processes

MDPI

Article

Cellulosic Ethanol: Improving Cost Efficiency by Coupling Semi-Continuous Fermentation and Simultaneous Saccharification Strategies

Patricia Portero Barahona [1,2], Bernardo Bastidas Mayorga [1], Jesús Martín-Gil [2], Pablo Martín-Ramos [3] and Enrique Javier Carvajal Barriga [1,*]

1 The Catholic University Yeast Collection in Quito (CLQCA), Neotropical Center for the Biomass Research, School of Biological Sciences, Pontifical Catholic University of Ecuador, Avda. 12 de Octubre 1076 y Roca, Quito 170525, Ecuador; pportero@puce.edu.ec (P.P.B.); bb158919@ohio.edu (B.B.M.)
2 Department of Agricultural and Forestry Engineering, ETSIIAA, Universidad de Valladolid, Avenida de 10 Madrid 44, 34004 Palencia, Spain; mgil@iaf.uva.es
3 Instituto Universitario de Investigación en Ciencias Ambientales (IUCA), EPS, Universidad de Zaragoza, Carretera de Cuarte, s/n, 22071 Huesca, Spain; pmr@unizar.es
* Correspondence: ejcarvajal@puce.edu.ec; Tel.: +593-2-2991700

Received: 3 October 2020; Accepted: 22 October 2020; Published: 15 November 2020

Abstract: A novel approach to improve ethanol production from sugarcane bagasse is proposed. Biomass was pretreated with sodium hydroxide, sulfuric, oxalic, and maleic acids (1% *w/v*) at different temperatures (130–170 °C) and times (10–30 min). The pretreatment with NaOH at 160 °C for 20 min was found to be the most efficient for further enzymatic saccharification. A semi-continuous fermentation system coupled with a simultaneous saccharification and fermentation strategy was used, attaining fermented liquor every 24 h. The amount of enzymes needed for saccharification was optimized, as well as the production time and ethanol concentration. The process occurred with near to complete depletion of glucose, obtaining ethanol concentrations ranging from 8.36 to 10.79% (*v/v*). The whole system, at bench scale, showed stability over 30 days, and ease of management and control. This strategy may improve cost efficiency in the production of cellulosic ethanol at industrial scale.

Keywords: bioethanol; enzymes; pretreatment; semi-continuous culture; sugarcane bagasse

1. Introduction

Fossil fuel depletion and climate change are problems that have encouraged the research and development of technologies aimed at obtaining possible substitutes for fossil fuel sources [1]. Biofuels that come from a renewable source, such as biomass, are regarded as a promising replacement for petroleum-based fuels [2]. Plant biomass is a raw material of interest because it is readily available, both as crops or as waste from industrial activities, and can be used for energy purposes [3].

Lignocellulosic ethanol refers to the product of fermentation of both glucose from cellulose and pentoses from hemicellulose, while cellulosic ethanol is obtained from cellulose as the sole source of glucose [4]. The leftovers and/or waste obtained from agriculture and industry, such as sugarcane bagasse (SCB), corn stover, wheat and rice straw, wood chips, and the like, can be regarded as suitable lignocellulosic materials [5,6]. The production of ethanol from such lignocellulosic biomass (primarily composed of cellulose, hemicellulose, and lignin) requires four steps: (i) Pretreatment of biomass, (ii) enzymatic saccharification to sugar monomers, (iii) fermentation of hydrolyzed sugars to ethanol, and (iv) distillation and dehydration [7].

A pretreatment is essential for the fractionation of lignocellulosic biomass. It contributes to removal of lignin, reduction of cellulose crystallinity, and rise in the material's porosity, thus resulting

in the increase of fermentable sugars released into the liquid medium [8]. Numerous technologies involving physical and chemical pretreatments—applied separately, simultaneously, or in a sequential manner—have been investigated. Physical pretreatments include: Mechanical comminution [9], extrusion [10], thermal methods (pyrolysis, steam explosion, and hydrothermal) [11,12], microwave irradiation [13], and ultrasonic treatment [14], among others. Alternatively, chemical pretreatments that make use of acidic catalysts [15], alkalis [16], ionic liquids [17], and organosolvs [18] have also received extensive attention in the literature. In any case, the combination of physical and chemical pretreatments is regarded as necessary to improve the permeation of enzymes into cellulose and hemicellulose in biomass [19].

As with any industrial process, there is interest in continuing to optimize ethanol production, with reducing production time as well as lowering operational costs as the main objectives [20,21]. Most prior research has focused on the improvement of separate hydrolysis and fermentation (SHF). As a result of enzymatic hydrolysis, glucose and cellobiose are released, and, if these saccharides are not consumed by yeast, they can accumulate in the medium, producing an inhibitory effect on the cellulase enzymes [22]. Conversely, in the simultaneous saccharification and fermentation (SSF) process, the released glucose can quickly be fermented into ethanol by the yeasts. This continuous removal of products minimizes the inhibition of enzymatic activity, prevents osmotic stress in yeast cells, and reduces the risk of contamination by immediate transformation of sugars into ethanol while maintaining low sugar levels and a high concentration of ethanol [23].

However, SSF also presents some drawbacks, mainly in terms of the temperature at which the process must be run. The optimal temperature for enzymatic hydrolysis is 50–55 °C, while the most adequate temperature for fermentation is between 35–37 °C [24], necessitating a compromise on temperature. Moreover, SSF presents difficulties in reusing both enzymes and yeast in subsequent processes; consequently, new yeast cultures and enzymes doses must be added to every new SSF batch [25]. The pre-saccharification and simultaneous saccharification and fermentation (PSSF) process was proposed to avoid the negative effects caused by suboptimal temperature in SSF. In this process, the ideal temperature for enzymatic hydrolysis is applied at the beginning (improving the saccharification), and later, the temperature is set at the optimum range for fermentation with yeasts. The PSSF generates more fermented products in comparison to SSF, but a precise control of temperature is required [26].

To overcome the aforementioned problems, researchers have focused their attention on the effects of a number of modifications aimed at attaining an increase in productivity and ethanol yield. The variations evaluated in other studies include: (i) The selection of fermenting microorganisms with high tolerance to factors such as inhibitors, high temperature, and the ability to uptake and/or co-ferment hexoses and pentoses [27]; (ii) the reduction of the viscosity of the lignocellulosic biomass/water/enzymes mix [28,29]; (iii) the optimization of enzyme loading [30]; and (iv) the design of novel fed-batch systems [31].

