to elephant grass that underwent acid-alkaline or only alkaline treatments (Scopel and Rezende, 2021).

Fixing the enzyme dosage at 8 FPU and increasing the solid content to 5 wt.% resulted in increased glucose concentration (from about 3 g/L to values closer to 30 g/L). This is an interesting result about achieving high glucose concentrations since HY are important but should be considered together with the glucose concentrations, which facilitates further conversion into ethanol. Finally, a test using a higher enzyme dosage (20 FPU) was evaluated, resulting in glucose concentrations between 35–37 g/L, while HY ranged from 72 to 82%. It suggests that increasing enzyme dosage and solid contents should be considered together, aiming at both HY and glucose concentration.

Comparing between the evaluated samples, higher glucose concentrations can be achieved using both acid-alkaline (S8 and S16) or only alkaline treatments (S15). It is important to mention that this assay was carried out as proof of concept to demonstrate the optimized pretreatment effectiveness. Sequential studies can be performed at high-solids conditions to consider specific questions due to the so-called high-solids effect (da Silva *et al.*, 2020). It includes a reduction in glucose production due to inhibition of enzymes because of high product concentration, high concentration of degradation products, and unproductive binding of enzymes, to cite a few, which are very dependent on process parameters, such as the type of reactors.

The results achieved at high solid concentrations are similar to those reported when rice straw was treated with sequential acid-alkaline, but here we generally achieved higher HY in a reduced residence time of enzymatic hydrolysis. Rice straw treated sequentially with H_2SO_4 (1 wt.%) and NaOH (1.5 wt.) and hydrolyzed with Zytex-Supercut (10 FPU/g) and in-house β-Glucosidase 100 IU enzymes resulted in HY 83.2 % after 48 h (Ashoor *et al.*, 2023). Similarly, rice straw treated with H_2SO_4 (1 wt.%) followed by NaOH (1.25 wt.%) resulted in HY of 70.9 % after enzymatic hydrolysis with 25 FPU/g Accellerase® for 72 h (Weeresai *et al.*, 2014).

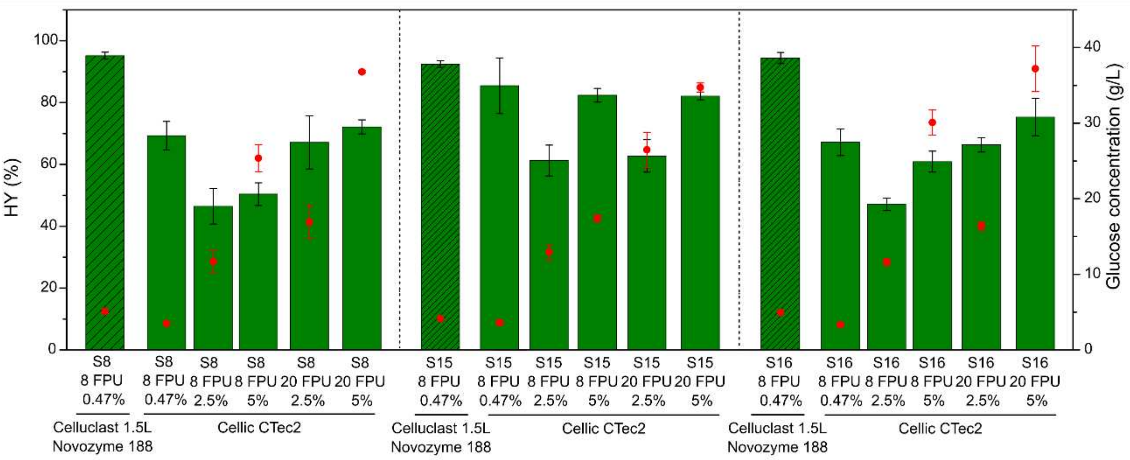

Figure 3.7. HY and glucose concentration obtained from samples S8, S15, and S16 hydrolyzed with the enzymatic cocktails Celluclast 1.5L/Novozyme 188 or Cellic CTec2 for 24 h. Green bars indicate HY and red circles indicate glucose concentration.

3.3 CONCLUSIONS

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

ACKNOWLEDGEMENTS

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APPENDIX B - Supplementary Material for the paper

SIMULTANEOUS OPTIMIZATION OF ALKALINE AND ACID-ALKALINE PRETREATMENTS APPLIED TO RICE STRAW TO PRODUCE GLUCOSE CORRELATED WITH CHEMICAL AND MORPHOLOGICAL EFFECTS

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ANOVA

Table S1. ANOVA table for the selected model based on 2^{5-1} FFD and describing the sugar release as a linear function of the selected coefficients (Response: HY after 12 h of enzymatic hydrolysis).

