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Effect of Steam Explosion Pretreatment on Improving Enzymatic Hydrolysis and Alcoholic Fermentation of Corn Stover

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Abstract

Corn stover (CS) is a promising feedstock for producing fermentable sugars (FS) and ethanol due to its high holocellulose content. This study investigated the enhancement of FS production via enzymatic hydrolysis (EH) following steam explosion (SE) pretreatment of CS, aiming to improve ethanol yields through alcoholic fermentation. SE was conducted at 160 °C, 180 °C, and 200 °C for 2.5 and 10 min. EH was performed using 15 and 30 filter paper unit (FPU) enzyme loadings and 1% and 2% (*w*/*w*) substrate concentrations, applied to both untreated CS and pretreated CS (PCS). Fermentations with *Saccharomyces cerevisiae* were carried out at 30 °C and 120 rpm. The optimal SE condition (200 °C, 10 min) yielded 4.25 g of total sugars per 100 g CS. Under 30 FPU and 2% PCS, EH yielded 41.57 g/100 g PCS and 18.51 g/100 g CS. The combined SE + EH process reached 45.82 g/100 g PCS, about 2.5 times more than EH alone. The highest ethanol concentration and production rate were 15.54 g/L and 1.30 g/L·h, with a yield of 0.47 g/g. Under optimal conditions, fermentation achieved a conversion efficiency of 92.33%.

Keywords: biomass; hydrothermal process; inhibitors; cellulosic ethanol

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1. Introduction

Glucose, cellobiose, xylose, and arabinose are fermentable sugars and chemical precursors to obtain 2G ethanol [1–3]. Lignocellulosic biomass residues are potential sources for obtaining these sugars as they are abundant and inexpensive [4] and not intended for human food purposes [5].

There are diverse primary crops cultivated around the world but just four account for half the global production: sugarcane (20%, 1.9 billion tons), corn (13%, 1.24 billion tons), wheat (8%, 0.80 billion tons), and rice (5%, 0.51 billion tons) [6]. Considering only the worldwide production of grain, corn is the most produced (44.6%, 1.24 billion tons), followed by wheat (28.4%, 0.80 billion tons) and rice (16.6% 0.51 billion tons) [7]. The largest corn producers are the United States (0.39 billion tons, 32%), China (0.29 billion tons, 24%), and Brazil (0.12 billion tons, 10%) [8].

The lignocellulosic residues of corn consist of leaf, tassel, stalk, straw, and corn cob (Figure 1). The amount generated by these residues, on a dry basis, is 1 $kg_{residue}/kg_{corn\ grain}$ [9]. Therefore, the production of 1.24 billion tons of corn grains will produce the same amount of residue in the form of leaf, tassel, stalk, straw, and corn cob.

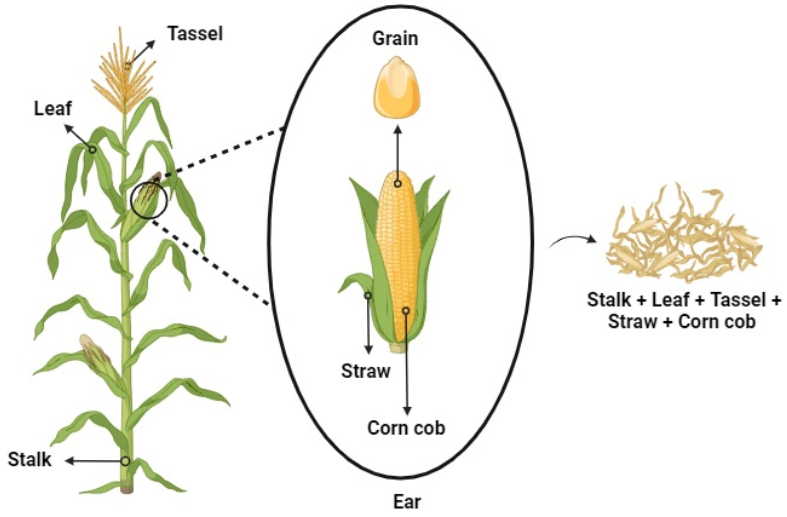


Figure 1. Components of the plant (*Gramineae* family and *Zea mays* species) and its residue.

