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Second Generation Bioethanol Production from Soybean Hulls Pretreated with Imidazole as a New Solvent

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Abstract: Soybean hulls (SH) are the main industrial waste from soybean processing, representing 5–8% of the whole grain. Imidazole was employed for the hydrothermal pretreatment of SH and further bioethanol production. Different pretreatment temperatures (120 and 180 °C) and times (1 and 3 h) were tested. Lignin removal and glucose yield were significantly influenced by temperature. After 48 h of enzymatic hydrolysis of imidazole-treated SH (120 °C, 1 h), 32.7 g/L of glucose and 9.4 g/L of xylose were obtained. A maximum bioethanol yield of 78.9% was reached after 12 h of fermentation by *Saccharomyces cerevisiae* using SH enzymatic hydrolysate. Imidazole appears to be a potential alternative to pretreat lignocellulosic wastes such as SH for the production of second-generation biofuels and other biomolecules.

Keywords: soybean hull; bioethanol; imidazole; enzymatic hydrolysis; pretreatment

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1. Introduction

Soybean (*Glycine max* (L.) Merrill) is one of the most produced crops worldwide, with a production of 371.7 million tons in 2021. Brazil is the leading soybean producer, followed by the Unites States and Argentina, with a production of 134.9, 120.7 and 46.2 million tons in 2021, respectively [1]. This crop can be used in several industries, including animal nutrition, food production, and the biofuel sector, where considerable potential has been observed due to its promising results [2].

The processing of soybean grains results in two main products: soybean meal (used for human and animal food) and soybean oil (used for human consumption and biodiesel production) [3]. Thus, soybean processing generates different wastes, such as hulls, molasses, and okara. Soybean hulls (SH) are the main residue of soybean processing, representing about 5–8% of the whole grain dry matter, and containing about 86% of complex carbohydrates [4].

Due to its renewable nature and global abundance, lignocellulosic biomass shows a promising potential for low cost production of medium to high value biomolecules [5]. However, these materials have a complex and rigid structure that makes them resistant to both chemical and biological degradation. Therefore, the pretreatment step is very important: to decrease biomass recalcitrance, which depends on the type and source of the material; to facilitate the access of biocatalysts to obtain fermentable sugar from polysaccharides; and increase the accessibility to enzymes which convert cellulose into glucose for further fermentation [6].

Several studies have analyzed different pretreatment techniques (physical, chemical, physicochemical, and biological methods) to recover fermentable sugars from lignocellulosic biomass [7]. Some of the reported methods involve thermo-mechanical extrusion [8],

dilute acid [9], and alkaline pretreatment [10]. However, a major disadvantage of conventional acid or alkaline pretreatment is that they cause corrosion in the equipment used in these operations, hence, corrosion resistant reactors are often expensive [11]. Other disadvantages include the cost to neutralize the pretreated material that generates solid salts, which are difficult to remove, and toxic residues with a high environmental impact. Furthermore, the dilute acid pretreatment produces some inhibitors, which hampers the enzymatic hydrolysis of cellulose [12].

The selection of a pretreatment method depends on the chemical composition and physical nature of the material. For this reason, an ideal pretreatment must: consider the sugar loss from pretreated fractions; reduce the production of inhibitory compounds for enzymatic hydrolysis and fermentation; minimize enzyme loading for efficient hydrolysis; decrease energy consumption; and permit the delignification and recovery of other promising compounds [6]. Lignocellulosic residues have considerable potential for the production of biofuels and other biomolecules. The use of these agro-industrial wastes in second generation (2G) bioethanol production could contribute to the decrease of up to 85% of greenhouse gas emissions, and reduce dependence on fossil fuels [13]. Furthermore, the choice of green solvents reduces or eliminates the use and production of hazardous substances, minimizes health and environmental damages, and moves towards cleaner and sustainable production from renewable sources [14].

Imidazole is an aromatic heterocycle compound used as a solvent in the pretreatment of different lignocellulosic biomasses, such as sugarcane bagasse [15], elephant grass [16] and wheat straw [17]. Imidazole has a low toxicity and high boiling point (non-flammability), and is considered a less aggressive solvent for humans and the environment [18]. It is a fairly stable compound with a low vapor pressure that facilitates its manipulation [17].

The objective of this work was to evaluate the effects of SH pretreatment with imidazole on the release efficiency of fermentable sugars through enzymatic hydrolysis with subsequent bioethanol production applying a biorefinery approach.