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	867.16	6	144.53	**20.61**	< 0.0001
A-[H_2SO_4] step 1	59.80	1	59.80	8.53	0.0119
B-[NaOH] step 2	360.66	1	360.66	51.43	< 0.0001
C-Temperature step 2	52.64	1	52.64	7.51	0.0169
D-Time step 2	134.62	1	134.62	19.20	0.0007
E- S/L step 2	165.40	1	165.40	23.59	0.0003
BC	94.03	1	94.03	13.41	0.0029
Curvature	0.5681	1	0.5681	0.081	0.7804
Residual	91.17	13	7.01		
Lack of Fit	64.31	9	7.15	**1.06**	0.5165
Pure Error	26.86	4	6.72		
Cor Total	958.89	20			

Table S2. ANOVA table of the model describing the sugar release as a linear function of the selected coefficients, based on 2^{5-1} fractional factorial design results (Response: HY after 24 h of enzymatic hydrolysis).

Source	**Sum of Squares**	**df**	**Mean Square**	**F-value**	**p-value**
Model	6991.93	13	537.84	**373.41**	< 0.0001
A-[H_2SO_4] step 1	65.61	1	65.61	45.55	0.0005
B-[NaOH] step 2	3540.25	1	3540.25	2457.94	< 0.0001
C-Temperature step 2	1486.10	1	1486.10	1031.78	< 0.0001
D-Time step 2	57.00	1	57.00	39.58	0.0008
E-S/L	121.00	1	121.00	84.01	< 0.0001
AC	220.52	1	220.52	153.11	< 0.0001
AD	14.82	1	14.82	10.29	0.0184
BC	976.56	1	976.56	678.01	< 0.0001
BD	16.40	1	16.40	11.39	0.0150
BE	88.36	1	88.36	61.35	0.0002
CD	20.25	1	20.25	14.06	0.0095
CE	267.32	1	267.32	185.60	< 0.0001
DE	117.72	1	117.72	81.73	0.0001
Curvature	**307.89**	**1**	**307.89**	**213.76**	**< 0.0001**
Residual	8.64	6	1.44		
Lack of Fit	6.97	2	3.49	8.34	0.0374
Pure Error	1.67	4	0.4180		
Cor Total	7308.46	20			