Corn stover (CS) refers to a mixture of straw, corn cob, leave, and stalks in mass proportions of 8%, 15%, 21–28%, and 48–56% [10–12], respectively. These residues are left on the field after harvest and can be used as soil cover or as fuel in combustion processes for energy generation [13]. Tassel is also considered a residue, but its mass percentage contribution is negligible compared with other corn residues. CS is classified as a lignocellulosic residue with a complex and recalcitrant structure [14]. The typical composition of CS is approximately 70% cellulose and hemicellulose, and 15–20% lignin [10]. Specifically, CS contains about 40.7% cellulose, 31.1% hemicellulose, and 11.7% lignin [15]. However, several studies have reported cellulose contents ranging from 32.4 to 40.0%, hemicellulose from 16.7 to 27.7%, and lignin from 13.0 to 21.2% [16–19].

The production of fermentable sugars from lignocellulosic residues involves pretreatment and hydrolysis. Pretreatment is necessary to reduce the biomass recalcitrance caused by lignin [20]. Steam explosion (SE) is a physicochemical pretreatment that involves exposing the biomass to high temperature and pressure, typically using water, followed by mechanical disruption through a rapid depressurization (explosion) process [21]. SE is carried out under relatively mild operating conditions, such as temperatures ranging from 160 to 260 °C, pressures between 0.69–4.83 MPa, and relatively short residence times (5–30 min) [22,23].

The biomass is initially exposed to saturated steam for a specific period, allowing high vapor-phase diffusion to enhance accessibility to its inner structures. Heating occurs through steam condensation, and the capillary-like microporous structure of the biomass becomes saturated with hot liquid water. This process leads to the release of acids from the hemicellulose fraction, lowering the pH to around 3–4 [24,25]. The acidic environment breaks down the lignocellulosic structure and hydrolyzes the hemicellulose (autohydrolysis), thereby improving enzymatic hydrolysis [26]. At the end of the steam treatment, the pressure is rapidly released, discharging the liquid fraction of the biomass. The sudden drop to atmospheric pressure causes flash evaporation of the internal superheated water, triggering an explosive decompression of the biomass. This results in fiber rupture, defibration, and defibrillation [27]. During steam explosion, acids released from the lignocellulosic

matrix autocatalyze the saccharification of hemicellulose. Therefore, steam explosion (SE) is considered both a physical method (pressure-driven explosion) and a chemical method (autohydrolysis) [10].

Chemical and thermochemical hydrolysis processes, such as dilute acid or alkaline treatments, generally require higher energy inputs and involve additional costs related to chemical neutralization and equipment corrosion. In contrast, enzymatic hydrolysis operates under milder conditions, consuming significantly less energy while maintaining high selectivity and yield, despite the higher enzyme cost. Enzymatic hydrolysis (EH) is a suitable treatment because it requires less energy, operates under mild conditions (temperatures of 40–50 °C, atmospheric pressure, and pH of 4–5), and generates few or no inhibitors [28–30]. On the other hand, enzymatic hydrolysis requires longer processing times (5–12 h), and enzyme costs are relatively high, although advances have been made in reducing these costs [31,32].

Enzymatic hydrolysis (EH) is a suitable treatment because it requires less energy, operates under mild conditions (temperatures of 40–50 °C and pH of 4–5), and generates few or no inhibitors [28–30]. On the other hand, enzymatic hydrolysis requires longer processing times (5–12 h), and enzyme costs are relatively high, although advances have been made in reducing these costs [31,32].

