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Enhanced Production of Clean Fermentable Sugars by Acid Pretreatment and Enzymatic Saccharification of Sugarcane Bagasse

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Abstract: Sugarcane bagasse (SCB), an agro-industrial byproduct generated by a sugar mill, holds a substantial carbohydrate content of around 70 wt.%, comprising cellulose and hemicellulose. Saccharification plays a pivotal role in the conversion of SCB into second-generation (2G)-ethanol and valuable compounds, which is significantly aided by thermochemical pretreatments. In this study, SCB underwent diluted sulfuric acid pretreatment (2% H_2SO_4, 80 rpm, 200 °C, 20 min), resulting in the removal of 77.3% of the xylan. The hemicellulosic hydrolysate was analyzed to identify the sugars and degraded products acting as microbial inhibitors. The acid hydrolysate showed a xylose yield of 68.0% (16.4 g/L) and a yield of 3.8 g/L of acetic acid. Afterward, the hemicellulosic hydrolysate was concentrated 2.37 times to obtain a xylose-rich stream (39.87 g/L). The sequential detoxification, employing calcium oxide and activated carbon, removed the inhibitory compounds, including acetic acid, while preserving the xylose at 38.10 g/L. The enzymatic saccharification of cellulignin at 5% and 10% of the total solids (TSs) yielded comparable reducing sugar (RS) yields of 47.3% (15.2 g/L) and 47.4% (30.4 g/L), respectively, after 96 h, employing a 10 FPU/g enzyme loading of Cellic® CTec3 (Novozymes Inc. Parana, Brazil). In summary, these findings outline an integrated green chemistry approach aimed at addressing the key challenges associated with pretreatment, concentration, detoxification, and enzymatic hydrolysis to produce fermentable sugars.

Keywords: sugarcane bagasse; detoxification; saccharification; reducing sugars; sugarcane hydrolysate

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1. Introduction

In recent decades, the global demand for renewable and sustainable sources of energy has surged, catalyzing the exploration of alternative methods to produce biofuels and green chemicals [1]. Amidst the array of biomass resources, sugarcane emerges as a crop of paramount economic significance [2], boasting an annual production surpassing 1.9 billion metric tons and playing a pivotal role in both the food and biofuel industries [3].

Brazil shines brightly as the foremost producer of sugarcane, commanding a substantial share at 677.6 million tons of the global production, thereby constituting a significant portion of its agricultural economy [4]. However, the sugarcane industry, while prolific, begets substantial waste in the form of straw and bagasse, which are byproducts of sugar and ethanol production. With sugarcane harvesting yielding 140 kg of dry bagasse and straw per ton of processed cane [5], these residues present environmental quandaries owing to their voluminous nature and management challenges [6]. Yet, bagasse and straw concurrently represent a cost-effective opportunity for their utilization as lignocellulosic material in biotechnological processes [7].

Comprising primarily of cellulose (38.4–45.5%), hemicellulose (22.7–27.0%), and lignin (19.1–32.4%) [8], sugarcane bagasse (SCB) comes to light as an appealing substrate for

bioconversion processes, yielding fermentable sugars and serving as an eco-friendly platform for producing value-added molecules. However, SCB presents a complex hierarchical structure consisting of crystalline cellulose nanofibrils intertwined within an amorphous matrix of cross-linked lignins and hemicelluloses [9]. This intricate arrangement poses a formidable barrier to enzyme and microbial accessibility, hindering the efficient breakdown of cellulose into fermentable sugars [10]. Given the heterogeneous nature of biomass feedstock, the development of a universal pretreatment process remains an arduous task [11].

Various pretreatment strategies for SCB have been delineated in the literature, encompassing chemical, physical, and biological treatments, albeit demanding considerable time and energy resources [12]. Dilute acid pretreatment has emerged as a potent approach for recovering xylose-rich hydrolysates and augmenting the enzymatic hydrolysis of lignocellulosic biomass by disrupting the lignin–hemicellulose–cellulose matrix and enhancing cellulose accessibility to hydrolytic enzymes [13]. Central to biorefineries are two sequential steps: biomass saccharification, followed by fermentation of the liberated sugars [14].