In order to fulfill a gap in the knowledge and technology currently being employed for cellulosic ethanol production, the work presented herein assesses an alternative system for the fermentation of glucose resulting from cellulose hydrolysis, which may be referred to as a "semi-continuous fermentation and simultaneous saccharification" (SFSS). This novel strategy involves the obtainment of concentrated worts by means of an optimized physical–chemical pretreatment of SCB (although the reported methodology may be applied to any cellulosic material) and a further enzymatic pre-liquefaction of the pretreated SCB (to solve the inherent problems associated with cellulosic biomass water absorption capacity), followed by a 24 h SSF run in continuous culture (shorter than the typical saccharification and fermentation times, 72 h). Particular emphasis is placed on an efficient use of cellulolytic enzymes, given that they represent a high percentage of the total cellulosic ethanol production costs [32].

2. Materials and Methods

2.1. Materials and Yeast Strain

SCB was provided by a local sugar mill (Ingenio Azucarero del Norte, Imbabura, Ecuador). Cellic® CTec2 and HTec2 enzymes for cellulosic ethanol were supplied by Novozymes (Franklinton, NC, USA). The *Saccharomyces cerevisiae* strain used in the study (CLQCA-INT-005) was supplied by the Catholic University Yeast Collection in Quito (CLQCA). This strain was selected for its high ethanol yield (0.49 G·g^{-1}, in grams of ethanol produced per gram of glucose) after screening 150 different *S. cerevisiae* yeasts (data not shown).

2.2. Chemical Analysis of SCB

SCB was characterized by three different methods: Neutral detergent fiber (NDF) (method MO-LSAIA-02.01), acid detergent fiber (ADF) (method MO-LSAIA-02.02), and acid detergent lignin (ADL) (method MO-LSAIA-02.03). For fiber analysis purposes, a Fibretec^{TM} 1020/FT122 analyzer was used. Cellulose, hemicellulose, and lignin were quantified in accordance with McIntosh et al. [33]. All these analyses were performed at the National Autonomous Institute of Agricultural Research of Ecuador (INIAP) laboratories.

2.3. Physical and Chemical Pretreatments of SCB

SCB was washed, dried, and ground to obtain particles smaller than 100 µm. Several solutions containing different catalyzers such as oxalic acid, maleic acid, sulfuric acid, and sodium hydroxide (all at a concentration of 1% *w/v*) were tested with SCB at 10% (*w/v*) to assess different thermal pretreatments. The trials were performed in triplicate in a pressure reactor (Parr 4848; Parr Instrument Co., Moline, IL, USA) according to the following variables: Temperature—130, 140, 150, 160, and 170 °C—and reaction time—10, 20, and 30 min. The pretreated samples of SCB were centrifuged at 7000 rpm for 5 min, and the solid fraction was washed with distilled water by centrifugation until a neutral pH was reached and then dried at 105 °C for 24 h. The solid fraction recovery was quantified according to Equation (1):

$$\text{Solid fraction recovery } (\%) = \frac{\text{Pf pretreated biomass}}{\text{Pi biomass}} \times 100, \quad (1)$$

where Pf is the weight of the insoluble part of pretreated SCB (g) and Pi is the biomass weight of SCB (g).

A vibrational analysis of the recovered solid fraction was conducted using a Nicolet iS50 FTIR spectrometer (Thermo Scientific, Waltham, MA, USA), equipped with an in-built diamond attenuated total reflectance (ATR) system. The spectra were collected in the 400–4000 cm^{-1} region with a 1 cm^{-1} spectral resolution; 128 scans were co-added, and the resulting interferogram was averaged.

2.4. Enzymatic Saccharification of the Holocellulosic Fraction of SCB

Enzymatic saccharification was performed in triplicate in 100 mL flasks at 15% solids (*w/v*) (dry pretreated biomass of SCB) suspended in 0.05 M citrate buffer (pH 4.5), with the addition of 10 FPU (filter paper unit) of Cellic® CTec2 per gram of dry biomass and Cellic® HTec2 at 20% of the volume of Cellic® CTec2.

The activity of Cellic® CTec2 was determined in accordance with the standard protocols of the National Renewable Energy Laboratory [34]. The saccharification conditions were: 55 °C in a stirring incubator at 200 rpm for 72 h. The samples were centrifuged at 7000 rpm for 5 min, the liquid fraction containing dissolved hydrolysis products was analyzed by high-performance liquid chromatography (HPLC) with an Agilent (Santa Clara, CA, USA) 1200 Series apparatus, and the hydrolysate was used in the batch fermentation.

The yield of saccharide production from pretreated SCB hydrolysate was calculated according to Equation (2), adapted from Li et al. [35]:

$$\text{Saccharide yield } (\%) = \frac{\left(\frac{C_{glucose} \times 0.9}{\text{Cellulose content}} + \frac{C_{xylose} \times 0.88}{\text{Xylan content}}\right) \times \text{solid fraction}}{C_{substrate}} \times 100, \quad (2)$$

where $C_{glucose}$ and C_{xylose} represent the concentrations of glucose and xylose in the enzymatic hydrolysates (in g·L^{-1}), respectively; 0.9 is the glucan-to-glucose content conversion factor; 0.88 is the xylan-to-xylose content conversion factor; and $C_{substrate}$ is the substrate loading (in g·L^{-1}). Cellulose and xylan contents correspond to the composition of SCB (in g·g^{-1}).

2.5. Yeast Strain Acclimation

The chosen *S. cerevisiae* strain (CLQCA-INT-005) was acclimated by a series of successive liquid cultures consisting of pretreated SCB hydrolysate and yeast extract peptone dextrose in proportions of 0:1; 0.25:0.75; 0.5:0.05; 0.75:0.25; and 1:0 *v/v*. The acclimated yeast culture was used in fermentation tests, both in batch and semi-continuous fermentation experiments.

2.6. Batch Fermentations (15% Solids w/w)

Batch fermentations were performed in tubes with 5 mL of hydrolyzed SCB as the sole carbon source, 0.67% (*w/v*) yeast nitrogen base, and 1×10^8 cells·mL^{-1} of acclimated *S. cerevisiae*. All fermentations were carried out in triplicate. The incubation was conducted at 30 °C, under stirring at 100 rpm, for 72 h. The fermented product was centrifuged at 7000 rpm for 10 min to separate yeast cells and remnant non-hydrolyzed biomass. The supernatant was then collected and filtered with a 0.22 µm membrane filter and stored at −20 °C.

2.7. Measurement of Sugar and Ethanol Yield

The sugar and ethanol concentrations were analyzed by HPLC at 79 °C with 5 mM H_2SO_4 as an eluent at a flow rate of 0.6 mL·min^{-1} and an injection volume of 10 µL. An organic acid H^+ ion exchange column (Rezex ROA; Phenomenex, Torrance, CA, USA) was used; sugars and ethanol were detected by a refractive index detector. The compounds were identified by their relative retention times and quantified based on a calibration curve prepared with standard sugars and fermentation products.