While several studies have investigated the use of corn residues, most have done so individually. However, the harvest and separation of each residue in the field is labor-intensive and costly. The main objective of this study is to evaluate the use of mixture corn residues and to evaluate the effect of steam explosion pretreatment on enhancing the enzymatic hydrolysis efficiency and subsequent ethanol production from corn stover biomass. Specifically, this study aims to (i) determine the influence of different temperatures and residence times during steam explosion on the chemical composition and structural characteristics of pretreated corn stover; (ii) assess the enzymatic hydrolysis performance under different enzyme loadings and substrate concentrations; and (iii) investigate the fermentation efficiency of the obtained hydrolysates using Saccharomyces cerevisiae. By integrating these stages—pretreatment, enzymatic hydrolysis, and fermentation—this study seeks to identify optimal operational conditions that maximize fermentable sugar recovery and ethanol yield, contributing to the development of efficient and sustainable processes for second-generation bioethanol production.

2. Materials and Methods

2.1. Feedstock Characterization

Corn stover (a mixture of leaf, tassel, stalk, straw, and corn cob) was obtained from the cultivation of the agronomy faculty of the Federal University of Santa Maria. The corn stover in natura was oven-dried for 4 h at 60 °C, homogenized through size reduction using a hammer mill, and separated by sieving using Tyler series sieves. All characterization and experiments were performed with CS from the 16, 24, and 32 mesh. The samples were packaged in plastic films and stored at −5 °C to preserve their characteristics until the experimental essay. The CS in natura was characterized following the protocols described by the National Renewable Energy Laboratory (NREL) for contents of cellulose, hemicellulose and lignin (NREL/TP-510-42618), extractives (NREL/TP-510-42619), moisture (NREL/TP-510-42620), and ashes (NREL/TP-510-42622).

2.2. Experimental Procedures

Figure 2 shows the experimental setup used to convert lignocellulosic residue into fermentable sugars and ethanol. The corn stover processing involves three main steps: (A) pretreatment of corn stover in natura by steam explosion (SE); (B) enzymatic hydrolysis

(EH) treatment of both untreated corn stover (referred to as CS) and steam-exploded corn stover (referred to as PCS); and (C) alcoholic fermentation of the EH hydrolyzed. Detailed descriptions of steps (A), (B), and (C) are provided in Section 2.2.1, Section 2.2.2, and Section 2.2.3, respectively.

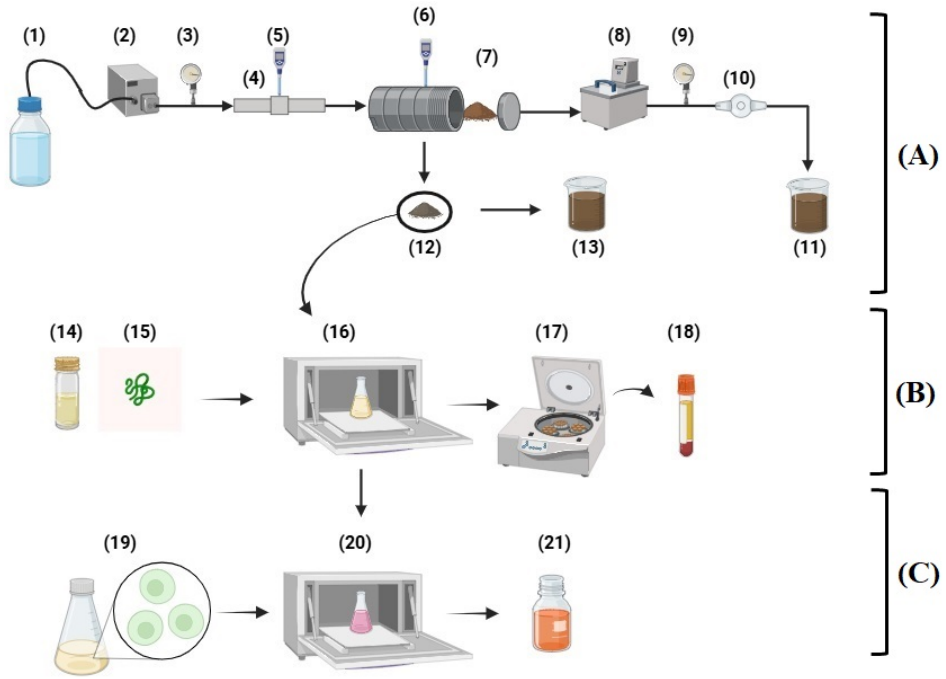


Figure 2. (**A**) Steam explosion (SE); (**B**) enzymatic hydrolysis (EH); and (**C**) alcoholic fermentation (AF).