Dilute acid pretreatment stands out as a practical and highly effective chemical method, offering significant economic and environmental benefits. These advantages include reduced process costs, minimal acid consumption, diminished bioreactor corrosion, and wastewater with a low acid residue concentration [15]. By fine-tuning operational parameters such as solid loading, acid concentration, temperature, and retention time, the recovery of sugars can be substantially enhanced from SCB via diluted sulfuric acid treatment [16,17]. Additionally, the maximization of C5 and C6 sugar acquisition can catalyze the production of derivatives from this carbon source, as highlighted in previous works like that by Dionoso et al. [18].

Nevertheless, xylose-rich hydrolysates often harbor inhibitory compounds, such as furfural, 5-hydroxymethylfurfural (5-HMF), acetic acid, and phenolic compounds, which can impede microbial fermentation and diminish bioproduct yields [13,19]. Thus, detoxification strategies for hydrolysates are imperative to alleviate the inhibitory effects of these compounds and enhance substrate fermentability efficiency [20–22].

In this context, this current study delves into the enhanced production of fermentable sugars from sulfuric acid-diluted SCB pretreatment under low-temperature and short reaction time conditions. The subsequent concentration and detoxification of xylose-rich hydrolysates were undertaken to safeguard the fermentable sugars and eradicate the inhibitory compounds. Furthermore, the cellulignin generated during pretreatment was assessed for its potential to produce reducing sugars (RSs) via saccharification, employing low enzyme loading and comparing low and high solid loadings to discern their impact on sugar production and yield.

By elucidating the synergistic effects of sulfuric acid pretreatment, detoxification, and enzymatic saccharification in SCB conversion, this research contributes to the advancement of efficient and sustainable processes for producing biofuels (e.g., ethanol and methane), enzymes, bioplastics, and chemicals from lignocellulosic biomass.

2. Materials and Methods

2.1. Feedstock and Chemicals

The SCB used in this study was kindly supplied by the Ipiranga Agroindustrial Plant (Descalvado, Sao Paulo, Brazil). Afterward, it underwent washing to remove all dust particles and was then sun-dried to eliminate moisture. Following this, the bagasse was finely ground in a high-speed stainless industrial blender and stored in plastic bags at room temperature. The commercial enzymatic cocktail Cellic® CTec3 (300.12 FPU/mL) was provided by the company Novozyme Inc. (Curitiba, Parana, Brazil). Activated carbon, calcium oxide (CaO), and sulfuric acid (H_2SO_4) were purchased from Labsynth, Diadema, SP, Brazil.

2.2. Pretreatment of Sugarcane Bagasse with Diluted Sulfuric Acid

The dilute acid hydrolysis of the SCB was conducted in a CR 80 Allbiom stainless steel reactor (Cajuru, Sao Paulo, Brazil) with an 80 L capacity. The hydrolysis reaction employed a 2% (v/v) H_2SO_4 (98%) catalyst, along with a ratio of 10% (w/v) TS (3 kg bagasse; 30 L acid solution) at 120 °C and 80 rpm for 20 min. Post-reaction, the treated biomass (cellulignin) was recovered via centrifugation (4000× g for 20 min) using a Sppencer Scientific Centrifuge, Sao Paulo, Brazil. The hemicellulosic hydrolysate obtained was stored at 4 °C. The cellulignin was rinsed with tap water until it reached a neutral pH, followed by drying at room temperature.

2.3. Chemical Composition

The compositional analysis for determining carbohydrates, lignin, ash, and total extractives in the native SCB and in the pretreated biomass (cellulignin) was carried out through H_2SO_4 hydrolysis, essentially following the methods outlined by the National Renewable Energy Laboratory (NREL) in the NREL/TP-510-42618 and NREL/TP-510-42619 protocols [23,24]. The inorganic ash present was measured by ashing the samples at 550 °C for 3 h using gravimetric analysis. The polysaccharide content was calculated based on the amount of released monosaccharides, determined through High-Performance Liquid Chromatography (HPLC) Waters 1515 (Milford, MA, USA).

2.4. Concentration and Detoxification of Sugarcane Hemicellulosic Hydrolysate

The hemicellulosic hydrolysate was concentrated twice at 80 °C to increase the sugar concentration, primarily xylose [25]. This process employed a Hipperquimica 32 L capacity vacuum concentrator (Iperó, Sao Paulo, Brazil). After concentration, the resulting xylose-rich hydrolysate underwent detoxification according to the method outlined by Marton et al. [26]. This detoxification process involved three stages: the neutralization of the pH to 7.0 using a solution with 50% by weight of CaO, the reduction in the pH to 5 with H_2SO_4, and the addition of activated carbon at a ratio of 2% (w/v). The mixture was then placed on a Thermo Scientific MaxQ 4000 rotary shaker (Waltham, MA, USA), set to operate at 100 rpm and 50 °C for 60 min. Vacuum filtration was performed after each processing step. Finally, upon completion of the detoxification process, the hydrolysate was stored at 4 °C for further analyses.