The theoretical yield of ethanol (%) was calculated with Equation (3) [36]:

$$\text{Ethanol yield } (\%) = \left(\frac{C_{ethanol}}{0.51 \times C_{glucose}}\right) \times 100, \quad (3)$$

where $C_{ethanol}$ corresponds to the ethanol concentration at the end of the fermentation (in g·L^{-1}); $C_{glucose}$ corresponds to the glucose concentration at the beginning of the fermentation (in g·L^{-1}); and 0.51 represents the conversion factor for glucose-to-ethanol based on the stoichiometry of fermentation.

2.8. Pretreatment and Prehydrolysis of SCB

The biomass (SCB) was pretreated with NaOH (1%, *w/v*) at 160 °C for 20 min and at a 1:10 (*w/v*, solids:NaOH) rate. The process was carried out in a 400 L-capacity pressure reactor heated by thermal oil. The pretreated SCB was filtered, washed, dried, and eventually ground. The resultant biomass was liquefied (prehydrolysis) in flasks by suspending the biomass in 0.05 M citrate buffer (pH 4.5). Finally, 6 FPU of Cellic® CTec2 per gram of dry biomass and Cellic® HTec2 were incorporated into the mix. Following this, 60% of the prehydrolyzed SCB (15 g) was incubated for 6 h at 55 °C at 200 rpm; once liquefied, the remaining 40% of the prehydrolyzed SCB (10 g) was added to the suspension. A second incubation period of 4 h was needed to reach full liquefaction of the SCB.

2.9. Semi-Continuous Fermentation System Coupled with Simultaneous Saccharification

The SFSS experiments were carried out in a 2 L Bioflo/Celligen 310 jar bioreactor (New Brunswick Scientific, Edison, NJ, USA) at 100 rpm, pH 4.5, 30 °C for 30 days. As a starter culture, 1 L of enzymatic

liquefied SBC was added with acclimated *S. cerevisiae* (1×10^8 cells·mL^{-1}). Every 24 h, 100 mL of the product was harvested from the jar, and 100 mL of enzymatically prehydrolyzed SCB was added. The concentration of fermentation products and residual glucose (as well as other saccharides) was determined by high-performance liquid chromatography.

2.10. Statistical Analyses

The enzymatic saccharification, fermentation, and SFSS data were analyzed using IBM SPSS statistics software (IBM, Armonk, NY, USA). An analysis of variance was performed on the results, and the means were compared by Tukey's honest significant difference test with a significance level of 0.05.

3. Results and Discussion

3.1. Characterization of SCB

SCB was characterized to determine the lignocellulosic fraction, obtaining 42.78% of cellulose, 32.07% of hemicellulose, and 14.16% of lignin. The overall content of polysaccharides (holocellulose) in SCB was 74.85%, which represents the biomass fraction that can be utilized for bioethanol production processes. Other studies show a variation of ±10% in the composition of cellulose, hemicellulose, and lignin in SCB [37]. Such variation in SCB composition can be ascribed to the sugarcane variety, age, type of crop, and method of harvesting [38].

3.2. Physical and Chemical Pretreatments of SCB

The effects of the chemical pretreatments on the recovery of the solid fraction of SCB are summarized in Table 1 (see Table S1 for all the different process conditions). The solid fraction left after NaOH pretreatment was always higher both in mild and strong conditions. In mild conditions, the remnant biomass after alkaline pretreatment was 10.51, 4.7, and 7.4% higher than the ones left after sulfuric, oxalic, and maleic acids pretreatments, respectively, whereas, after the strong alkaline pretreatment, the remnant biomass was 5.5, 0.6, and 4.8% higher than the ones left after sulfuric, oxalic, and maleic acids pretreatments. This result was in accordance with other studies, which have shown that, in comparison to acid pretreatments, alkaline pretreatments can remove lignin more efficiently with a relatively mild attack to the holocellulose fraction. In consequence, the hydrolytic enzymes' accessibility to the polysaccharide fraction is highly increased [39].

Table 1. Percent of solid fraction recovery of sugarcane bagasse (SCB) after pretreatments.

Chemical Pretreatment	Range of Solid Fraction Recovery (% *w/w*)	
	Min. Value	Max. Value
NaOH	54.3 ± 1.5	63.7 ± 0.6
Sulfuric acid	51.3 ± 1.5	57.0 ± 1.0
Oxalic acid	54.0 ± 1.0	60.7 ± 0.6
Maleic acid	51.7 ± 1.2	59.0 ± 1.0

The data correspond to the maximum and minimum values obtained at temperatures 130, 140, 150, 160, and 170 °C and reaction times of 10, 20, and 30 min for each temperature.

The ATR-FTIR spectra of the solid fractions recovered showed the higher removal of lignin by the pretreatment with NaOH (Figure S1). On the other hand, it could be observed that the H_2SO_4 pretreatment led to the lowest solid fraction recovery, indicating a greater solubilization of lignin and hemicellulose and even some degradation of the cellulose fraction [40]. Similar studies have reported that the solid fraction of lignocellulosic materials decreases as the pretreatment's severity increases, causing a loss of sugars, which are converted into non-fermentable by-products such as fermentation inhibitors or saccharinic acids [41].

These results coincide with those reported by Qing et al. [40], who found that alkaline pretreatments produce strong delignification on lignocellulosic materials, whereas acid pretreatments can mainly hydrolyze and remove hemicellulose from the solid fraction of pretreated SCB, resulting in a higher concentration of sugar-degradation by-products.

The aim of a pretreatment is to facilitate the accessibility of cellulase enzymes to convert cellulose into fermentable sugars via hydrolysis. Since an adequate pretreatment is closely related to the digestibility of the material, pretreatment choice has a noticeable impact on ethanol yield after fermentation. Apart from the type of chemical catalyst, other pretreatment conditions, such as temperature and reaction time, also have a significant influence on the overall process [12].

At this point, it should be clarified that the drying of pretreated cellulose fibers (performed at 105 °C in the present study) may cause hornification, meaning that the accessibility of enzymes may be hindered by the agglomeration of cellulose fibers [42]. However, for industrial ethanol production, the drying step would not be required, so the enzymatic accessibility of cellulose should not be compromised.

3.3. Enzymatic Saccharification of the Holocellulosic Fraction of SCB

The pretreated SCB hydrolysates showed the presence of saccharides such as glucose, xylose, cellotriose, and cellobiose (detailed for all process conditions in Table S2). The results in this study showed that the type of chemical catalyst exerted a more pronounced effect on the saccharide composition and concentration than temperatures or reaction times. For instance, a 33% higher glucose production was observed for NaOH pretreatments compared to the acid pretreatments (Table 2). Likewise, higher xylose contents were obtained from the NaOH pretreatment than for those consisting of diluted acid; this result is attributed to the lack of a degradation effect from NaOH on hemicellulose. Conversely, the acid pretreatments resulted in lower concentrations of xylose [16]. In all reactions, partial digestion products of cellulose, i.e., cellobiose and cellotriose, were detected (at concentrations between 2.22–10.41 and 0.84–4.43 $g{\cdot}L^{-1}$, respectively).