2.2.1. Pretreatment by Steam Explosion (SE)

SE was carried out in a multipurpose unit equipped with a tank with distilled water (1), a pump (high-pressure) (2), a preheater (4), a reactor (81 mL) with heating system (7), a cooler (8), and accessories such as thermocouples (5, 6), manometers (3, 9), and a valve to control the system pressure (10) and to collect samples (11). The flow lines and the preheater are insulated to prevent heat loss. The multipurpose unit is capable of operating at ambient temperatures up to 400 °C, pressures from 0.1 to 35 MPa, and flow rates from 1 to 50 mL/min.

Steam explosion tests were carried out using 11.25 g of CS in natura. The CS was fed into the reactor and connected to the experimental unit. The operational conditions evaluated were temperatures of 160, 180, and 200 °C and reaction times of 2.5 and 10 min under a constant pressure of 5 MPa. The temperature range was selected based on typical values reported in previous studies [22,23,33], while the residence time included both typical and shorter durations to assess whether the effect on the steam explosion process was significant [22,23,33]. SE consisted of turning on the preheater (4) and reactor heating jackets (7) and setting the desired temperature of the reaction. Next, the system was fed at a water flow rate (2) of 20 mL/min and stood until the reactor water outlet temperature reached the temperature determined under the operating pressure (5 MPa). Afterward, the water outlet valve (10) was closed, the water flow was turned off (2), and the reaction time was started. After the reaction time ended, the valve (10) was quickly opened to instantly depressurize the system and consequently collect the liquid fraction (referred to as prehydrolyzed or P) (11). The solid fraction pretreated (PCS) (12) was collected, washed, and filtered with distilled water to neutralize and recover free sugars (the liquid fraction recovered from PCS was referred to as recovered prehydrolyzed or RP) (13).

The procedures were performed in duplicates. Untreated corn stover (without steam explosion pretreatment—CS in natura) was used as a negative control for comparison with pretreated samples.

The remaining solid fraction (PCS) was analyzed to determine the lignocellulosic content, and X-ray diffraction (XRD) (Rigaku Miniflex 300 X-ray) analysis was utilized to verify the crystallinity of the lignocellulosic structure. The radiation source was of the Cu kα type with 30 kV and 10 mA of 5–99.98°(2θ) and a step of 0.03°.

The crystallinity index (CrI) was determined by the peak height method [34].

$$\mathrm{CrI} = \left(\frac{\mathrm{I_{002}} - \mathrm{I_{am}}}{\mathrm{I_{002}}}\right) \cdot 100 \quad (1)$$

In addition, Equation (2) was used for the CrI calculation through the amorphous subtraction method [35].

$$\mathrm{CrI} = \left(\frac{\mathrm{A_c}}{\mathrm{A_c} + \mathrm{A_a}}\right) \cdot 100 \quad (2)$$

where I_{002} is the intensity of the peak of the crystalline region, I_{am} is the intensity of the peak of the amorphous region, A_c is the integrated area below the crystalline peaks, and A_a is the integrated area of the amorphous region. I_{002} and I_{am} are the intensity of the peaks for CS at 2θ near 22.0–22.6° and 18.7° [36–38], respectively, close to those indicated by Segal et al. [34], Sun et al. [39], and Nasir et al. [40] (22.0° and 18°). For the amorphous subtraction method, the XRD was decomposed using peak-fitting functions in Origin® 9.3 to obtain a broad amorphous halo and sharp peaks from reflections of the crystalline peaks based on Khan et al. [41] and Navarro-Pardo [35].