Additional surface responses – 12 h of enzymatic hydrolysis

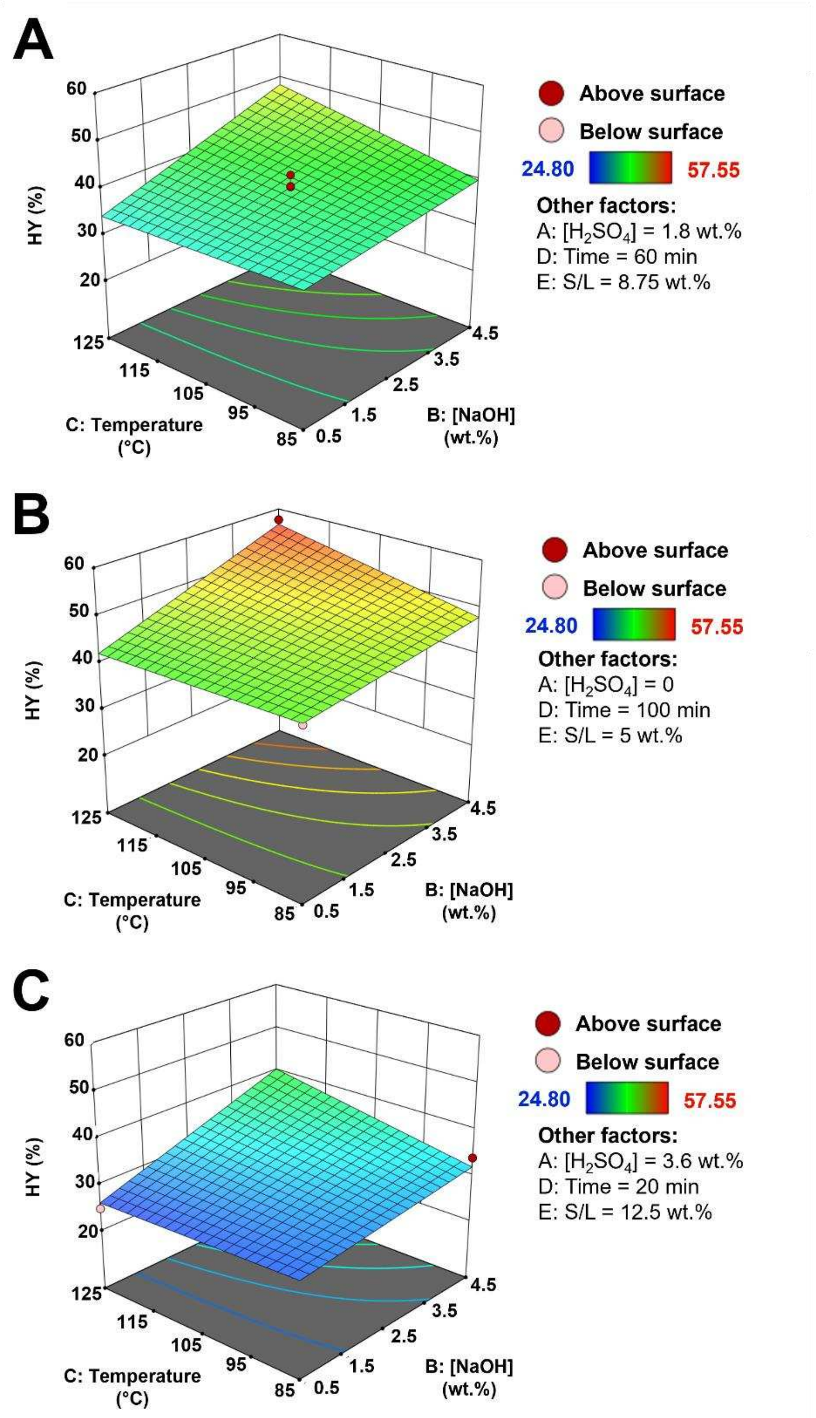

Figure S1. Response surface (HY after 12 h of enzymatic hydrolysis) of [NaOH] and Temperature for HY in rice straw samples: A: with all the other factors kept at their center points ([H_2SO_4] = 1.8 wt.%, Time = 60 min, S/L = 8.75 wt.%); B: under the conditions that resulted in the highest conversion ([H_2SO_4] = 0, Time = 100 min; S/L = 5 wt.%); and C:

under the conditions that resulted in lowest conversion ([H_2SO_4] = 3.6 wt.%, Time = 20 min, S/L = 12.5 wt.%).

Kinetic assay of enzymatic hydrolysis

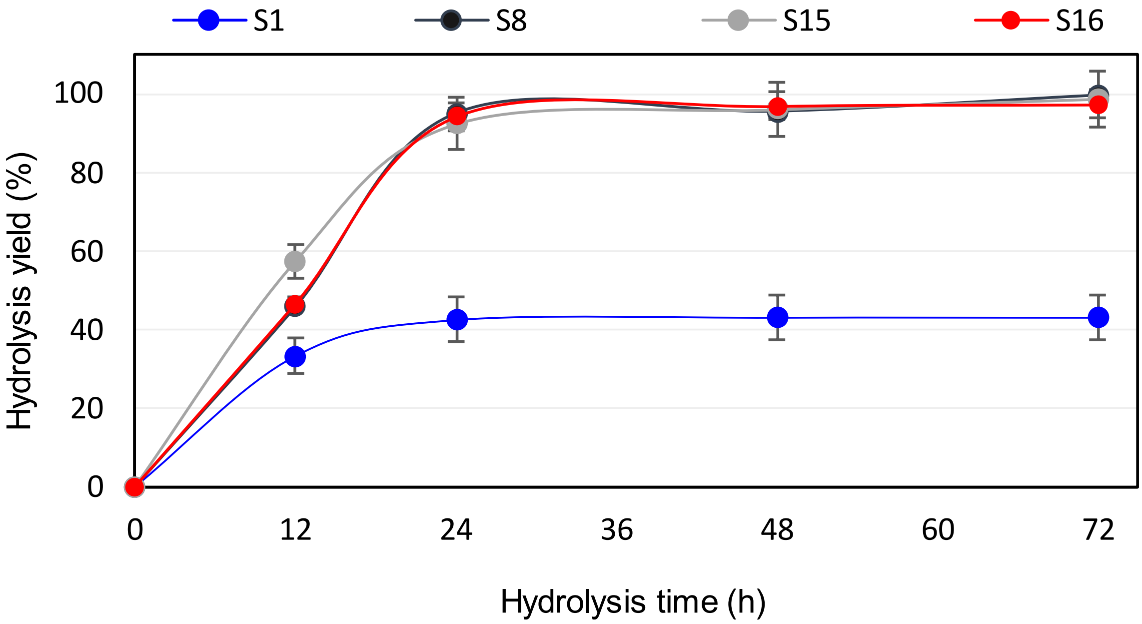

Figure S2. Hydrolysis yields (%) as a function of hydrolysis time (0 to 72h) for rice straw samples S1, S8, S15, and S16.

Chemical composition of rice husks *in natura* (raw material)

Table S3. Percentage of cellulose, lignin, hemicellulose, and ash from rice straw samples.