2. Materials and Methods

2.1. Lignocellulosic Biomass

Soybean hulls were acquired from Imcopa, located in Araucaria, Paraná, Brazil. The biomass drying process was performed in an air-circulating oven at 65 °C for 48 h, followed by grinding in a knife mill (Marconi, MA580/E). The SH particle size was between ASTM No. 20 (0.85 mm) and ASTM No. 45 sieves (0.35 mm) for all experiments. Imidazole 99%, ethanol 96% (*v/v*), HCl P.A. (37%) and H_2SO_4 P.A. (95–98%) were employed. Sugars and standard chemicals were: D-(+)-cellobiose, glucose (>99%), D-(+)-xylose (>99%), D-(-)-arabinose (>99%), and acetic acid (>99%) from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Chemical Composition of Untreated and Imidazole-Pretreated Soybean Hull

The physicochemical characteristics of untreated and imidazole-pretreated SH, such as solid content, ashes, extractives, acid insoluble lignin (AIL), acid soluble lignin (ASL) and structural carbohydrates, were analyzed according to the National Renewable Energy Laboratory (NREL) protocols and instructions [19–22]. Samples were filtered through 0.22 µm pore size filters (Millipore Corp., Billerica, MA, USA) and evaluated in a high-performance liquid chromatograph (HPLC) equipped with an Aminex Bio-Rad HPX-87H column at 60 °C, aqueous H_2SO_4 (5 mM) as mobile phase, a flow rate of 0.6 mL/min and a reflection index (RI) detector. The injection volumes were 10 µL.

2.3. Imidazole Pretreatment of Soybean Hulls

SH imidazole pretreatment followed the procedure described by Morais et al. (2016) [17] with modifications. The pretreatment reaction was carried out using 5 g of dried biomass with an imidazole/biomass ratio of 9 (*w/w*) in a 150 mL stainless steel reactor with mechanical stirring (Parr, Moline, IL, USA). The reactions were performed at 120 and 180 °C with 1

and 3 h of reaction time. After each pretreatment, samples were washed with 135 mL of deionized water and agitated for 1 h. Precipitated solid fractions (cellulose-rich fractions), which were mainly composed of polysaccharides, were vacuum filtrated and washed with 96% (*v/v*) ethanol. After each pretreatment, the recovered solid fractions were dried at 45 °C in an air-circulating oven and further submitted to enzymatic hydrolysis. Solid yield represents the weight recovered after imidazole pretreatment and determined according to Equation (1).

$$Solid\ yield\ (\%) = \left(\frac{Biomass\ weight_{pretreated}}{Biomass\ weight_{untreated\ dry}} \right) \times 100 \quad (1)$$

2.4. Enzymatic Digestibility

After imidazole pretreatment, the recovered cellulose-rich fractions were subjected to enzymatic hydrolysis. Experiments were performed using 125 mL Erlenmeyer flasks with 5% (*w/v*) biomass (dry weight) in 12.5 mL of 50 mM sodium citrate buffer solution (pH 4.8), 0.02% (*w/v*) of sodium azide and 1/9 ratio of Cellic HTec2®/Cellic CTec2® (*v/v*) from Novozymes–Araucaria, Brazil. Cellulase and xylanases were used at the enzyme loading of 20 FPU and 1206.7 U per gram of solid biomass, respectively. Samples were incubated at 50 °C for 48 h in an orbital shaker at 150 rpm. Sample aliquots were heated up to ~95 °C for 10 min in a water bath to stop enzymatic hydrolysis. Samples were then filtered through 0.22 µm cellulose acetate membrane syringe filters followed by HPLC analysis. All assays were performed twice or more. The glucose and xylose yield after enzymatic hydrolysis of untreated and pretreated SH were determined according to Equations (2) and (3):

$$Glucose\ yield\ (\%\ w/w) = \left(\frac{(GLU) \times V}{m_{biomass} \times F_g \times 1.11} \right) \times 100 \quad (2)$$

$$Xylose\ yield\ (\%\ w/w) = \left(\frac{(XYL) \times V}{m_{biomass} \times F_x \times 1.13} \right) \times 100 \quad (3)$$

where (*GLU*) and (*XYL*) are the concentrations of glucose and xylose (g/L) determined by HPLC, respectively; V is the volume (L) of hydrolysate; $m_{biomass}$ is the biomass dry weight (g); F_g and F_x are the glucan and xylan fraction in the biomass (%), respectively; 1.11 and 1.13 are the glucan to glucose and xylan to xylose conversion factor, respectively.

2.5. Bioethanol Production

The enzymatic hydrolysate of SH was employed in bioethanol production in 125 mL Erlenmeyer flasks with 5 g/L peptone, 5 g/L yeast extract, 2 g/L KH_2PO_4 and 1 g/L $MgSO_4$. The pH was settled to 5.5 in a final volume of 10 mL. The medium sterilization was performed at 121 °C for 15 min. For hydrolysate fermentation 5% *v/v* of 24 h old *Saccharomyces cerevisiae* inoculum was applied with an optical density (O.D) of 2.4 at 600 nm. The reactions occurred in an incubator at 35 °C with stirring at 150× *g*. Samples were withdrawn at 3, 6 and 12 h intervals for up to 48 h and centrifuged (10,000× *g*, 15 min, 25 °C). The chemical composition of the supernatant was determined by HPLC and the experiments were executed in duplicate. Bioethanol theoretical yield was calculated according to Equation (4):

$$Ethanol\ yield\ (\%) = \left(\frac{(bioethanol)}{0.511 \times (Glu_i - Glu_r)} \right) \times 100 \quad (4)$$

where 0.511 represents the conversion factor of glucose to bioethanol; (*bioethanol*) is the produced concentration of bioethanol, and (Glu_i) and (Glu_r) are initial and residual glucose concentration, respectively.