Table 2. Summary of the saccharide composition of SCB hydrolysate as a function of the different chemical pretreatments.

Saccharide		Chemical Pretreatment			
		NaOH	**Sulfuric Acid**	**Oxalic Acid**	**Maleic Acid**
Glucose ($g{\cdot}L^{-1}$)	Min. value	64.32 ± 0.07	22.56 ± 0.12	29.67 ± 0.09	25.71 ± 0.06
	Max. value	87.18 ± 4.63	63.83 ± 4.31	57.87 ± 0.84	69.40 ± 2.38
Xylose ($g{\cdot}L^{-1}$)	Min. value	29.51 ± 0.18	0.96 ± 0.30	0.52 ± 0.10	6.83 ± 0.08
	Max. value	35.88 ± 0.15	8.49 ± 0.17	6.76 ± 0.14	23.33 ± 0.68
Cellotriose ($g{\cdot}L^{-1}$)	Min. value	2.33 ± 0.08	0.84 ± 0.15	0.00 ± 0.00	0.00 ± 0.00
	Max. value	4.43 ± 0.18	2.08 ± 0.14	2.48 ± 0.14	3.17 ± 0.06
Cellobiose ($g{\cdot}L^{-1}$)	Min. value	5.48 ± 0.21	2.22 ± 0.42	3.32 ± 0.12	2.81 ± 0.11
	Max. value	10.41 ± 0.20	4.33 ± 0.11	4.25 ± 0.26	3.76 ± 0.15
Saccharide yield (%)	Min. value	91.14 ± 0.65	22.29 ± 0.33	30.94 ± 0.50	28.51 ± 0.47
	Max. value	99.78 ± 2.88	47.38 ± 3.37	44.33 ± 0.57	67.02 ± 3.13

The data correspond to maximum and minimum values obtained at different temperatures (130, 140, 150, 160, and 170 °C) and different reaction times (10, 20, and 30 min) for each temperature.

Xylose released from hemicellulose hydrolysis can be fermented to produce ethanol by certain yeast strains and bacteria. A great number of genetically modified yeast capable of fermenting both hexoses and pentoses have been developed in recent years [43]. However, in this study, we tested a non-genetically modified *Saccharomyces cerevisiae* strain that can only convert glucose to ethanol. Thus, the calculations of ethanol yields in this study were only focused on glucose fermentation (although it should be noted that the hemicellulose fraction that yields predominantly pentose sugars in the context of a biorefinery may be converted into ethanol by using recombinant yeasts or bacteria).

The impact of the pretreatment temperature and reaction times was also studied. The results showed that an increase in temperature and reaction time resulted in an increase in the amount of glucose and xylose released from cellulose and hemicellulose, respectively. Glucose and xylose release was higher when using biomass pretreated at a high temperature and extended time. The increase factor was 3.86 and 69 times higher in the case of glucose and xylose, respectively. These figures were obtained taking into account the maximum and minimum values experimentally registered from all the chemical pretreatments. However, the statistical analysis showed no significant differences ($p > 0.05$) as a function of the reaction time (in the two-way and three-way interactions), showing a maximum variation of 0.2% for glucose and xylose.

Conversely, there were significant differences ($p < 0.05$) depending on the temperature of the pretreatments: The highest saccharide yields were attained with NaOH at 160 and 170 °C. According to Tukey's HSD tests (based on saccharide yield), the combination of NaOH (1% *w/v*), 160 °C, and 20 min may be considered the most suitable conditions for SCB pretreatment.

The saccharide yield was determined after 72 h of enzymatic saccharification, showing higher values obtained from NaOH pretreatment (data are summarized in Table 2). The results were similar to those reported by Qing et al. [40], who found that soybean hull and straw pretreated with alkali showed better yields as compared to acid-pretreated samples (86.9 and 75.6%, respectively).

3.4. Batch Fermentation

To establish which process performed the best in terms of yield, it was necessary to determine the ethanol production efficiency. The influence of the different pretreatments on ethanol yield is summarized in Table 3 (detailed in Table S3 for all process conditions). All the maximum fermentation yield values are very similar as seen in Table 3, which demonstrates the satisfactory performance of the yeast strain. Nevertheless, the ethanol concentration is a function not only of the yeast's fermentation yield, but also of the fermentable sugar concentration in the wort. According to our experiments, the biomass that was enzymatically hydrolyzed and previously pretreated with NaOH, yielded more fermentable sugar concentration compared to the three different acid pretreatment experiments above reported in Table 2. The ethanol yield of this experiment was calculated based on the theoretical 0.51 g ethanol produced per g glucose consumed.

Table 3. Summary of ethanol produced by fermentation of pretreated SCB hydrolysate for the different pretreatment conditions.

		Chemical Pretreatment			
		NaOH	**Sulfuric Acid**	**Oxalic Acid**	**Maleic Acid**
Ethanol ($g \cdot L^{-1}$)	Min. value	29.53 ± 0.11	9.50 ± 1.22	11.42 ± 0.10	12.5 ± 0.10
	Max. value	43.91 ± 1.86	32.17 ± 1.81	24.76 ± 0.64	34.03 ± 1.52
Ethanol yield (%)	Min. value	78.63 ± 0.16	82.64 ± 10.92	75.47 ± 0.63	93.37 ± 0.67
	Max. value	99.85 ± 0.38	99.42 ± 0.21	99.55 ± 0.43	97.92 ± 1.43

The data correspond to maximum and minimum values obtained at different temperatures (130, 140, 150, 160, and 170 °C) and different reaction times (10, 20, and 30 min) for each temperature.

According to Tyagi et al. [44], alkaline pretreatments of SCB can result in a cellulose recovery of 81% and delignification of 68.5%. The comparison of the means of the treatments used in this study showed significant differences ($p < 0.05$) in ethanol concentration influenced by different chemical pretreatments. The results showed that the NaOH-pretreated SCB hydrolysates exhibited the highest glucose concentrations, yielding up to 43.91 $g \cdot L^{-1}$ of ethanol, as expected.

Regarding the theoretical ethanol yields, a range between 97.92 and 99.85% was reached, and no significant differences among pretreatments were found ($p > 0.05$). The attainment of these high yields may be ascribed to: (i) Selection of an adequate pretreatment, which does not release by-products that may act as fermentation inhibitors for *S. cerevisiae* [45]; (ii) selection of yeast strains that can

efficiently convert sugars to ethanol [46]; (iii) acclimation of yeasts in pretreated biomass hydrolysates as culture medium; and (iv) proper carbon:nitrogen proportion in the wort. The combination of the aforementioned factors made it possible to eliminate the inhibitory effect, resulting in a satisfactory yeast performance.