Sample	**Cellulose** (%)	**Lignin** (%)	**Hemicellulose** (%)	**Ash** (%)	**Extractives** (%)	**Total**
SIN	36.9 ± 0.0	12.9 ± 0.4	24.9 ± 0.4	7.7 ± 0.2	17.4 ± 0.6	100.0 ± 1.0
SH1	59.3 ± 0.1	22.4 ± 0.5	8.8 ± 0.2	10.9 ± 0.1	-	101.2 ± 0.1
SH2	55.1 ± 0.9	24.5 ± 1.1	6.1 ± 0.1	10.3 ± 0.3	-	96.0 ± 1.8
S1	40.3 ± 0.6	15.6 ± 0.7	35.6 ± 0.3	8.7 ± 0.4	-	100.2 ± 0.5
S2	78.8 ± 0.7	12.4 ± 0.4	5.9 ± 0.9	1.3 ± 0.0	-	98.3 ± 1.9
S3	67.0 ± 0.8	9.2 ± 0.1	18.0 ± 0.2	2.9 ± 0.1	-	97.2 ± 1.0
S4	85.8 ± 0.0	6.6 ± 0.4	4.2 ± 0.1	0.7 ± 0.0	-	97.3 ± 0.2
S5	57.4 ± 1.3	8.7 ± 0.5	30.7 ± 1.0	3.6 ± 0.1	-	100.3 ± 0.9
S6	72.4 ± 1.0	11.5 ± 0.2	4.1 ± 0.4	11.2 ± 0.2	-	99.1 ± 0.3
S7	69.4 ± 0.9	4.2 ± 0.1	17.7 ± 0.4	4.5 ± 0.3	-	95.9 ± 0.3
S8	90.3 ± 1.6	2.9 ± 0.2	2.6 ± 0.2	1.4 ± 0.1	-	97.2 ± 0.1
S9	48.9 ± 1.3	13.7 ± 0.4	32.7 ± 0.9	3.5 ± 0.1	-	98.8 ± 0.7
S10	64.8 ± 0.2	13.3 ± 0.1	7.4 ± 0.5	11.2 ± 0.1	-	96.7 ± 0.9
S11	66.5 ± 1.7	8.5 ± 0.1	19.5 ± 0.0	3.3 ± 0.4	-	97.8 ± 1.5
S12	81.4 ± 0.1	9.2 ± 0.1	3.5 ± 0.5	1.0 ± 0.0	-	95.1 ± 0.2
S13	46.6 ± 1.8	16.0 ± 0.3	28.0 ± 1.4	9.0 ± 0.1	-	99.6 ± 2.8
S14	79.4 ± 0.1	9.8 ± 0.0	2.2 ± 0.2	7.7 ± 0.3	-	99.1 ± 0.6
S15	75.6 ± 0.6	4.0 ± 0.0	14.1 ± 0.7	5.0 ± 0.1	-	98.7 ± 0.2
S16	88.7 ± 0.4	3.7 ± 0.0	2.4 ± 0.6	1.5 ± 0.2	-	96.3 ± 0.3
S17	87.7 ± 0.1	4.7 ± 0.9	3.4 ± 0.4	1.1 ± 0.1	-	96.9 ± 0.3
S18	87.9 ± 0.2	5.5 ± 0.4	3.4 ± 0.4	0.9 ± 0.0	-	97.7 ± 0.6
S19	87.9 ± 0.4	4.9 ± 0.0	3.0 ± 0.1	1.2 ± 0.0	-	97.0 ± 0.3
S20	87.8 ± 0.1	5.1 ± 0.3	3.4 ± 0.3	1.0 ± 0.1	-	97.3 ± 0.4
S21	87.9 ± 0.3	5.2 ± 0.2	3.2 ± 0.1	1.1 ± 0.1	-	97.4 ± 0.4