2.6. Fourier Transforming Infrared Spectroscopy (FTIR), X-ray Diffraction (XRD) and SCANNING Electron Microscopy (SEM)

For FTIR analysis, 2 mg of dried samples were mixed with 100 mg KBr to form discs. The spectra were obtained between 4000 and 400 cm^{-1} with a 4 cm^{-1} resolution in a Vertex 70 spectrometer (Bruker, Billerica, MA, USA). The absorption bands at 1430 and 896 cm^{-1} were analyzed for cellulose crystallinity index calculation according to Equation (5) [17].

$$LOI = \frac{A_{1430}}{A_{898}} \quad (5)$$

where *LOI* represents the lateral order index and *A* the absorbance value of the corresponding band.

Biomass crystallinity was measured by XRD using an X-ray diffractometer 700 Maxima (Shimadzu, Columbia, MD, USA) operated at 40 kV and 20 mA and Ni-filtered copper radiation (CuKα, λ = 1.5418 Å). Samples were scanned in the 2θ range of 5–30° at a rate of 2° min^{-1}. The crystallinity index (*CrI*) was calculated according to Equation (6).

$$CrI = \left(\frac{(I_c - I_a)}{I_c}\right) \times 100 \quad (6)$$

where I_c is the intensity peak at maximum of 2θ between 22° and 23° and I_a is the intensity peak at minimum of 2θ around 18°.

SEM analyses were performed utilizing dried biomass samples, which were mounted on supports using copper foil tape with double-sided adhesive. The surfaces of the samples were sputter-coated with gold layer, and in a scanning electron acceleration of 15 kV. The scanning and acquisition of microphotographs were carried out using a Jeol JSM 6360-LV (Oxford Instruments, Abingdon, UK).

3. Results and Discussion

3.1. Effect of Imidazole Pretreatment on Soybean Hull's Composition

Lignocellulosic biomass consists fundamentally of cellulose, hemicellulose, lignin and small amounts of ashes and extractives (waxes, fatty acids, essential oils, terpenes, aromatic compounds and residual sucrose) [23]. Untreated SH had a composition of 32.5% *w/w* glucan, 12.2% *w/w* xylan, 1.8% *w/w* lignin, 9.2% *w/w* moisture, 4.3% *w/w* ashes, 4.0% *w/w* ethanol extractives,13.5% *w/w* water extractives, and solid yield represents the weight recovered after imidazole pretreatment (Table 1). Variation in the chemical composition of SH is common and usually depends on the cultivation characteristics, type of grain processing, genetic factors, and different soybean processing industries [23].

Table 1. Chemical composition of untreated and imidazole-pretreated SH.

Composition (%)	Untreated [a]	120–1	120–3	180–1	180–3
Glucan	32.5	57.1	57.9	69.9	68.3
Xylan	12.3	16.3	17.4	21.3	20.6
Anhydroarabinose	3.2	2.7	2.0	0.0	0.0
Acetyl groups	0.0	0.0	0.0	0.0	0.0
Acid-soluble lignin	1.1	1.4	1.5	1.4	1.6
Acid-insoluble lignin	0.7	1.3	0.7	0.5	0.3
Ash	4.3	-	-	-	-
Extractives-Water	13.5	-	-	-	-
Extractives-EtOH	4.0	-	-	-	-
Solid Yield (%)	-	61.38	59.23	44.48	44.14

[a] Dried biomass-based.

The removal of lignin and hemicellulose has a direct correlation with cellulose digestibility. Lignin is known to be a barrier to efficient lignocellulosic biomass bioconversion to sugars and, consequently, their fermentation to biofuels [6]. As it is possible to observe,

SH present a low lignin content compared to other agro-industrial residues, which could facilitate the saccharification process for the production of biochemicals [24].

It is possible to observe in detail the composition of imidazole-pretreated SH samples in Table 1. The temperature significantly influenced the recovery of solid yields. At the lower temperature (120 °C), the solid recovery yield was around 60% *w/w* while at 180 °C, it was 44% *w/w*, showing higher biomass loss. The solid recovery yield is directly influenced by the pretreatment severity, mainly because the higher conditions dissolve the extractive fraction and part of the amorphous fraction (hemicellulose and lignin). The same behavior was previously reported when other pretreatment methods and biomasses, such as sugarcane bagasse [15,25], wheat straw [24], and others, were employed.

Furthermore, after pretreatment at 180 °C, the obtained cellulose-rich fraction, presented a glucan content of approximately 70% *w/w*, which corresponded to 2.1- and 1.7-times higher than untreated SH (32.5% *w/w*) and SH pretreated at 120 °C (57.9% *w/w*), respectively (Figure 1). On the other hand, the processing time (1 and 3 h) exerted negligible effect. In all probability this was due to the chemical composition and structure of SH, as well as the effect of the imidazole, which facilitated the quick dissociation of the lignocellulosic fractions. Therefore, it is possible to suggest that the disaggregation of lignocellulosic biomass by imidazole facilitated the recovery of cellulose-rich fractions with minimal glucose loss. Other authors have also observed the dominant effect of temperature in wheat straw treatment with imidazole [17]. As the reaction temperature increased from 110 °C to 170 °C using the same reaction time, the glucan content increased. In addition, cotton residue treated with imidazole confirmed that temperature is an important factor influencing the cellulose content [26]. Likewise, the cellulose content increased with the severity of the reaction that was also shown by other pretreatment methods, such as sequential hydrothermal-imidazole pretreatment of elephant grass [16], ionic liquid ([bmin][Ac]) pretreatment of SH [27], and alkaline pretreatment of SH [28]. Therefore, to be a viable process within the context of lignocellulosic material reuse, it is important that the biomass choice and treatment type are allied to lower time and energy used in the process, such as was observed for SH imidazole pretreatment.