3.5. The SFSS System

The kinetics of glucose and ethanol production for the SFSS system of the prehydrolyzed SCB with high solids load (25% *w/v*) during a period of 30 days are shown in Figure 1. The yeast strain (CLQCA-INT-005) was added to the system after an acclimation process. A lag phase of 5 days occurred before the fermentation attained the steady state. Then, during the following 25 days of fermentation, glucose consumption was almost complete, remaining less than 1% in the medium.

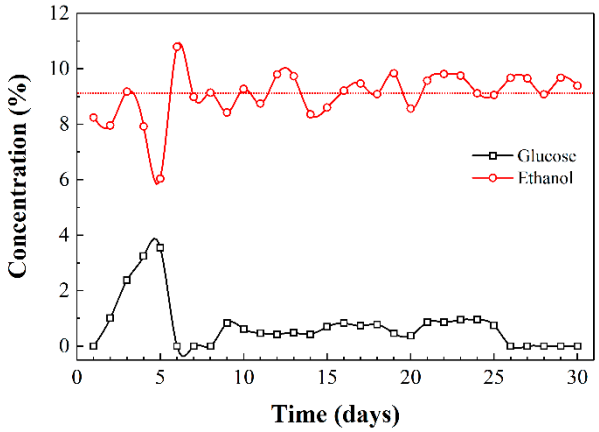

Figure 1. Semi-continuous fermentation and simultaneous saccharification (SFSS) kinetics of the cellulosic fraction of SCB using *Saccharomyces cerevisiae*: Ethanol concentration (% *v/v*) and glucose concentration (%).

Consequently, with glucose consumption, the production of ethanol was constant, showing an average concentration of 9.07% *v/v* (which corresponded to 71.57 $g{\cdot}L^{-1}$), with a standard deviation of 0.85, during the whole experiment. Ethanol concentration did not show significant differences ($p > 0.05$) in production until the end of the experiment.

At an industrial scale, a compromise between concentration and fermenter residence time for ethanol production needs to be found. A minimum of 40 $g{\cdot}L^{-1}$ of ethanol is necessary for the process to be profitable. Xu et al. [29] and Dahnum et al. [22] reported that SSF was more efficient in terms of ethanol productivity, exhibiting advantages—such as shorter production time and better operational and financial feasibility—compared to SHF systems.

Some studies have reported that the gradual addition of substrate and/or enzymes improves enzymatic hydrolysis, which, in turn, allows the increase in substrate load (>10% solids); thus, the increase in sugar concentration and ethanol production was possible [28]. According to Sotaniemi et al. [47], a 10% increase in enzymatic hydrolysis can be reached in SSF systems in fed-batch compared to batch systems. However, previous studies have shown an unfavorable correlation between the initial solids loading and cellulose conversion, which mainly results in high viscosity of the hydrolyzed sludge in the reactor [48]. To overcome these limitations, the prehydrolysis of biomass prior to SSF allows high loads of solids to be handled with lower sludge viscosity, resulting in higher ethanol productivity.

Over the past two years, other approaches have been developed by modifying SSF in different manners. Aiming at increasing ethanol yields, the mix of first and second generation sources, such as sweet sorghum with its stalks or corn cobs with tea seed cake [49,50], has been proposed. Other variations to the SSF method have included pre-saccharification, fed batch, and a high-density yeast inoculum [51–53]. A summary of the raw materials, main process parameters, and ethanol yields

in these studies and in other recent reports on SSF modification strategies is presented in Table 4. Reported fermentation times varied from 36 to 216 h; the amounts of enzymes used ranged from 6 to 30 FPU; and the ethanol percentages attained were in the 1.9 to 15.26% interval.

Table 4. Comparison of different strategies for ethanol production using *Saccharomyces cerevisiae* strains reported in literature and in this study.

Process [a]	Raw Material	Enzymes (FPU/g cellulose)	Fermentation Time (h)	Ethanol Concentration (% *v/v*) [b]	References
SFSS	Sugarcane bagasse	6	24	9.07	This study
SSJcF	Sweet sorghum (stalk and juice)	20	216	15.26	[49]
Co-feeding SSF	Corncob residues and tea-seed cake	10	120	10.96	[50]
Fed batch SSF	Sugarcane bagasse and waste *Dioscorea composita*	15	120	10.4	[51]
SSF	Pine stumps	15	72	10.01	[54]
mSSF	Switchgrass	28.5 [c]	72	9.16	[29]
Fed non-isothermal SSF	Chips of paper mulberry	8.33	72	8.09	[55]
Fed batch SSCF	Corn stover	6	48	7.57	[56]
SSF	Corncob residues	15	60	5.94	[57]
PSSSF	Sugarcane straw	14.5	45	5.7	[52]
Fed batch SSF	Sugarcane bagasse	10	72	5.46	[58]
PSSF	Corn cobs	30	48	4.67	[59]
Fed batch SSF	Sugarcane bagasse	15	40	3.72	[60]
HCDC and SSF	Corn stover	15	36	2.15	[53]
dSSF	Oat-hull pulp	– [d]	72	1.90	[61]

[a] SFSS: Semi-continuous fermentation and simultaneous saccharification; SSJcF: Simultaneous saccharification and juice co-fermentation; SSF: Simultaneous saccharification and fermentation; mSSF: Modified simultaneous saccharification and fermentation; SSCF: Simultaneous saccharification and co-fermentation; PSSSF: Pre-saccharification and simultaneous saccharification and fermentation; PSSF: Prehydrolysis and simultaneous saccharification and fermentation; HCDC and SSF: High cell-density culture and simultaneous saccharification and fermentation; dSSF: Simultaneous saccharification and fermentation with delayed yeast inoculation. [b] Ethanol concentration in % (*v/v*) was calculated from ethanol concentration in $g{\cdot}L^{-1}$ (specific gravity of ethanol 0.789 at 25 °C). [c] Taking an average FPU for Accellerase 1500 (52.0–62.0 FPU/mL) and considering the reported dose (0.5 mL/g cellulose). [d] Not reported in FPU/g substrate. A combination of CelloLux-A (0.04 g/g substrate) and BrewZyme BGX (0.2 mL/g substrate) was used.

It is worth noting that the ethanol concentration obtained in this work was among the highest ones. If operational factors (viz., enzyme concentration and time) are weighted in, the SFSS strategy (6 FPU cellulases, 24 h process) has an unmatched performance (9.07% ethanol yield). In the works in which higher ethanol yields were attained (Table 4), fermentation times were at least three times longer, and enzyme concentration was at least 67% higher. In view of its efficiency, the proposed strategy holds enough promise for cellulosic ethanol production to deserve further examination in a pilot-scale.