Correlation between saccharification results and biomass chemical composition

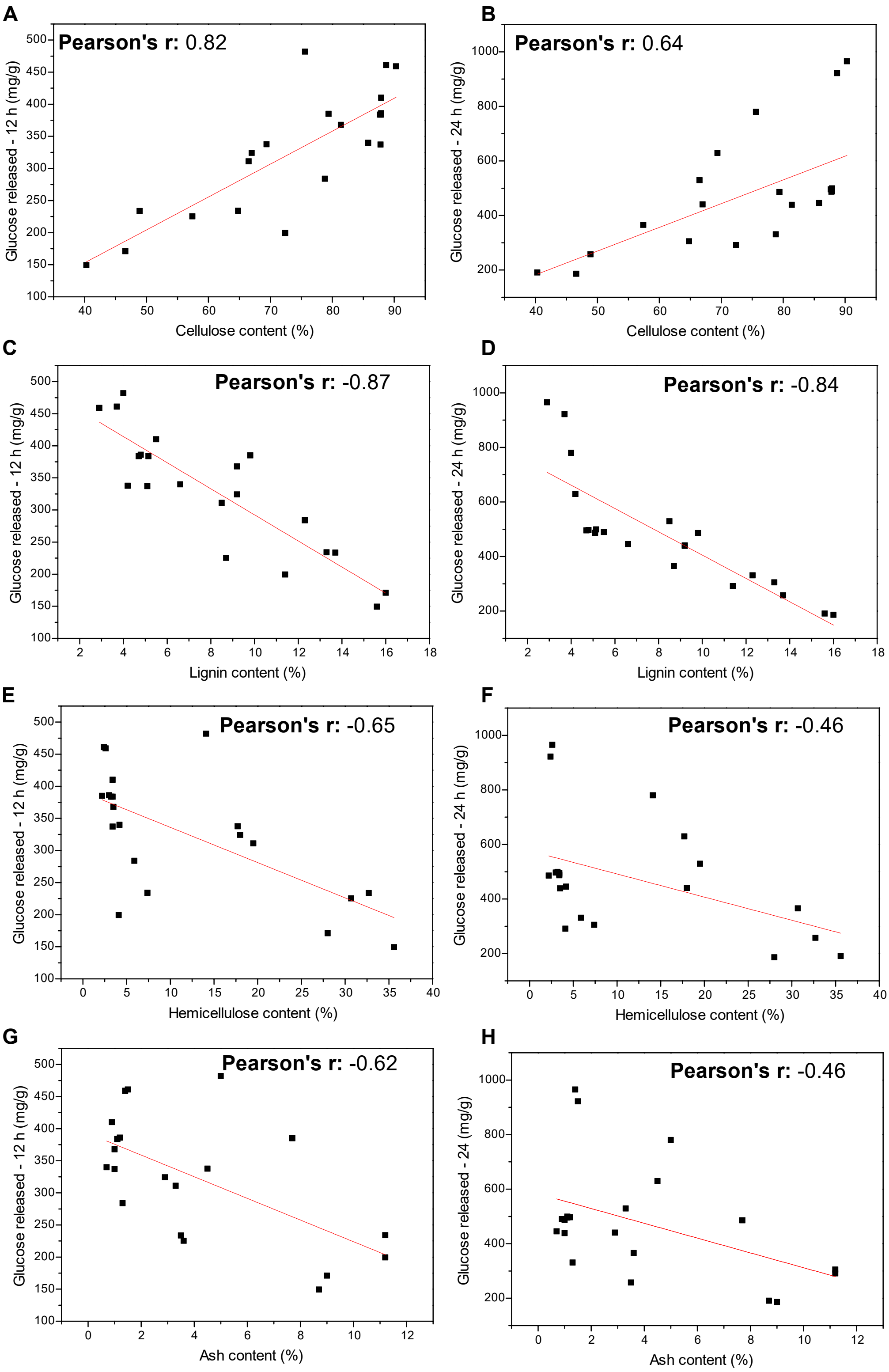

Figure S3. Correlation between the amount of glucose released by enzymatic hydrolysis of rice straw samples after acid-alkaline treatment (FFD 2^{5-1}) and: A) Cellulose after 12 h; B) Cellulose after 24 h; C) Lignin after 12 h; D) Lignin after 24 h; E) Hemicellulose after 12 h; F) Hemicellulose after 24 h; G) Ashes after 12 h; H) Ashes after 24 h.

Lignin extracted by alkaline and acid-alkaline treatments

Table S4 describes the quantity of lignin removed by acid-alkaline or only alkaline treatments. The lines highlighted in green indicate the conditions in which the acid step was carried out, while the lines highlighted in green indicate the central point. Percentual values were calculated based on the lignin composition determined for each sample (Table S3) and the remaining mass in solid fraction after each treatment.

Table S4. Quantity of lignin extracted by alkaline and acid-alkaline treatments. Green lines indicate the central point

Sample	Lignin extracted (total) (%)	Lignin extracted by alkaline step (%)
1	5	5
2	69	62
3	69	69
4	85	78
5	63	63
6	71	64
7	89	89
8	95	88
9	32	32
10	61	54
11	74	74
12	81	74
13	11	11
14	79	72
15	90	90
16	94	87
17	91	77
18	89	75
19	91	77
20	90	76
21	89	75

X-Ray Diffraction (XRD) and crystallinity index (CrI)

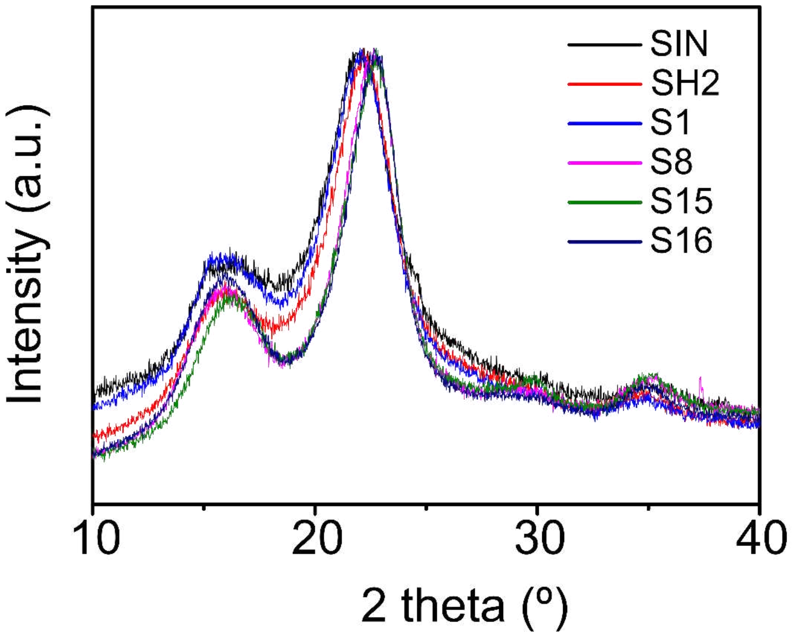

Figure S4. XRD analysis of selected samples.

The Crystallinity Index (CrI) was calculated based on the peak height method, as follows:

$$CrI = \frac{I_{002} - I_{am}}{I_{002}}$$

Where: I_{002} is the intensity of the crystalline peak and the I_{am} is the intensity of the amorphous area [1].