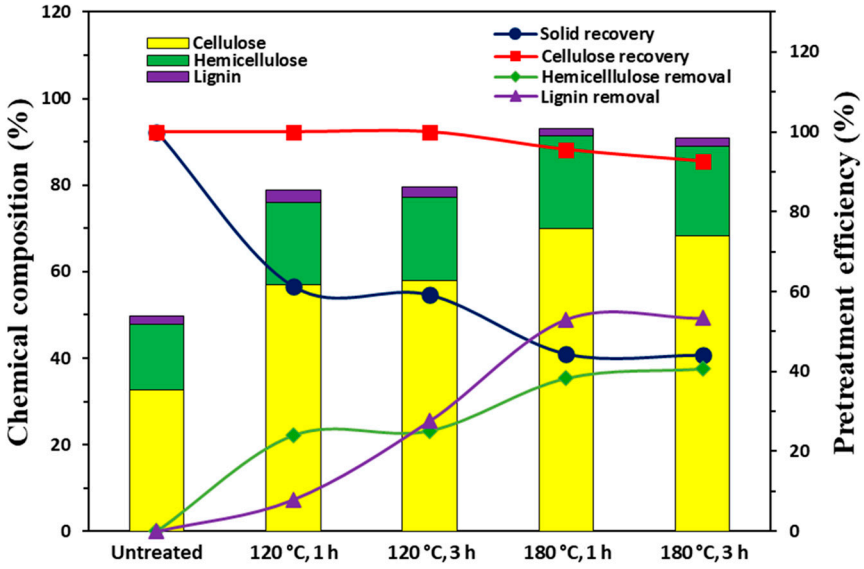

Figure 1. Chemical composition and efficiency of SH imidazole pretreatment.

Comparable to the glucan content, the xylan content increased after imidazole pretreatment, reaching up to ~20% *w/w* compared with untreated SH (12.3% *w/w*) (Figure 1). Unlike cellulose, hemicellulose is easily released under alkaline conditions from lignocellulosic materials [29]. Imidazole has alkaline characteristics, and for this reason the hemicellulose removal increases significantly with more severe conditions, such as high temperature

(Figure 1). Additionally, it was observed that the increase in pretreatment temperature promoted higher lignin removal (almost 50%). This could be due to partial degradation through dissolution and depolymerization of lignin at higher temperatures and longer reaction times [30].

The imidazole pretreatment has been shown to be effective for biomass delignification, which occurs by breaking the ester bonds cross-linkage in lignin and hemicellulose, and consequently increasing its porosity [31]. Higher temperatures (180 °C) promoted higher lignin removal, recording 53.03% and 53.45%, after 1 and 3 h of pretreatment time, respectively (Figure 1). Due to the planar structure of imidazole, it easily interacts with the aromatic structure of lignin, promoting the dissolution of lignin and further depolymerization [32]. The use of imidazole promotes the recovery of lignin-rich fractions that could be utilized to produce valuable biobased materials and biochemicals, such as a potential resource for vinyl phenolics production [33].

3.2. Effect of Imidazole Pretreatment on the Morphology of Soybean Hull

The morphological profiles of untreated and imidazole-pretreated SH were analyzed by SEM (Figure 2). Untreated SH presented a smooth and compact structure (typical of lignocellulosic material) and presented three main layers: the palisade layer (outer surface of SH), the hourglass layer (middle layer), and the parenchyma tissue (inner surface of SH) [34,35]. Normally, the native state of the lignocellulosic material presents waxes and other encrusting substances that form a thick and smooth outer layer in the raw residue [36]. These were affected by the imidazole treatment, along with palisade layer. The degradation and disorganization of the pretreated SH resulted in the loss of biomass structure according to the severity of the treatment. Some changes were observed in the morphology characteristics, as well as in crystallinity, which was increased, suggesting that imidazole would have the ability to remove part of the amorphous fraction of lignocellulosic biomass.