2.5. Enzymatic Saccharification of the Cellulignin Fraction

The enzymatic hydrolysis (E.H.) of cellulignin was conducted using 5% and 10% TS, following the method outlined by Ascencio et al. [27]. Saccharification tests were performed by adding 5 and 10 g of the pretreated SCB to 250 mL Erlenmeyer flasks containing 100 mL of sodium citrate buffer (50 mM; pH 4.8) [27]. The Cellic® CTec3 was utilized with an enzyme loading of 10 FPU (Filter Paper Units) per 0.55 g of cellulose from the pretreated biomass at 50 °C and 180 rpm for 96 h in a Thermo Scientific MaxQ 4000 rotary shaker (Ashville, OH, USA). Using the same experimental conditions, cellulignin without cellulase enzymes served as a substrate blank, while cellulase enzymes without cellulignin were employed as a control. Samples of the enzymatic hydrolysate were collected periodically at 0, 12, 24, 48, 72, and 96 h. These samples were boiled for 5 min at 100 °C to deactivate the cellulase enzymes and then centrifuged at 10,000× g for 10 min. The supernatant was collected and frozen for the subsequent analysis of RSs using the method by Miller [28].

2.6. Analytical Techniques

The xylose, glucose, arabinose, cellobiose, and acetic acid were determined using a Waters 1515 HPLC (Milford, MA, USA) equipped with a Bio-rad aminex HPX-87H column (300 × 7.8 mm) coupled to an index detector with a 2414 refraction. A 5 mM H_2SO_4 eluent was used at a flow rate of 0.6 mL/min, with a column temperature of 45 °C and an injected volume of 20 µL. Before analysis via HPLC, the samples were filtered through a Waters Sep-Pak C18 filter (WAT051910). The total phenolics (g/L) in the hemicellulosic

hydrolysates were determined using the method described by Singleton et al. [29]. All analyses in this work were performed in triplicate. The xylan removal [30], xylose yield in the hemicellulosic hydrolysate [31], and the RSs yield in the enzymatic hydrolysate [27] were calculated using the following formulas:

$$Xylan\ removal\ (\%) = \frac{Final\ mass\ of\ xylose\ in\ the\ acid\ hydrolysate\ (\mathrm{g}) - Initial\ mass\ of\ xylose\ (\mathrm{g})}{Initial\ mass\ of\ xylose\ contained\ in\ the\ xylan\ SCB(\mathrm{g})} \times 100\%$$

$$Xylose\ yield\ \ from\ biomass\ pretreated\ (\%) = \frac{Xylose\ in\ the\ acid\ hydrolysate\ (\mathrm{g})}{Xylaninthe\ SCB\ (\mathrm{g})} \times 0.88 \times 100\%$$

$$RS\ yield\ from\ E.H.(\%) = \frac{Sugar\ concentration\ \left(\frac{\mathrm{g}}{\mathrm{L}}\right) \times enzymatic\ hydrolysate\ (\mathrm{L})}{Glucan + Xylan\ in\ the\ cellulignin\ (\mathrm{g})} \times 100\%$$

3. Results and Discussion

3.1. Diluted Sulfuric Acid Pretreatment

SCB, a highly abundant industrial by-product utilized in Brazil for 2G-ethanol production, was pretreated with 2% (*v*/*v*) sulfuric acid, serving as a catalyst for acid hydrolysis, as depicted in Figure 1. Both the liquid fraction (hemicellulosic hydrolysate) and the solid biomass (cellulignin) were retrieved for subsequent sugar concentration–detoxification and enzymatic hydrolysis, respectively.