Table S5. Crystallinity index (CrI)

Sample	CrI (%)
SIN	46
SH2	54
S1	52
S8	64
S15	63
S16	63

Quantity of glucose released starting from 100 g of raw biomass after pretreatments and enzymatic hydrolysis

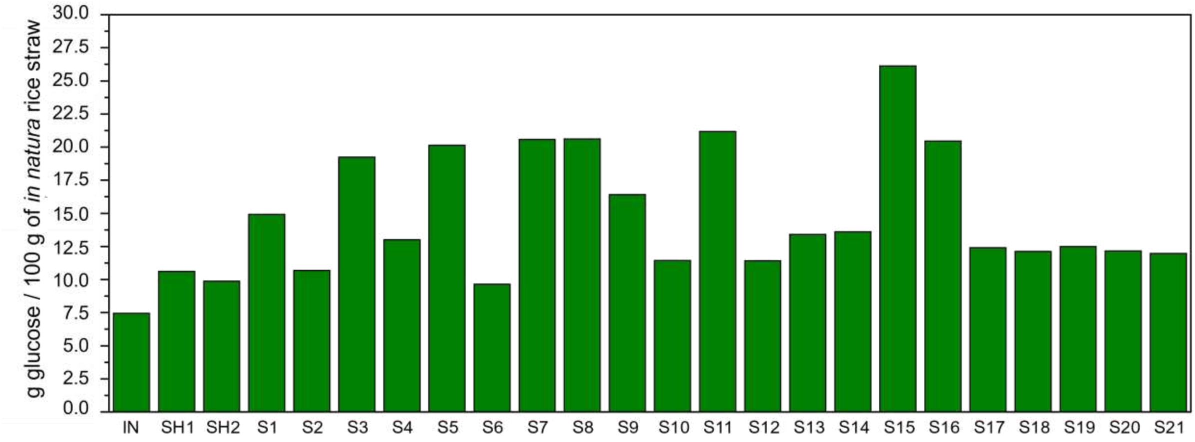

Figure S5. Glucose mass released from 100 g of raw rice straw after pretreatments and enzymatic hydrolysis (24 h).