4. Conclusions

The pretreatment with NaOH (1%, *w/v*) at 160 °C for 20 min was the most efficient for further conversion of cellulose into fermentable sugars. The SFSS system was used for enzymatic saccharification and fermentation of SCB, using a high proportion of solid feedstock to liquid (25% solids), and harvesting the fermented product every 24 h. Monitoring of the SFSS system over 30-days showed an average ethanol concentration of 9.07% (*v/v*) and <1% of residual glucose, demonstrating that the system can reach a steady state with no significant variations during the whole process. The proposed SFSS system would have potential advantages in terms of: (i) Reduction of the cellulase/xylanase enzymes use; (ii) the addition of high solids load; (iii) continuous production of ethanol in a stable system; (iv) ability for daily processing of ethanol; and (v) a more practical and simplified operational system. All these improvements would have a highly positive impact on the economy of cellulosic ethanol production at the industrial scale, and call for further studies.

Supplementary Materials: The following are available online at http://www.mdpi.com/2227-9717/8/11/1459/s1, Figure S1: ATR-FTIR spectra of the solid fraction recovered after alkali and acid pretreatments at 160 °C for 20 min; Table S1: Solid fraction recovery of pretreated SCB as a function of pretreatment conditions; Table S2: Saccharide composition of SCB hydrolysate as a function of pretreatment conditions; Table S3: Ethanol produced by fermentation of pretreated SCB hydrolysate as a function of pretreatment conditions.

Author Contributions: Conceptualization, E.J.C.B. and P.P.B.; methodology, E.J.C.B. and J.M.-G.; validation, E.J.C.B.; P.M.-R., and J.M.-G.; formal analysis, P.P.B., J.C.B, J.M.-G., and P.M.-R.; investigation, P.P.B. and B.B.M.; resources, E.J.C.B. and J.M.-G.; writing—original draft preparation, P.P.B., E.J.C.B., J.M.-G., and P.M.-R.; writing—review and editing, P.P.B., E.J.C.B., J.M.-G., and P.M.-R.; visualization, P.M.-R.; supervision, E.J.C.B. and J.M.-G.; project administration, E.J.C.B.; funding acquisition, E.J.C.B. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Acknowledgments: The authors would like to thank the Neotropical Center for Biomass Research (CNIB) of the Pontifical Catholic University of Ecuador (PUCE) for financial support during this investigation. The authors also thank Novozymes for providing the enzymes used in this study. Instituto Universitario de Investigacion en Ciencias Ambientales de Aragón (IUCA)—Universidad de Zaragoza is gratefully acknowledged for covering the APCs.

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

References

1. Binod, P.; Gnansounou, E.; Sindhu, R.; Pandey, A. Enzymes for second generation biofuels: Recent developments and future perspectives. *Bioresour. Technol. Rep.* **2019**, *5*, 317–325. [CrossRef]
2. Chen, H.; Fu, X. Industrial technologies for bioethanol production from lignocellulosic biomass. *Renew. Sustain. Energy Rev.* **2016**, *57*, 468–478. [CrossRef]
3. Rastogi, M.; Shrivastava, S. Recent advances in second generation bioethanol production: An insight to pretreatment, saccharification and fermentation processes. *Renew. Sustain. Energy Rev.* **2017**, *80*, 330–340. [CrossRef]
4. Lynd, L.R.; Liang, X.; Biddy, M.J.; Allee, A.; Cai, H.; Foust, T.; Himmel, M.E.; Laser, M.S.; Wang, M.; Wyman, C.E. Cellulosic ethanol: Status and innovation. *Curr. Opin. Biotechnol.* **2017**, *45*, 202–211. [CrossRef]
5. Manochio, C.; Andrade, B.; Rodriguez, R.; Moraes, B. Ethanol from biomass: A comparative overview. *Renew. Sustain. Energy Rev.* **2017**, *80*, 743–755. [CrossRef]
6. Nanda, S.; Azargohar, R.; Dalai, A.K.; Kozinski, J.A. An assessment on the sustainability of lignocellulosic biomass for biorefining. *Renew. Sustain. Energy Rev.* **2015**, *50*, 925–941. [CrossRef]
7. Robak, K.; Balcerek, M. Review of Second-Generation Bioethanol Production from Residual Biomass. *Food Technol. Biotechnol.* **2018**, *56*, 174–187. [CrossRef] [PubMed]
8. Hassan, S.S.; Williams, G.; Jaiswal, A.K. Emerging technologies for the pretreatment of lignocellulosic biomass. *Bioresour. Technol.* **2018**, *262*, 310–318. [CrossRef] [PubMed]
9. Da Silva, A.S.; Inoue, H.; Endo, T.; Yano, S.; Bon, E.P.S. Milling pretreatment of sugarcane bagasse and straw for enzymatic hydrolysis and ethanol fermentation. *Bioresour. Technol.* **2010**, *101*, 7402–7409. [CrossRef]
10. Duque, A.; Manzanares, P.; Ballesteros, M. Extrusion as a pretreatment for lignocellulosic biomass: Fundamentals and applications. *Renew. Energy* **2017**, *114*, 1427–1441. [CrossRef]
11. Kumar, M.; Oyedun, A.O.; Kumar, A. A review on the current status of various hydrothermal technologies on biomass feedstock. *Renew. Sustain. Energy Rev.* **2018**, *81*, 1742–1770. [CrossRef]
12. Zhang, X.; Yuan, Q.; Cheng, G. Deconstruction of corncob by steam explosion pretreatment: Correlations between sugar conversion and recalcitrant structures. *Carbohydr. Polym.* **2017**, *156*, 351–356. [CrossRef]
13. Aguilar-Reynosa, A.; Romaní, A.; Rodríguez-Jasso, R.M.; Aguilar, C.N.; Garrote, G.; Ruiz, H.A. Microwave heating processing as alternative of pretreatment in second-generation biorefinery: An overview. *Energy Convers. Manag.* **2017**, *136*, 50–65. [CrossRef]
14. Liyakathali, N.A.M.; Muley, P.D.; Aita, G.M.; Boldor, D. Effect of frequency and reaction time in focused ultrasonic pretreatment of energy cane bagasse for bioethanol production. *Bioresour. Technol.* **2016**, *200*, 262–271. [CrossRef]
15. Nair, R.B.; Kalif, M.; Ferreira, J.A.; Taherzadeh, M.J.; Lennartsson, P.R. Mild-temperature dilute acid pretreatment for integration of first and second generation ethanol processes. *Bioresour. Technol.* **2017**, *245*, 145–151. [CrossRef] [PubMed]
16. Kim, J.S.; Lee, Y.; Kim, T.H. A review on alkaline pretreatment technology for bioconversion of lignocellulosic biomass. *Bioresour. Technol.* **2016**, *199*, 42–48. [CrossRef] [PubMed]