The CS in natura and PCS under the mildest steam explosion condition (SE/2.5/160) and the most severe condition (SE/10/200) were subjected to scanning electron microscopy (SEM) to evaluate the topographic structure of the biomass after steam explosion pretreatment. The images were captured at magnitudes and resolutions of 1000× and 10 µm and 5000× and 2 µm using a Sigma 300 VP scanning electron microscope (Carl Zeiss, Germany), equipped with a gemini column and a Schottky-type field emission filament (FEG) (tungsten filament coated with zirconium oxide). To obtain high-resolution images, a secondary electron detector was used, operating at an excitation voltage of 1 kV in high vacuum mode, with a vacuum greater than 10^{-9} mbar.

The prehydrolyzed (P) and recovered prehydrolyzed (RP) were frozen at −5 °C to preserve fermentable sugars until analyzed by high-performance liquid chromatography (HPLC) for characterization of the formation of sugars and inhibitors. HPLC (Proeminence UFLCXR, Shimadzu, Kyoto, Japan) was used for the determination of fermentable sugars and organic acids, through use of an Aminex HPX-87H chromatographic column and refractive index detector (RID) (Shimadzu, Kyoto, Japan). Inhibitors were determined by chromatographic column (Shimadzu, Kyoto, Japan) using a Shim-pack CLC-ODS(M) C18 and a photodiode detector (SPD-M20-A, Shimadzu, Kyoto, Japan).

Total yield of fermentable sugars ($Y_{SE,T}$), yield of hemicellulosic fermentable sugars ($Y_{SE,Hem}$), and yield of cellulosic fermentable sugars ($Y_{SE,Cel}$) were calculated by Equations (3)–(5), respectively. The results are in wt% which is equivalent to g/100 g.

$$\mathrm{Y_{SE,T}} = \frac{\mathrm{m_{SE,T}}}{\mathrm{m_{CS}}} \quad (3)$$

$$\mathrm{Y_{SE,Hem}} = \frac{\mathrm{m_{SE,Hem}}}{\mathrm{m_{CS}}} \quad (4)$$

$$\mathrm{Y_{SE,Cel}} = \frac{\mathrm{m_{SE,Cel}}}{\mathrm{m_{CS}}} \quad (5)$$

where m_{CS} represents the mass (g) of untreated CS, $m_{SE,T}$ corresponds to the total mass (g) of xylose, arabinose, cellobiose, and glucose in P and RP, $m_{SE,Hem}$ corresponds to the total mass (g) of xylose and arabinose in P and RP, and $m_{SE,Cel}$ corresponds to the total mass (g) of cellobiose and glucose in P and RP.

2.2.2. Treatment by Enzymatic Hydrolysis (EH)

EH experiments were carried out using different CS and PCS loads in combination with varying cellulase filter paper unit (FPU) activities. The assays were conducted with 1 and 2 g (1 and 2% *w*/*v*) of CS or PCS (12), using sodium citrate buffer (0.05 M, pH 4.8) (14), and the Cellic CTec2 enzymatic cocktail (15) at dosages of 15 or 30 FPU, which enables the hydrolysis of cellulose and hemicellulose. Cellulase activity was measured prior to enzyme application following the NREL/TP-510-42629 protocol, and the reaction mixtures were incubated in an orbital shaker (16) at 250 rpm and 50 °C for 48 h [42]. During the EH, aliquots of 1 mL samples were collected at time intervals of 1, 3, 6, 12, and 48 h. The aliquots were centrifuged (17) and the supernatant liquid was collected and separated from the liquid fraction (referred to as hydrolyzed or H). The liquid fraction samples (18) were placed in 2 mL vials and refrigerated at −20 °C until analyzed. The kinetic data for fermentable sugars were obtained from aliquots collected according to the previously mentioned time intervals. H was analyzed similarly to P and RP, as previously mentioned. The assays were performed in duplicate.