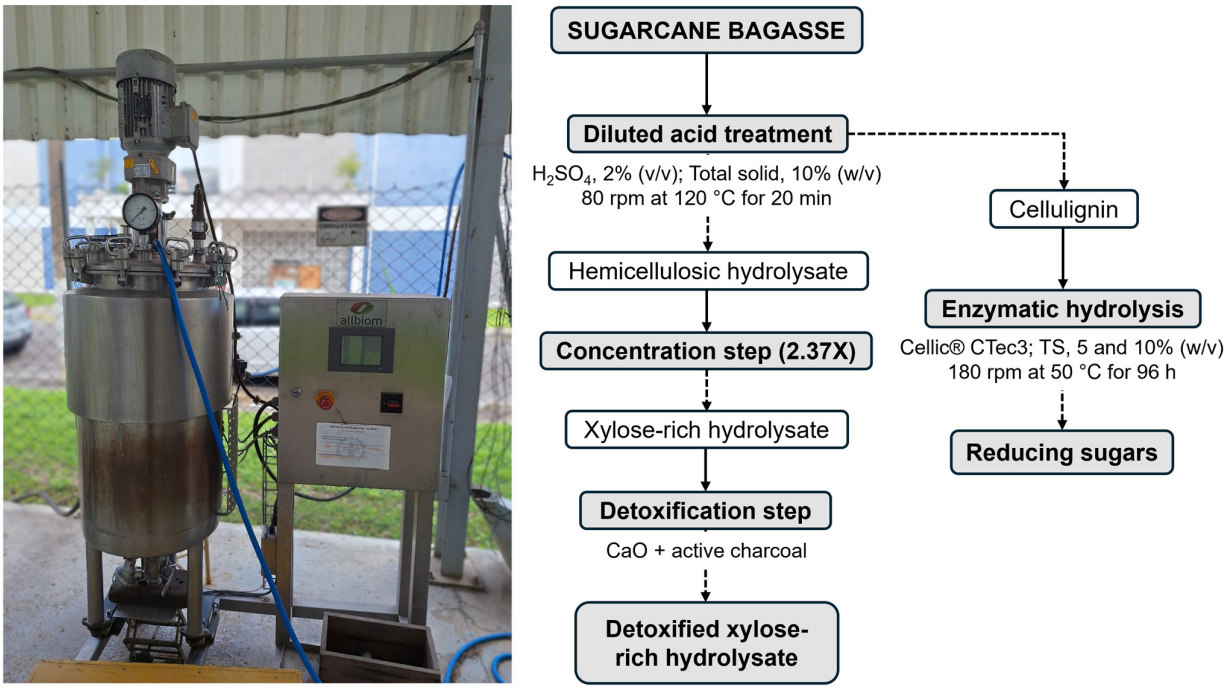

Figure 1. General overview of the retrieval of reducing sugars found in the hemicellulosic and enzymatic hydrolysate derived from acid-pretreated sugarcane bagasse, employing a mechanical reactor.

Untreated SCB revealed the following composition (%, dry weight): 40.6 ± 0.4 glucan, 21.3 ± 0.0 xylan, 1.9 ± 0.0 arabinan, 21.4 ± 0.3 total lignin, 3.2 ± 0.0 acetic acid, 1.5 ± 0.1 ash, and 2.0 ± 0.2 total extractives. The reported values fell within the ranges documented in a comprehensive study that examined sixty bagasse samples collected from the primary sugarcane-producing regions in Brazil [32]. Furthermore, the compositional analysis of the pretreated SCB demonstrated an increase in glucan and lignin content under the examined reaction conditions (2% H_2SO_4, 80 rpm at 200 °C) compared to the untreated bagasse, while reductions were observed for xylan, arabinan, acetic acid, total extractives, and ash. Notably, xylan exhibited a significant decrease from 21.3% to 9.1% post-acid treatment, as

illustrated in Table 1. Certainly, Philippini et al. [33] presented similar data in the chemical characterization of sulfuric acid-treated SCB cellulignin, encompassing xylan (8.8%), glucan (53.8%), lignin (36.1%), and ash (0.5%).

Table 1. Compositional analysis (%, dry weight) of untreated and dilute sulfuric acid-pretreated SCB.

Biomass	Lignin	Glucan	Xylan	Arabinan	Acetic Acid	Total Extractives	Ash	Total
SCB untreated	21.4 ± 0.3	40.6 ± 0.4	21.3 ± 0.0	1.9 ± 0.0	3.2 ± 0.0	2.0 ± 0.2	1.5 ± 0.1	92.2 ± 0.5
SCB pretreated	27.1 ± 0.3	55.1 ± 3.0	9.1 ± 0.8	0.5 ± 0.0	1.1 ± 0.0	ND	0.8 ± 0.0	93.8 ± 3.8

ND, not detectable.