Proposal of routes for rice straw processing

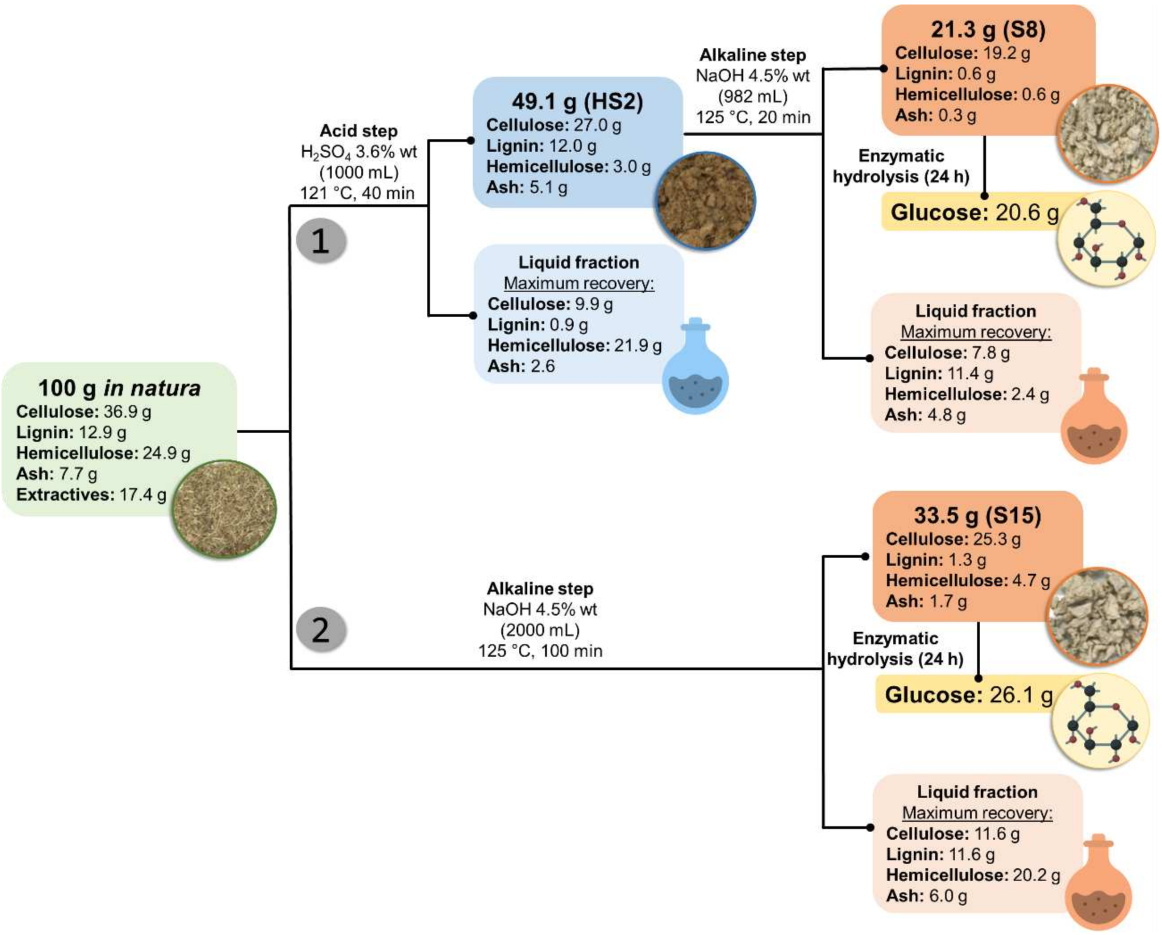

Figure S6. Summary of the processes to produce glucose from rice straw considering the two potential routes: 1) using acid-alkaline pretreatments to fractionate hemicellulose and lignin in different streams and 2) only alkaline pretreatment. Note that values described in “Liquid fraction” estimates the maximum quantity of each component that can be recovery based on the mass balance of the solid substrates, considering pretreatment yields and chemical compositions.

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GENERAL CONCLUSIONS

The results obtained in this work confirmed that pretreatment methods with well-known effects for one biomass can have different effects on others. Rice husks, undergoing the same Experimental Design of sequential acid-alkaline pretreatments as straw, achieved hydrolysis yields much lower (approximately 16%, after 72 h of enzymatic hydrolysis) than straw (more than 92%, after only 24 h of enzymatic hydrolysis).

Furthermore, the hydrolysis yields for rice straw obtained here were higher than the values for corn straw and sugar cane bagasse under acid-alkaline pretreatments using conditions similar to those used in this work, as previously reported in the literature. Therefore, it is concluded that rice straw has a great potential to be used in the production of second-generation ethanol. As for the rice husks, although the hydrolysis yields were low, the use of the silica removed in the alkaline pretreatment stage (which was recovered in this work with high purity and high yield) can contribute to the viability of the process as a whole.

The correlations between chemical and morphological changes obtained in this work should contribute to a better understanding of the relevant parameters for the use of rice straw in the production of biofuels and chemical products. Finally, future work could focus on optimizing different pre-treatments for specific biomasses and also on ways to fully utilize their components with a biorefinery approach.

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ANNEX

Regarding the article presented in chapter 2 "IMPROVED HYDROLYSIS YIELD AND SILICA RECOVERY BY DESIGN OF EXPERIMENTS APPLIED TO ACID-ALKALI PRETREATMENT IN RICE HUSKS", according to Elsevier Copyright Policy[1], authorization is not required for its reproduction in a thesis or dissertation written by the author of the article. See the image below:

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

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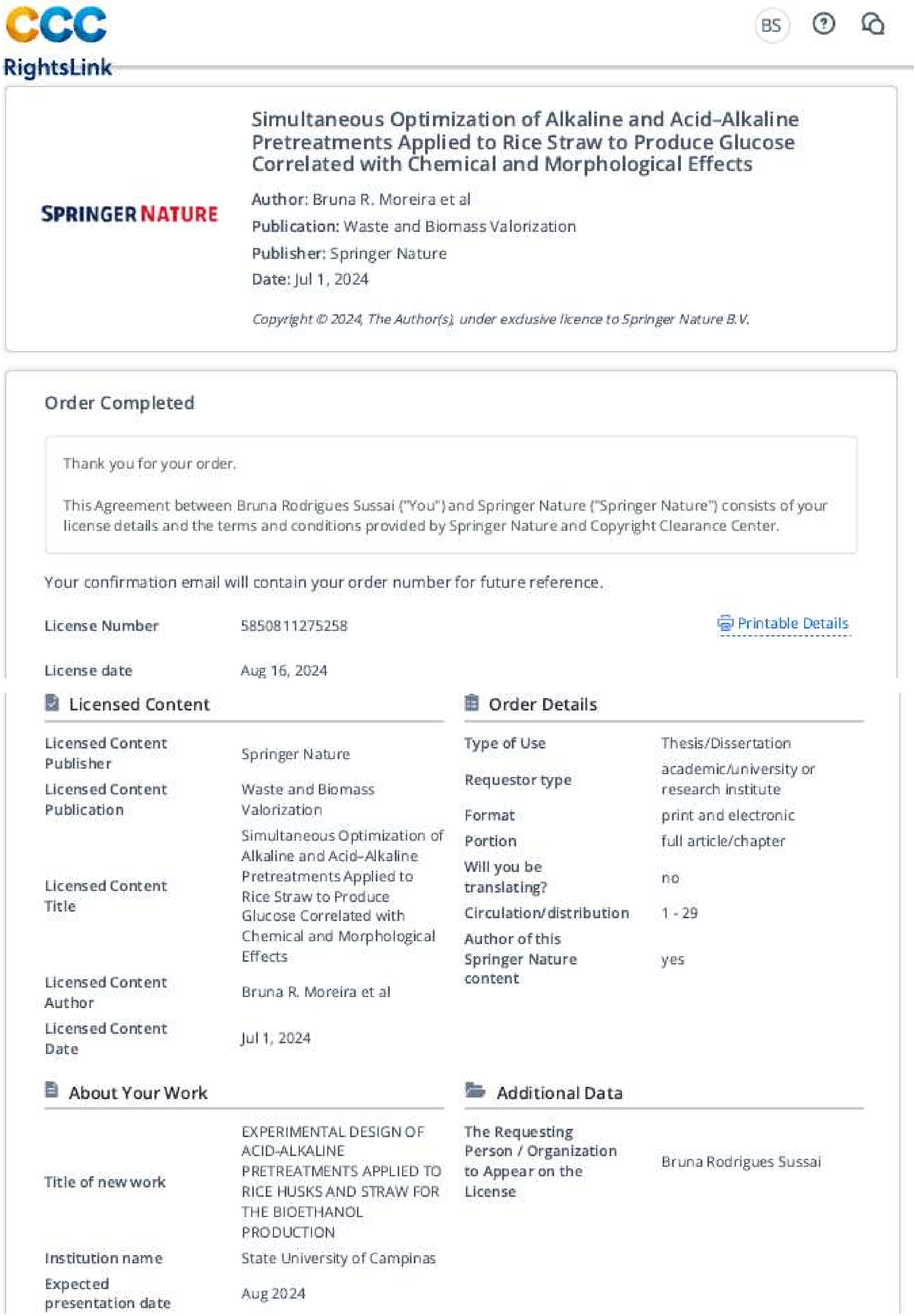