Total yield of fermentable sugars ($Y_{EH,T}$), yield of hemicellulosic fermentable sugars ($Y_{EH,Hem}$), and yield of cellulosic fermentable sugars ($Y_{EH,Cel}$) were calculated by Equations (6)–(8), respectively. Total efficiency of enzymatic hydrolysis ($E_{EH,T}$) was calculated by Equation (9), and hemicellulose ($E_{EH,Hem}$) and cellulose ($E_{EH,Cel}$) efficiency conversions were calculated by Equations (10) and (11), respectively. The same equations were used in EH with CS, where the denominator becomes the CS mass instead of PCS. The yields and efficiencies are expressed in wt% which is equivalent to g/100 g.

$$Y_{EH,T} = \frac{m_{EH,T}}{m_{PCS}} \quad (6)$$

$$Y_{EH,Hem} = \frac{m_{EH,Hem}}{m_{PCS}} \quad (7)$$

$$Y_{EH,Cel} = \frac{m_{EH,Cel}}{m_{PCS}} \quad (8)$$

$$E_{EH,T} = \frac{m_{EH,T}}{m_{(Cel+Hem)P}} \quad (9)$$

$$E_{EH,Hem} = \frac{m_{EH,Hem}}{m_{(Hem)P}} \quad (10)$$

$$E_{EH,Cel} = \frac{m_{EH,Cel}}{m_{(Cel)P}} \quad (11)$$

where m_{PCS} is the mass (g) of PCS, $m_{EH,}$ corresponds to the total mass (g) of xylose, cellobiose, and glucose in hydrolyzed, $m_{EH,Hem}$ corresponds to the total mass (g) of xylose in hydrolyzed, $m_{EH,Cel}$ corresponds to the total mass (g) of cellobiose and glucose in hydrolyzed, $m_{(Cel+Hem)P}$ corresponds to the total mass (g) of cellulose and hemicellulose in PCS, $m_{(Cel)P}$ corresponds to the mass (g) of cellulose in PCS, and $m_{(Hem)P}$ corresponds to the mass (g) of hemicellulose in PCS.

2.2.3. Alcoholic Fermentation

To perform the fermentations, EH was performed similarly to the procedures described in "Section 2.2.2 Treatment by Enzymatic Hydrolysis (EH)", using a reaction time

of 48 h with 5 g of PCS (5% *w*/*v*) in 0.1 L. Fermentation was performed by adding a mixture of 1 g of peptone (10 g/L), 1 g of yeast extract (10 g/L), and 0.1 g (1 g/L) of lyophilized *Saccharomyces cerevisiae* yeast (19) to fermentable sugars obtained in the EH of the PCS (16), maintaining the solid fraction. The Erlenmeyer flasks were transferred to an orbital shaker (20) at 30 °C and 120 rpm. Samples (21) were collected at 0, 6, 12, 24, 48, and 72 h for ethanol and sugar content analysis by HPLC. The procedures were performed in duplicates. The ethanol production rate and yield were calculated using Equations (12) and (13), respectively.

$$R_{et} = \frac{C_{et}}{t} \tag{12}$$

$$Y_{et,g} = \frac{C_{et}}{C_g} \tag{13}$$

where R_{et} is the ethanol production rate over the fermentation period (g/L·h), C_{et} is the concentration of ethanol at the sampling (g/L), t is time elapsed between the start of fermentation and the sampling (h), $Y_{et,g}$ is the ethanol yield (g/g), and C_g is the glucose concentration of the hydrolyzed from EH (g/L).

The maximum theoretical ethanol concentration (g/L) was estimated using Equation (14).

$$C_{et,max} = \frac{Y_{et,max} \cdot m \cdot x_{cel} \cdot 1.11}{V} \tag{14}$$

where $Y_{et,max}$ is the maximum theoretical ethanol yield (0.51 g/g) [43], m is the mass of biomass mass used (g), x_{cel} is the fraction of cellulose in the biomass (%), V is the fermentation volume (L), and 1.11 is the stoichiometric conversion factor of cellulose into glucose [44].