The reduction in xylan by 77.3% in the cellulignin indicates the effectiveness of the pretreatment using a low-concentration of sulfuric acid (2% w/v) and a short reaction time (20 min) in our study. In fact, Alves et al. [34] reported a 71.4% xylan removal using 2% (w/v) H_2SO_4 to treat the 10% SCB at 121 °C, but with autoclaving for a longer reaction time (60 min). Similarly, Hans et al. [35] optimized the SCB treatment using 1.5% H_2SO_4, 12.5% (w/v) solid loading, and 37 min in an autoclave, removing 75% of xylan.

Meanwhile, Canilha et al. [15] reported low yields regarding xylan removal in acid treatment of 15% (w/v) SCB using 2.5% (w/v) H_2SO_4 at 120 °C, even with shorter times of 10 min (3.6%) or longer times of 30 min (22.8%) in stainless steel reactors, compared to 20 min in the present study. However, the best xylan removal (61.9%) was observed when the pretreatment was carried out with 1.5% (w/v) acid at 135 °C for 20 min [15].

It is worth mentioning that previous works lacked agitation, a key factor in dilute acid pretreatment processes to increase the xylan removal efficiency. Effective agitation improves heat and mass transfer within the reactor, facilitating the redistribution of the catalytic agent throughout the biomass [36]. Additionally, agitation applies physical forces to the biomass, contributing directly to fiber wear. As fibers mix, impact forces hitting the bottom of the reactor cause the mechanical breakdown of lignocellulose [37]. The current work did not optimize factors such as solid loading or the variation in the H_2SO_4 concentration; however, the results were promising. As demonstrated by Rocha et al. [38], using a rotary reactor to pretreat SCB with an acid mix (1% sulfuric acid and 1% acetic acid) at 190 °C for 10 min, and solid loadings of 6.6% and 10%, achieved a xylan removal exceeding 90% in both cases.

This treatment primarily aims at solubilizing xylan, which possesses a slightly amorphous and branched structure resistant to hydrolysis. The resulting hemicellulosic hydrolysate holds potential applications in biofuel and green chemical production within a sugarcane-based biorefinery, catering to formulations requiring suitable sources of C5 sugars.

3.2. Concentration and Detoxification of Hemicellulosic Hydrolysate

The hemicellulosic hydrolysate, post-acid treatment, yielded 19.9 g/L of total RSs, comprising 16.4 g/L xylose, 1.6 g/L glucose, 1.7 g/L arabinose, and 0.5 g/L cellobiose. Additionally, the analysis revealed 2.1 g/L of acetic acid and 0.8 g/L of total phenolics as fermentation-inhibitory compounds that were produced during pretreatment. Likewise, comparable concentrations of xylose (19.1 g/L) have been measured in the hemicellulosic hydrolysate of sulfuric acid-treated sugar cane bagasse, along with glucose (0.9 g/L), arabinose (1.8 g/L), acetic acid (3.4 g/L), and total phenolics (1.9%) [39].

In this study, the total xylose yield reached 68.0% of the theoretical xylose content found in the untreated SCB. It is worth mentioning that this figure aligns with the results reported in other studies of 63.3% [18], 65% [38], and 74% [39]. To enhance the reducing sugar concentration, the hemicellulosic hydrolysate underwent a 2.37-fold increase, resulting in a significant increase to 47.4 g/L, with xylose as the predominant sugar at 38.1 g/L. Consequently, there was also an observed increase in inhibitory compounds, reaching 3.8 g/L for acetic acid and 1.6 g/L for phenolic compounds. Figure 2 provides a clear overview of the chemical composition, including the RSs and inhibitory compounds found in both the unconcentrated and concentrated hemicellulosic hydrolysates.