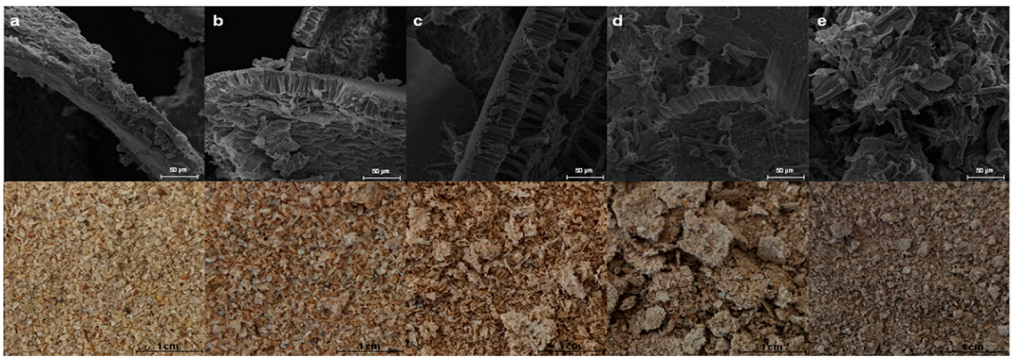

Figure 2. SEM images of SH samples: (**a**) untreated; (**b**) 120 °C 1 h; (**c**) 120 °C 3 h; (**d**) 180 °C 1 h, and (**e**) 180 °C 3 h.

Structural changes were observed in all pretreated materials, such as size reduction and destruction of fibers, which increased at higher temperature and processing time. The biomass treated at 120 °C showed a rougher surface fiber. This could indicate a partial removal of the outer non-cellulosic layer composed of hemicellulose and lignin [35]. The fibers seemed more intact after 1 h of imidazole pretreatment than those after 3 h of processing time. At the higher temperature (180 °C) and processing time, a significant modification of the fibers was observed, presenting a much less compact and disrupted structure, mainly after 3 h. In fact, the severe degree of imidazole pretreatment promoted the disorganization of fibers with more aggressive conditions, which may have generated an increase in the surface area of biomass facilitating the enzymatic attack that led to a better release of fermentable sugars [15].

3.3. Effect of Imidazole Pretreatment on Crystallinity of Soybean Hull

The CrI of both untreated and pretreated SH were analyzed by XRD patterns (Figure 3A). Overall, semicrystalline materials, such as lignocellulosic biomass, are composed of amorphous broad hump and crystalline peaks [34].

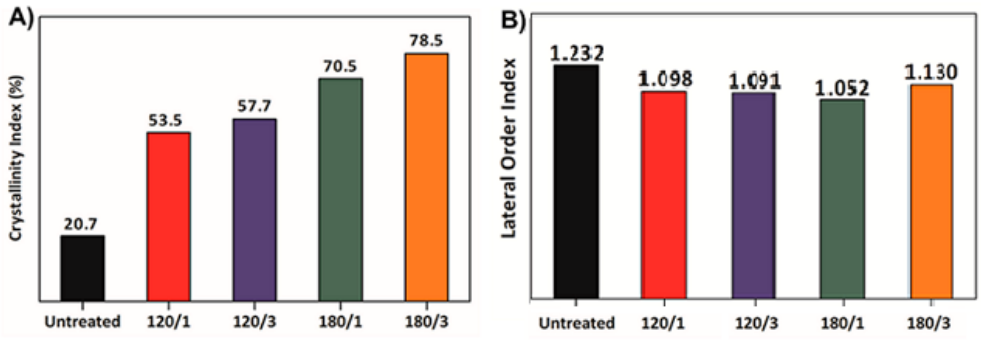

Figure 3. Crystallinity index (**A**) and Lateral Order Index (**B**) of untreated and imidazole pretreated samples.

The CrI of untreated SH was 20.7% and this value increased after pretreatment with imidazole, presenting up to 78.5% of CrI under the most severe conditions (180 °C for 3 h). The increase in CrI values occurred due to the partial removal of disordered cellulose, hemicellulose, lignin (amorphous fraction), and the change of cellulose crystallinity (Figure S1). However, some researchers have shown that imidazole does not interfere directly with cellulose crystallinity [17]. The CrI value is also sensitive to cellulose crystal size and preferential orientation (texture) of the cellulose crystallites [37,38]. All analyzed samples exhibited peaks at approximately 2θ = 22° and 34.2°, which are related to cellulose type I. Both peaks were sharper for imidazole-pretreated SH. A peak at 2θ = 15.7° was also observed in all pretreated samples, which was associated to cellulose type I [15,34]. Moreover, the XRD patterns suggest a strong influence of preferred orientation, which can be inferred from the varied proportions between the (004) peak (at ~35°) and the (200) peak (at ~22°) [37,38]. Compared to the other lignocellulosic biomass, SH presents wider cellulose crystallites, and these characteristics are noticed by the sharper (200) diffraction peak. In addition, wider crystallites cellulose is an indication of relatively pure and well aligned cellulose that are present in both the palisade and the hourglass layer from SH [39]. The amount of cellulose in pretreated SH increased from 57% *w/w* to 69% *w/w* after pretreatment at 120 °C and 180 °C, respectively. These results suggest that imidazole pretreatment could be effective for cellulose exposition, contributing to the increase in CrI values.

The lateral order index (LOI) was determined from FTIR patterns and used to characterize the untreated and imidazole-pretreated SH, as shown in Figure 3B. The spectroscopic analysis was used to investigate the structure of constituents and the chemical changes that occurred after pretreatment (Figure S2), while the LOI is related to the presence of cellulose crystalline [40].