17. Zhang, Q.; Hu, J.; Lee, D.-J. Pretreatment of biomass using ionic liquids: Research updates. *Renew. Energy* **2017**, *111*, 77–84. [CrossRef]
18. Chen, H.; Zhao, J.; Hu, T.; Zhao, X.; Liu, D. A comparison of several organosolv pretreatments for improving the enzymatic hydrolysis of wheat straw: Substrate digestibility, fermentability and structural features. *Appl. Energy* **2015**, *150*, 224–232. [CrossRef]
19. Elumalai, S.; Agarwal, B.; Runge, T.M.; Sangwan, R.S. Advances in Transformation of Lignocellulosic Biomass to Carbohydrate-Derived Fuel Precursors. *Biofuel Biorefi. Technol.* **2017**, 87–116. [CrossRef]
20. Behera, S.; Arora, R.; Nandhagopal, N.; Kumar, S. Importance of chemical pretreatment for bioconversion of lignocellulosic biomass. *Renew. Sustain. Energy Rev.* **2014**, *36*, 91–106. [CrossRef]
21. Ravindran, R.; Jaiswal, A.K. A comprehensive review on pre-treatment strategy for lignocellulosic food industry waste: Challenges and opportunities. *Bioresour. Technol.* **2016**, *199*, 92–102. [CrossRef] [PubMed]
22. Dahnum, D.; Tasum, S.O.; Triwahyuni, E.; Nurdin, M.; Abimanyu, H. Comparison of SHF and SSF Processes Using Enzyme and Dry Yeast for Optimization of Bioethanol Production from Empty Fruit Bunch. *Energy Procedia* **2015**, *68*, 107–116. [CrossRef]
23. Carvajal, Y.A.-; Corsano, G.; González-Cortés, M.; Suárez, E.G. Preliminary design for simultaneous saccharification and fermentation stages for ethanol production from sugar cane bagasse. *Chem. Eng. Res. Des.* **2017**, *126*, 232–240. [CrossRef]
24. Olofsson, K.; Bertilsson, M.; Lidén, G. A short review on SSF—An interesting process option for ethanol production from lignocellulosic feedstocks. *Biotechnol. Biofuels* **2008**, *1*, 7. [CrossRef] [PubMed]
25. Hasunuma, T.; Kondo, A. Consolidated bioprocessing and simultaneous saccharification and fermentation of lignocellulose to ethanol with thermotolerant yeast strains. *Process. Biochem.* **2012**, *47*, 1287–1294. [CrossRef]
26. Guo, H.; Chang, Y.; Lee, D.-J. Enzymatic saccharification of lignocellulosic biorefinery: Research focuses. *Bioresour. Technol.* **2018**, *252*, 198–215. [CrossRef] [PubMed]
27. Choudhary, J.; Singh, S.; Nain, L. Bioprospecting thermotolerant ethanologenic yeasts for simultaneous saccharification and fermentation from diverse environments. *J. Biosci. Bioeng.* **2017**, *123*, 342–346. [CrossRef] [PubMed]
28. Du, J.; Cao, Y.; Liu, G.; Zhao, J.; Li, X.; Qu, Y. Identifying and overcoming the effect of mass transfer limitation on decreased yield in enzymatic hydrolysis of lignocellulose at high solid concentrations. *Bioresour. Technol.* **2017**, *229*, 88–95. [CrossRef]
29. Xu, Y.; Li, J.; Zhang, M.; Wang, D. Modified simultaneous saccharification and fermentation to enhance bioethanol titers and yields. *Fuel* **2018**, *215*, 647–654. [CrossRef]
30. Ramos, L.P.; Da Silva, L.; Ballem, A.C.; Pitarelo, A.P.; Chiarello, L.M.; Silveira, M.H.L. Enzymatic hydrolysis of steam-exploded sugarcane bagasse using high total solids and low enzyme loadings. *Bioresour. Technol.* **2015**, *175*, 195–202. [CrossRef]
31. Cheng, N.; Koda, K.; Tamai, Y.; Yamamoto, Y.; Takasuka, T.E.; Uraki, Y. Optimization of simultaneous saccharification and fermentation conditions with amphipathic lignin derivatives for concentrated bioethanol production. *Bioresour. Technol.* **2017**, *232*, 126–132. [CrossRef] [PubMed]
32. Dale, B.; Leong, C.; Pham, T.; Esquivel, V.; Ríos, I.; Latimer, V. Hydrolysis of lignocellulosics at low enzyme levels: Application of the AFEX process. *Bioresour. Technol.* **1996**, *56*, 111–116. [CrossRef]
33. McIntosh, S.; Vancov, T. Optimisation of dilute alkaline pretreatment for enzymatic saccharification of wheat straw. *Biomass Bioenergy* **2011**, *35*, 3094–3103. [CrossRef]
34. Adney, B.; Baker, J. *Measurement of Cellulase Activities Laboratory Analytical Procedure (LAP)*; National Renewable Energy Laboratory: Golden, CO, USA, 2008.
35. Li, H.; Xiong, L.; Chen, X.; Wang, C.; Qi, G.; Huang, C.; Luo, M.; Chen, X. Enhanced enzymatic hydrolysis and acetone-butanol-ethanol fermentation of sugarcane bagasse by combined diluted acid with oxidate ammonolysis pretreatment. *Bioresour. Technol.* **2017**, *228*, 257–263. [CrossRef]
36. Qin, L.; Li, X.; Liu, L.; Zhu, J.-Q.; Guan, Q.-M.; Zhang, M.-T.; Li, W.-C.; Li, B.-Z.; Yuan, Y. Dual effect of soluble materials in pretreated lignocellulose on simultaneous saccharification and co-fermentation process for the bioethanol production. *Bioresour. Technol.* **2017**, *224*, 342–348. [CrossRef] [PubMed]
37. Hilares, R.T.; Ienny, J.V.; Marcelino, P.F.; Ahmed, M.A.; Antunes, F.A.F.; Da Silva, S.S.; Dos Santos, J. Ethanol production in a simultaneous saccharification and fermentation process with interconnected reactors employing hydrodynamic cavitation-pretreated sugarcane bagasse as raw material. *Bioresour. Technol.* **2017**, *243*, 652–659. [CrossRef]