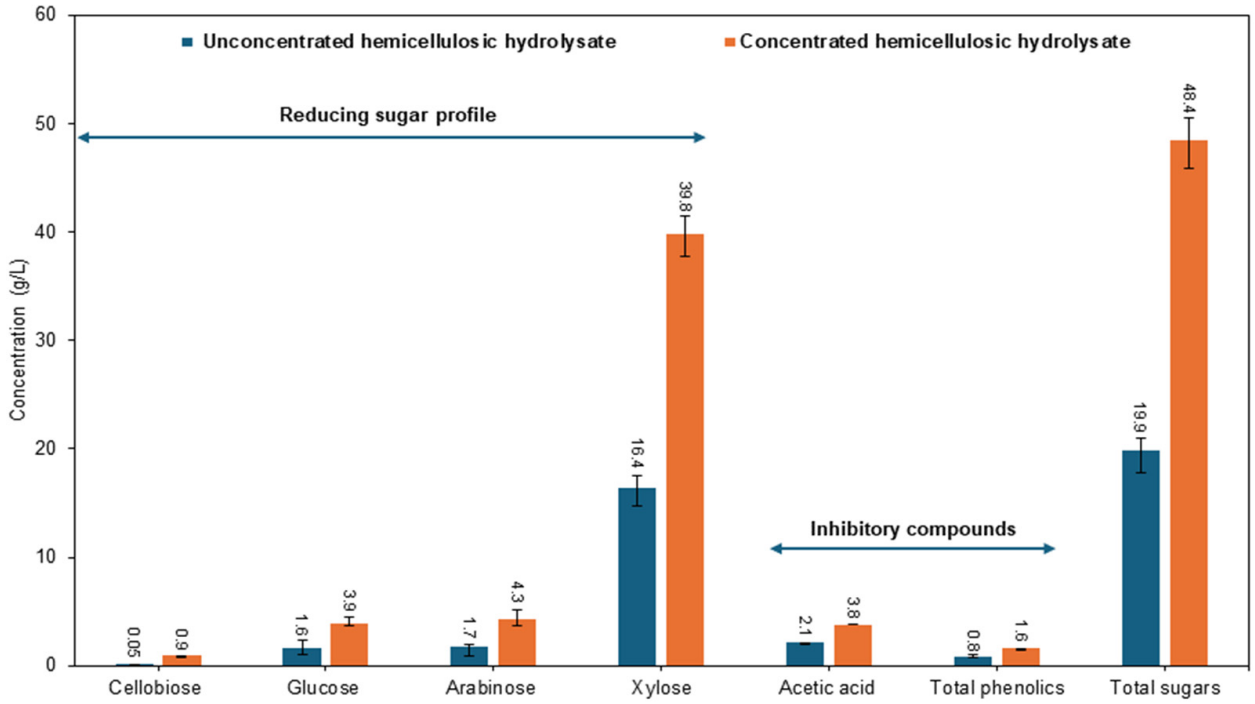

Figure 2. Chemical composition of hemicellulosic hydrolysate derived from sulfuric acid pretreatment. There is no statistical difference according to the Tukey test (95% confidence level).

Fermentation inhibitors, including furfural, 5-HMF, formic acid, acetic acid, and phenolic compounds, pose significant challenges by impeding microbial growth and sugar consumption [22,40]. The detoxification of the concentrated hemicellulosic hydrolysate was carried out to mitigate the adverse effects of these inhibitors to optimize the fermentation conditions and maximize the bioproduct yield [21,39,41].

The visual representation in Figure 3A depicts the detoxification process, wherein the acid hemicellulosic hydrolysate post-acid treatment (1) was concentrated (2) and detoxified [pH neutralization with 50% (*w*/*v*) CaO, pH reduction to 5 with H_2SO_4, and addition of 2% (*w*/*v*) activated carbon] (3), resulting in a xylose-rich hydrolysate after filtration (4). The detoxification step effectively removed the acetic acid, which was the predominant inhibitor identified in hydrolysates originating from xylan deacetylation [42]. In contrast, detoxification methods for sugar cane bagasse hydrolysate using various resins (A-103 S, A-860 S, Applexion cation, and Applexion anion) failed to remove any acetic acid whatsoever [43].

However, a novel approach to enhance the removal of undesirable byproducts and phenolic compounds from SCB hydrolysates involves utilizing a combination of activated carbon and macroporous adsorption resin, resulting in impressive removal efficiencies of 70.9% and 92.0%, respectively [44]. Our findings demonstrate that the CaO–activated carbon process significantly reduced phenolic compound levels by 56%, from 1.6 to 0.7 g/L, as depicted in Figure 3B, while fully preserving the RSs. Lignin-derived phenolic compounds are common in the hydrolysate of pretreated lignocellulosic biomass, which can reduce the efficiency of enzymatic hydrolysis [45]. Importantly, the detoxification process kept the RSs intact, ensuring the preservation of their full potential.