The 1430 cm^{-1} peak refers to the CH_2 scissoring motion in cellulose, which is related to cellulose crystalline structure. It has high intensity in cellulose type I but it shifts and is very weak in cellulose type II and amorphous cellulose. On the other hand, the peak at 898 cm^{-1} refers to C–O–C stretching at β-1,4-glycosidic linkage. It becomes weak and broad in cellulose type I and intense and sharp in cellulose type II and amorphous cellulose [41]. The peaks at 1430, 1370, 1320, 1246, 1166, 1116, 1060 and 896 cm^{-1} are associated with cellulose in lignocellulosic materials [42].

The LOI was determined with peaks 898 and 1430 cm^{-1} to evaluate the conversion of cellulose I to cellulose II and amorphous cellulose [17]. The untreated biomass showed a slightly higher LOI (1.23) when compared to cellulose-rich fractions (LOI ranging from 1.09 to 1.13), and the reduction of these LOI values reflects a more disordered structure [43]. This pattern was also observed in the literature using imidazole treated wheat straw [17]. The

temperature and reaction time resulted in lower LOI values, which means a more disordered structure and the promotion of cellulose I conversion to cellulose II or amorphous cellulose [17]. Amorphous cellulose and cellulose II are more enzymatically hydrolysable than cellulose I, and both types of cellulose contribute to an increase in cellulose digestibility [44].

3.4. Enzymatic Hydrolysis of Untreated and Imidazole-Pretreated Soybean Hull

Glucose and xylose release profiles during enzymatic hydrolysis of pretreated SH at 120 °C and 180 °C by commercial enzymes (Figure 4) were similar, revealing the high susceptibility of SH to imidazole. After 48 h of enzymatic hydrolysis of imidazole treated SH at 120 °C for 1 and 3 h, 32.7 and 35.7 g/L of glucose and 9.4 and 10.1 g/L of xylose were obtained, respectively. In the same way, for the material, which was pretreated at 180 °C for 1 and 3 h, glucose concentrations of 37.7 and 36.0 g/L and xylose concentrations of 10.6 and 9.4 g/L were reached, respectively.

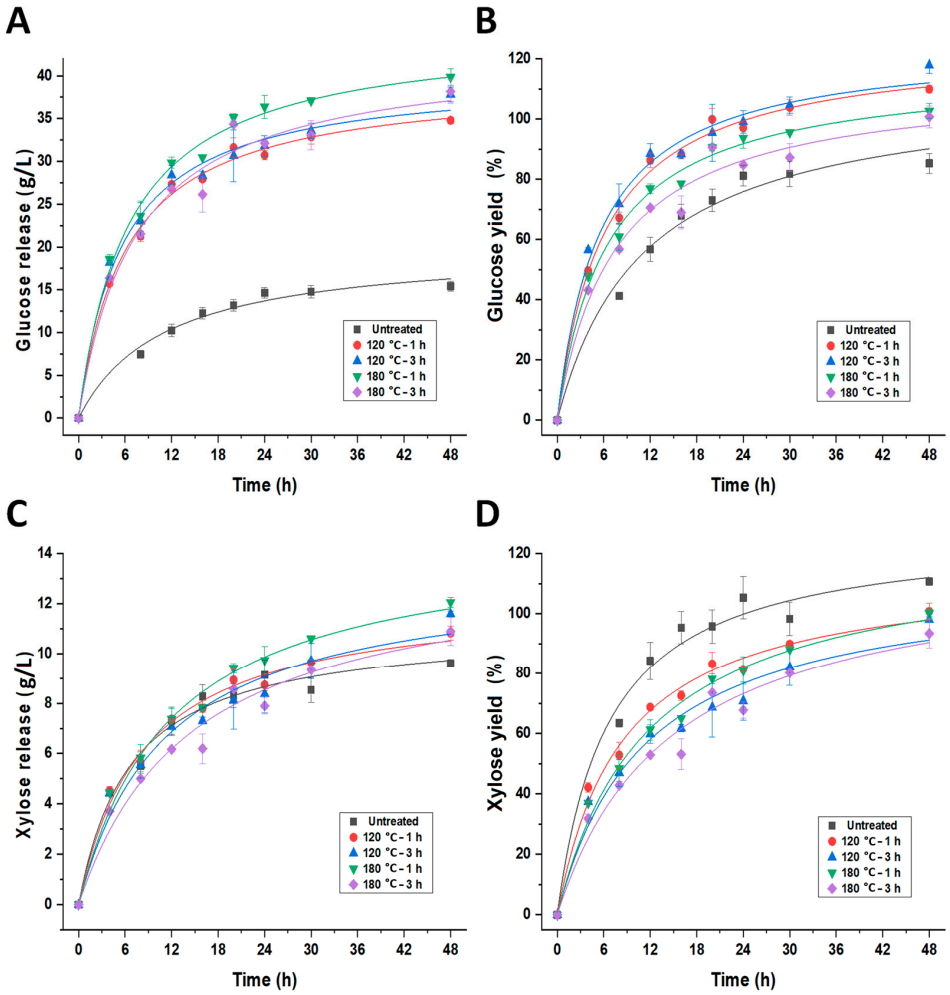

Figure 4. Enzymatic hydrolysis of untreated and imidazole-pretreated SH. (**A**): Glucose release; (**B**): Glucose yield; (**C**): Xylose release and (**D**): Xylose yield.