Equation (15) was used to calculate fermentation efficiency.

$$E_{exp} = \frac{Y_{et,g}}{Y_{et,max}} \cdot 100 \tag{15}$$

3. Results

3.1. Feedstock Characterization Results

The composition of CS in natura was 27.78 ± 2.35% cellulose, 17.48 ± 1.55% hemicellulose, 11.66 ± 2.29% lignin, 7.47 ± 0.20 moisture, 3.62 ± 0.04% ash, and 16.06 ± 0.19% extractives. According to Aghaei et al. (2022) [10], the content of holocellulose in corn stover is 70%, while, in this study, it was 45.26%. Buruiana et al. [16], Öhgren et al. [17], Wan and Li [18], Weiss et al. [19], Nasir et al. [40], and Mosier et al. [24] reported contents of cellulose, hemicellulose, and lignin between 32.4–46.0%, 16.7–27.7%, and 13.0–21.2%, respectively. The difference consists of factors such as corn species, constituents of corn stover, and climatic factors according to region/country. These studies consider an average holocellulose content varying between 49.1–73.7%. The lignin content obtained in this study was similar to previously mentioned literature studies.

3.2. Steam Explosion (SE) Results

Figure 3 shows the changes in the chemical composition of CS after SE under operating conditions of 160, 180, and 200 °C, time intervals of 2.5 and 10 min, and constant pressure of 5 MPa. Operational conditions were represented as "steam explosion/time/temperature". An increase in cellulose and lignin content and a reduction in hemicellulose content were observed under all conditions.

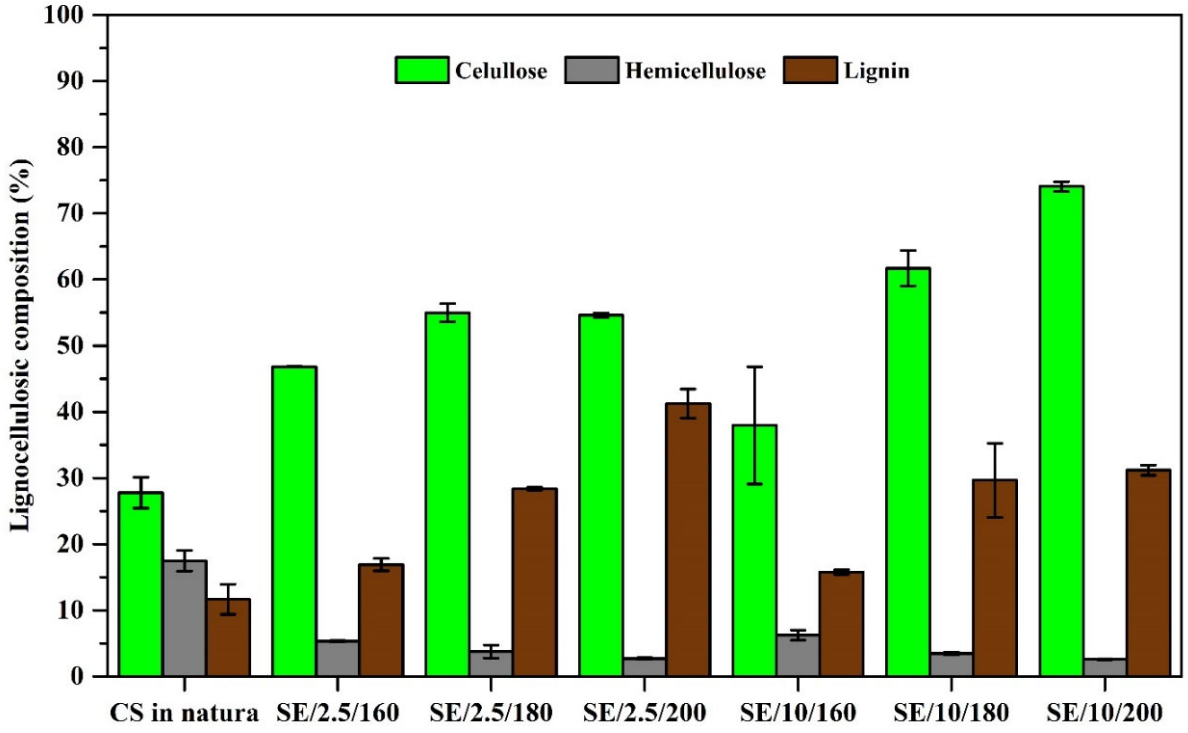


Figure 3. Lignocellulosic composition of the untreated corn stover (CS in natura) and pretreatment (PCS) by SE at different conditions (steam explosion/time (min)/temperature (°C)) under 5 MPa.