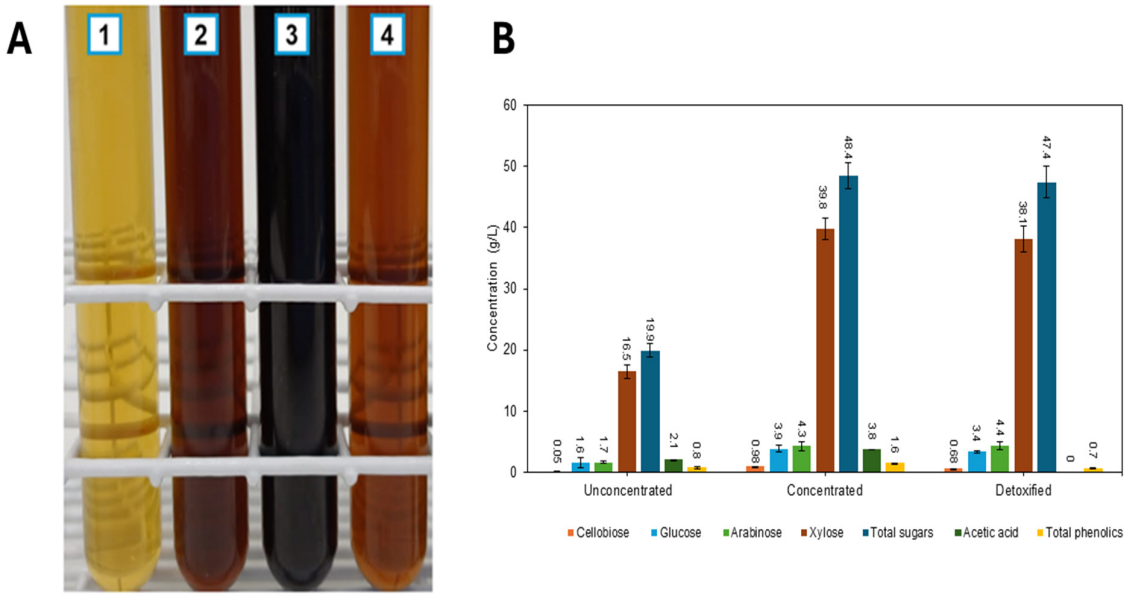

Figure 3. Concentration and detoxification of SCB hemicellulosic hydrolysate. (**A**) Visual progression for xylose-rich hydrolysate production: (1) hemicellulosic hydrolysate after acid pretreatment (unconcentrated), (2) hemicellulosic hydrolysate concentrated 2.37X, (3) detoxification step of hemicellulosic hydrolysate, and (4) xylose-rich hydrolysate after detoxification–filtration. (**B**) Analysis of sugar profiles and inhibitory compounds throughout the main stages of hemicellulosic hydrolysate processing. There is no statistical difference according to the Tukey test (95% confidence level).

3.3. Enzymatic Saccharification of Cellulignin

This assay evaluated the efficiency of dilute acid pretreatment through a saccharification reaction using 5% and 10% TS of SCB cellulignin. The time course of the enzymatic hydrolysis, conducted for 96 h using an enzyme loading of 10 FPU/g of dry biomass, is presented in Figure 4. A dramatic increase in RS yields was observed in both cellulignin hydrolysates compared to the substrate blank, which consisted solely of biomass without enzymes. Enzymatic hydrolysis with 5% and 10% TSs achieved similar yields, approximately 47.3% and 47.4%, respectively. Cellulignin lacks a significant portion of xylan, yet lignin remains abundant in the cell wall (Table 1), resulting in lower sugar recovery.

The lower sugar recovery at a 10% solid loading may be attributed to the inhibition of cellulolytic enzymes by the high lignin content in cellulignin and/or the inadequate mass transfer during the hydrolysis process, as the SCB suspension was only stirred in the incubator [46–48].

A similar experimental setup, using a 20% TS cellulignin from five SCB varieties but employing a blend of the commercial enzymes Celluclast 1.5 L and Novozyme 188, demonstrated an average RSs production of 27.2 g/L within 48 h [33]. Despite a TS reduction of 10% in this study, the results still showed a significant production of 23.5 g/L of RSs after 48 h. This finding underscores the efficiency of the process and suggests that even with a lower amount of raw material, considerable yields of sugars can be obtained, which could have positive implications in terms of production costs and efficiency on an industrial scale.

However, it was noted that the highest RSs (30.4 g/L) were obtained under the 10% TS condition at 96 h. This was attributed to a higher amount of dry biomass with a greater carbohydrate content, compared to 15.2 g/L obtained with 5% TS (yield 47.3%). However, after 48 h, the saccharification of 5% cellulignin showed a 52% RS yield when utilizing Avicel cellulase [49], a higher value compared to a yield of 33.1% RSs within the same timeframe in this work.