Glucose and xylose release profiles were approximately equal for all evaluated pretreatment conditions. The effectiveness of biomass delignification by imidazole favored the enzyme accessibility in the cellulose-rich fraction. Moreover, the LOI value showed that pretreatment promoted a low reduction of cellulose crystallinity, which also promoted a better enzymatic action. For the imidazole pretreated sugarcane bagasse, the increase in the severity of the reaction improved the enzymatic conversion to glucose [15]. This difference is likely related to biomass composition and structure.

When analyzing the concentration of glucose released during the saccharification process, an almost 3-fold increase in glucose (36 g/L) was observed when compared with untreated SH (12 g/L) (Figure 4A). The enzymatic hydrolysis yield of pretreated SH was over 95%, reaching 100% for imidazole treated samples at 120 °C (Figure 4B). Enzymatic hydrolysis of SH pretreated with ILs (ionic liquids) promoted a glucose yield of 91.7% [27]. However, there was no significant difference in hemicellulose conversion, present approximately 1.3 times more xylose released than in the untreated SH (Figure 4C), and after enzymatic hydrolysis the hemicellulose conversion was slightly higher for untreated SH than for pretreated SH (Figure 4D). In all likelihood, Xylose was released from the extractive fraction of untreated SH.

In a previous study, *Banana pseudostem* was pretreated with green liquor followed by saccharification with cellulase (20 FPU/g) over 48 h, achieving an enzymatic efficiency of 99% [45]. Soybean residue was pretreated with sulphuric acid, with further saccharification at 36 h reaching 92.7% of enzymatic efficiency [46]. Deep eutectic solvent was used in the pretreatment of *Eucalyptus camaldulensis*, and after 72 h of saccharification, using Cellic CTec^2 (15 FPU/g), the authors achieved 94.3% of efficiency [47]. The use of imidazole has also been tested for elephant grass [32] and cotton residue [26], at 135.6 °C for 308 min, and also at 140 °C for 120 min, respectively. This work presented 100% enzymatic efficiency, with a considerable performance of SH imidazole pretreatment as a previous saccharification step for lower process temperature and time, representing lower operation costs. In fact, imidazole pretreatment presents some advantages, not only for a better production of fermentable sugars, but also for minimum degradation of the cellulose in the recovered fractions. Moreover, the solubilization and separation of lignin may reduce cellulose loss due to the linkage between enzymes and lignin, which interferes in the interaction of cellulose and substrate, resulting in a decrease in the enzymatic hydrolysis efficiency [48]. Consequently, the removal of lignin promotes a better conversion of cellulose to glucose through enzymatic hydrolysis [32].

3.5. Bioethanol Production

Bioethanol production by *S. cerevisiae* was carried out using the SH enzymatic hydrolysate after imidazole-pretreatment (Figure 5). After 12 h of fermentation, the maximum bioethanol concentration and efficiency reached 12.9 g/L and 78.9%, respectively. Glucose consumption was over 95%. On the other hand, only 30% of xylose was consumed by the yeast.

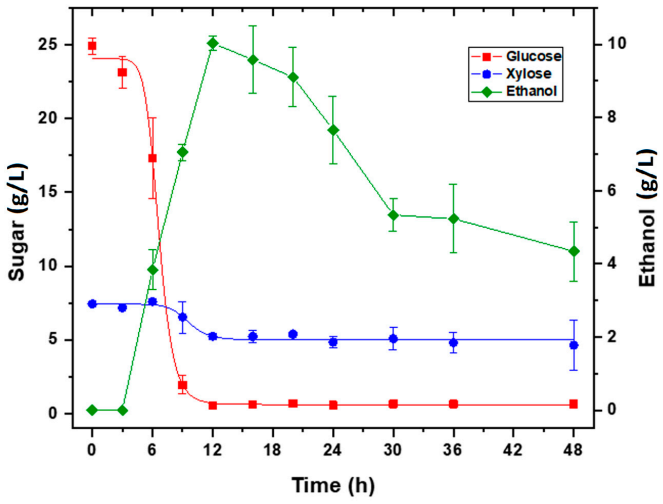

Figure 5. Bioethanol fermentation profile by *Saccharomyces cerevisiae*.

A study using SH enzymatic hydrolysate pretreated with ionic liquids (ILs) showed a production of 6 g/L of bioethanol by *Candida shehatae* in 24 h fermentation, representing

60.8% of efficiency compared to the theoretical yield [27]. The ILs have been studied as green alternatives for dissolving the complete biomass rather than individual subcomponents [49]. However, some ILs can remain in the hydrolyzed solution and may inhibit yeast growth, resulting in a negative effect on the bioethanol fermentation process [50]. These results reinforce the feasibility of using imidazole in lignocellulosic biomass pretreatment, due not only to its high efficiency, but also for the economic advantage compared to ILs.