Simultaneous Optimization of Alkaline and Acid-Alkaline Pretreatments Applied to Rice Straw to Produce Glucose Correlated with Chemical and Morphological Effects

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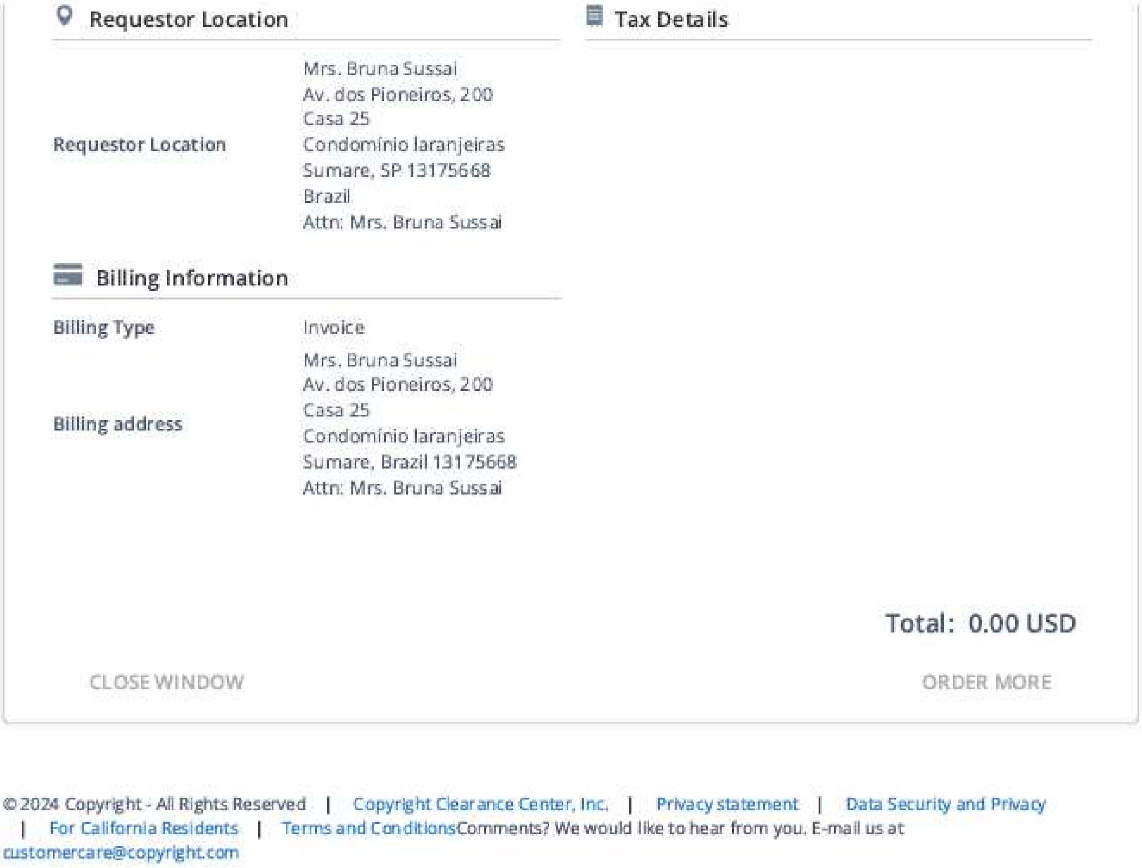

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