Our results are consistent with studies on the enzymatic hydrolysis of SCB cellulignin, although higher enzyme loadings are typically used. For instance, Ascencio et al. [27]

conducted enzymatic hydrolysis with 5% and 10% SCB cellulignin using 15 FPU/g of Cellic® CTec2, resulting in approximately 14 g/L and 22 g/L yields at 72 h, respectively. Hence, future studies could investigate using higher %TS concentrations in enzymatic hydrolysis to observe their impact on sugar yield, ultimately maximizing the RS production.

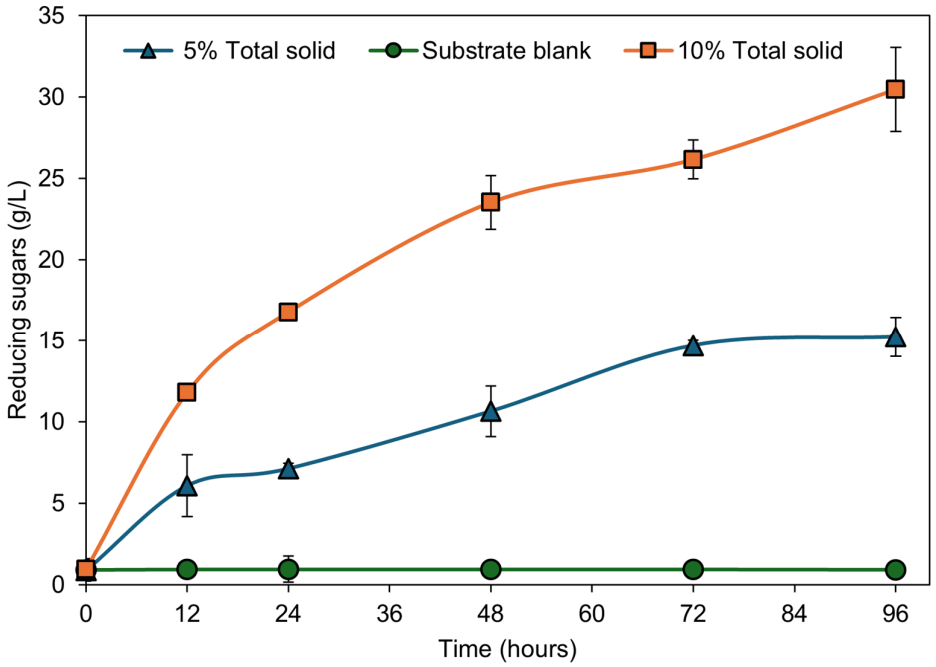

Figure 4. The kinetic profile of RSs released during the enzymatic hydrolysis of cellulignin under 5 and 10% TS. The substrate blank comprises cellulignin without the Cellic® CTec3 enzyme. There is no statistical difference according to the Tukey test (95% confidence level).

4. Conclusions

The pretreatment of SCB with 2% sulfuric acid at a low temperature and reaction time (120 °C, 20 min) induced significant alterations in its chemical composition, resulting in an increased glucan and lignin content and a noticeable reduction in xylan. This process effectively solubilized 77.3% of the xylan, facilitating subsequent acid hydrolysis. Acid hydrolysis generated a hemicellulosic hydrolysate abundant in RSs, predominantly xylose, with a yield of 68.06%. Although the concentration of the xylose-rich hydrolysate substantially augmented the RSs, it also elevated the levels of inhibitory compounds, such as acetic acid and phenolic compounds. Nevertheless, detoxification (CaO and activated carbon) of the concentrated hydrolysate efficiently removed the acetic acid and reduced the phenolic compounds by 56%, thereby maintaining the RS concentrations for valorization. The enzymatic hydrolysis of the SCB cellulignin exhibited enhanced RS yields, especially under the conditions of a higher %TS content. A fundamental aspect of this research lies in the comprehensive recovery of all sugar content present in SCB. These findings underscore the potential of SCB biomass for fermentable sugar production, which is crucial for obtaining green chemicals and fuels in biorefineries. Stressing the significance of the pretreatment and detoxification processes is essential for maximizing their efficiency.

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Data Availability Statement: Data are contained within the article.

Conflicts of Interest: The authors declare no conflicts of interest.

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