38. Castañón-Rodríguez, J.; Torrestiana-Sánchez, B.; Montero-Lagunes, M.; Portilla-Arias, J.; De León, J.R.; Aguilar-Uscanga, M. Using high pressure processing (HPP) to pretreat sugarcane bagasse. *Carbohydr. Polym.* **2013**, *98*, 1018–1024. [CrossRef] [PubMed]
39. Chang, M.; Li, D.; Wang, W.; Chen, D.; Zhang, Y.; Hu, H.; Ye, X. Comparison of sodium hydroxide and calcium hydroxide pretreatments on the enzymatic hydrolysis and lignin recovery of sugarcane bagasse. *Bioresour. Technol.* **2017**, *244*, 1055–1058. [CrossRef]
40. Qing, Q.; Guo, Q.; Zhou, L.; Gao, X.; Lu, X.; Zhang, Y. Comparison of alkaline and acid pretreatments for enzymatic hydrolysis of soybean hull and soybean straw to produce fermentable sugars. *Ind. Crop. Prod.* **2017**, *109*, 391–397. [CrossRef]
41. Mathew, A.K.; Parameshwaran, B.; Sukumaran, R.K.; Pandey, A. An evaluation of dilute acid and ammonia fiber explosion pretreatment for cellulosic ethanol production. *Bioresour. Technol.* **2016**, *199*, 13–20. [CrossRef]
42. Luo, X.; Zhu, J.Y. Effects of drying-induced fiber hornification on enzymatic saccharification of lignocelluloses. *Enzym. Microb. Technol.* **2011**, *48*, 92–99. [CrossRef]
43. Lee, Y.-G.; Jin, Y.-S.; Cha, Y.-L.; Seo, J.-H. Bioethanol production from cellulosic hydrolysates by engineered industrial Saccharomyces cerevisiae. *Bioresour. Technol.* **2017**, *228*, 355–361. [CrossRef] [PubMed]
44. Tyagi, S.; Lee, K.-J.; Mulla, S.I.; Garg, N.; Chae, J.-C. *Chapter 2—Production of Bioethanol from Sugarcane Bagasse: Current Approaches and Perspectives*; Shukla, P., Ed.; Academic Press: Cambridge, MA, USA, 2019; pp. 21–42.
45. Tesfaw, A.; Assefa, F. Current Trends in Bioethanol Production by Saccharomyces cerevisiae: Substrate, Inhibitor Reduction, Growth Variables, Coculture, and Immobilization. *Int. Sch. Res. Not.* **2014**, *2014*, 532852. [CrossRef] [PubMed]
46. Tomás-Pejó, E.; Olsson, L. Influence of the propagation strategy for obtaining robust Saccharomyces cerevisiae cells that efficiently co-ferment xylose and glucose in lignocellulosic hydrolysates. *Microb. Biotechnol.* **2015**, *8*, 999–1005. [CrossRef]
47. Sotaniemi, V.-H.; Taskila, S.; Ojamo, H.; Tanskanen, J. Controlled feeding of lignocellulosic substrate enhances the performance of fed-batch enzymatic hydrolysis in a stirred tank reactor. *Biomass- Bioenergy* **2016**, *91*, 271–277. [CrossRef]
48. Hodge, D.B.; Karim, M.N.; Schell, D.J.; McMillan, J.D. Soluble and insoluble solids contributions to high-solids enzymatic hydrolysis of lignocellulose. *Bioresour. Technol.* **2008**, *99*, 8940–8948. [CrossRef]
49. Zhang, C.; Wen, H.; Chen, C.; Cai, D.; Fu, C.; Li, P.; Qin, P.; Tan, T. Simultaneous saccharification and juice co-fermentation for high-titer ethanol production using sweet sorghum stalk. *Renew. Energy* **2019**, *134*, 44–53. [CrossRef]
50. Zheng, T.; Yu, H.; Liu, S.; Jiang, J.; Wang, K. Achieving high ethanol yield by co-feeding corncob residues and tea-seed cake at high-solids simultaneous saccharification and fermentation. *Renew. Energy* **2020**, *145*, 858–866. [CrossRef]
51. Ye, G.; Zeng, D.; Zhang, S.; Fan, M.; Zhang, H.; Xie, J. Ethanol production from mixtures of sugarcane bagasse and Dioscorea composita extracted residue with high solid loading. *Bioresour. Technol.* **2018**, *257*, 23–29. [CrossRef]
52. Pratto, B.; Dos Santos-Rocha, M.S.R.; Longati, A.A.; Júnior, R.D.S.; Cruz, A.J.G. Experimental optimization and techno-economic analysis of bioethanol production by simultaneous saccharification and fermentation process using sugarcane straw. *Bioresour. Technol.* **2020**, *297*, 122494. [CrossRef]
53. Shi, X.; Liu, Y.; Dai, J.; Liu, X.; Dou, S.; Teng, L.; Meng, Q.; Lu, J.; Ren, X.; Wang, R. A novel integrated process of high cell-density culture combined with simultaneous saccharification and fermentation for ethanol production. *Biomass Bioenergy* **2019**, *121*, 115–121. [CrossRef]
54. Mendes, C.V.T.; Vergara, P.; Carbajo, J.M.; Villar, J.C.; Rocha, J.M.D.S.; de Sousa Carvalho, M.D.G.V. Bioconversion of pine stumps to ethanol: Pretreatment and simultaneous saccharification and fermentation. *Holzforschung* **2020**, *74*, 212–216. [CrossRef]
55. Wang, Z.; Ning, P.; Hu, L.; Nie, Q.; Liu, Y.; Zhou, Y.; Yang, J. Efficient ethanol production from paper mulberry pretreated at high solid loading in Fed-nonisothermal-simultaneous saccharification and fermentation. *Renew. Energy* **2020**, *160*, 211–219. [CrossRef]
56. Zhu, J.-Q.; Zong, Q.-J.; Li, W.-C.; Chai, M.-Z.; Xu, T.; Liu, H.; Fan, H.; Li, B.-Z.; Yuan, Y.-J. Temperature profiled simultaneous saccharification and co-fermentation of corn stover increases ethanol production at high solid loading. *Energy Convers. Manag.* **2020**, *205*, 112344. [CrossRef]
57. Zhao, W.; Zhao, F.; Zhang, S.; Gong, Q.; Chen, G. Ethanol production by simultaneous saccharification and cofermentation of pretreated corn stalk. *J. Basic Microbiol.* **2019**, *59*, 744–753. [CrossRef]

58. Yu, N.; Tan, L.; Sun, Z.-Y.; Nishimura, H.; Takei, S.; Tang, Y.-Q.; Kida, K. Bioethanol from sugarcane bagasse: Focused on optimum of lignin content and reduction of enzyme addition. *Waste Manag.* **2018**, *76*, 404–413. [CrossRef] [PubMed]
59. Sewsynker-Sukai, Y.; Kana, E.G. Simultaneous saccharification and bioethanol production from corn cobs: Process optimization and kinetic studies. *Bioresour. Technol.* **2018**, *262*, 32–41. [CrossRef]
60. Guilherme, A.D.A.; Dantas, P.V.F.; Padilha, C.E.D.A.; Dos Santos, E.S.; De Macedo, G.R. Ethanol production from sugarcane bagasse: Use of different fermentation strategies to enhance an environmental-friendly process. *J. Environ. Manag.* **2019**, *234*, 44–51. [CrossRef]
61. Mironova, G.; Skiba, E.A.; Kukhlenko, A.A. Optimization of pre-saccharification time during dSSF process in oat-hull bioethanol technology. *3 Biotech* **2019**, *9*, 455. [CrossRef]