Rojas et al. (2014) [23] applied a dilute acid pretreatment to SH, obtaining a bioethanol productivity of 0.20 g/Lh using *S. cerevisiae*, while the productivity reached in this work was 1.07 g/Lh. Dilute acid hydrolysis is commonly applied to lignocellulosic material, however, it generates inhibitory compounds, for example, acetic acid, HMF (5-hydroxymethyl-2-furaldehyde), furfural, and phenol. All of these compounds negatively affect the fermentation step [51].

In the absence of glucose, bioethanol can be consumed by the yeast for the formation of other organic acids, such as acetic acid or succinic acid, which may indicate that organic acids were produced through the Krebs or glyoxylate cycle by the oxidation of bioethanol [25].

A mass balance was conducted, showing an increase in bioethanol production at 12 h for all imidazole pretreated samples of SH compared to the untreated material (Figure 6). At 120 °C the final bioethanol production increased by 43%, which was mainly influenced by the conservation of glucan that produced a higher release of glucose after enzymatic hydrolysis. Conditions of 120 °C and 1 h showed a better performance for bioethanol production with 145 g of bioethanol from 1 kg of dry SH. With these optimal results a minimization of energy consumption is attained because, when compared to more aggressive treatments, the condition of 120 °C at 1 h would require lower energy demand and costs. Also, with these pretreatment conditions it is possible to recover a rich hemicellulose fraction, obtaining 235 g of this fraction from 1 kg of dry SH with a composition of 16.9 g of glucan, 78.5 g of xylan and 8.0 g of lignin. There are reports of the use of soybean by-products for bioethanol production, such as the production of 59.1 g of bioethanol from 1 kg of soybean okara [52]. This fact demonstrates the high potential of SH pretreatment with imidazole to increase the production of second-generation bioethanol. Additionally, it is important to emphasize the amount of xylose produced in the process, which could be used in the production of bioethanol with C5 adapted yeasts. Thus, fermentation results showed that imidazole was suitable for lignocellulosic biomass delignification for further enzymatic hydrolysis of cellulose-rich fraction and, consequently, second-generation ethanol production. Moreover, imidazole pretreatment did not require detoxification steps [32].

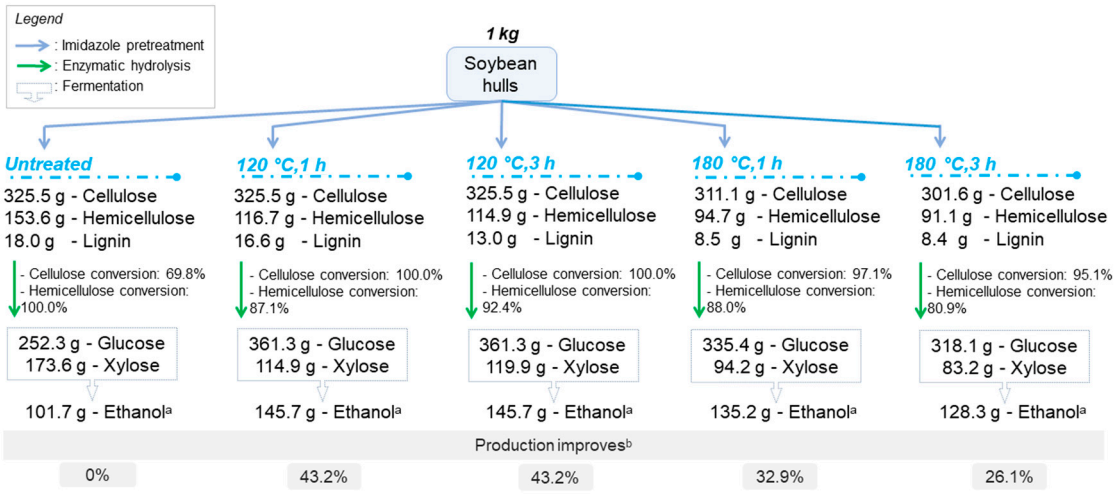

Figure 6. Mass balance of second-generation bioethanol production from 1 kg of dried SH [a] Calculated with bioethanol yield value (78.9%) and [b] Calculated from bioethanol production of untreated material.

In order to increase bioethanol production, it is imperative to continue investigating other strategies, such as simultaneous saccharification and fermentation (SSF), fed-batch fermentation or fermentation using genetically-modified microorganisms capable of consuming both glucose and xylose for bioethanol production [53].

4. Conclusions

This study has demonstrated that imidazole is a potential alternative for lignocellulosic waste pretreatment, such as soybean hulls, with considerable efficiency when compared to other methods. Moreover, imidazole presents low toxicity and is considered a less aggressive solvent for humans and the environment. In addition, the solvent is a stable compound with low vapor pressure that facilitates its manipulation. The use of imidazole promoted a high biomass delignification with a minimum negative effect on cellulose degradation, favoring glucose recovery by enzymatic hydrolysis. Thus, the obtained hydrolysate can be used in the production of second-generation ethanol and other economically interesting biomolecules from SH.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation9020093/s1, Figure S1: XRD spectra of native and imidazole pretreated samples; Figure S2: FTIR spectra of native and imidazole pretreated samples.

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