The increase in cellulose occurred because the hemicellulosic fraction was removed by hydrolytic reaction in accordance with results mentioned by Sun et al. [39]. The removal and breakdown of hemicellulose weakens the interactions between lignin and cellulose, thereby enhancing cellulose accessibility and increasing its likelihood of being efficiently hydrolyzed during enzymatic hydrolysis [45].

An increase in lignin content was also observed, though to a lesser extent than cellulose. The combined effect of higher temperature and extended reaction time promoted the disruption of the lignocellulosic matrix and facilitated hemicellulose removal and degradation, given its lower thermal stability. Hemicellulose has a degradation temperature between 150 °C and 350 °C, while cellulose and lignin have degradation temperatures between 275–350 °C and 250–500 °C, respectively [46,47]. The temperature and reaction time are important parameters for SE, ranging between 120 and 220 °C and 2 and 8 min [48]. The optimal SE conditions obtained in this study were a reaction time of 10 min at a temperature of 200 °C, where the content of cellulose increased from 27.78% (CS in natura) to 74.06% (SE/10/200). At this condition, the content of hemicellulose decreased from 17.48% (CS in natura) to 2.59% (SE/10/200), while lignin increased from 11.66% (CS in natura) to 31.19% (SE/10/200). Zhao et al. [49] evaluated the SE for pretreatment of CS and reported optimal conditions at 1.5 MPa, 200 °C, and 3 min. The content of cellulose increased from 32.31 to 34.12%, hemicellulose decreased from 28 to 13%, and lignin increased from 4.61 to 8.09%. Chen et al. [50] evaluated the pretreatment of CS by SE at 150 °C for 3 min and reported a reduction in the hemicellulose content (23.8% to 8.10%) and an increase in cellulose (40.02% to 49.07%), while the lignin content increased slightly (17.46% to 17.52%). Nasir et al. [40] pretreated CS by steam explosion under conditions of 1.0 MP, 184 °C, and 15 min. Their results showed a decrease in hemicellulose content from 19.26 to 12.50% and an increase in cellulose and lignin content from 46.00 to 53.30% and 20.31 to 25.56%, respectively. The studies mentioned demonstrate similar behavior of SE pretreatment on CS, but this study showed a higher percentage increase in cellulose content in the PCS.

However, it is not uncommon to find studies that report a decrease in cellulose, hemicellulose, and lignin content after SE. Chang et al. [51] used SE for CS pretreatment under conditions of 2.5 MPa for 3.33 min and reported a reduction of 8.47%, 50.45%, and 36.65% of cellulose, hemicellulose, and lignin in the PCS, respectively. However, most studies reporting this behavior have used acid catalysts in the SE, such as sulfuric acid and sulfur dioxide. An increase in cellulose content and a decrease in lignin content in the pretreatment of corn stalks by SE catalyzed by H_2SO_4 and by NaOH was observed in the study of Sun et al. [39]. Thus, the effect of the acid may result in greater degradation of the lignocellulosic components, but, from an environmental point of view, the use of acids may be unfavorable.

Figure 3 shows that the increase in the temperature and time reaction increases hemicellulose degradation and its removal to a greater extent than cellulose and lignin. Sui et al. [52] reported that high temperatures break down and hydrolyze the hemicellulose. Chang et al. [51] showed that SE primarily removes hemicellulose. The steam explosion also causes the release of organic acids such as acetic acids and other acids, which leads to the hydrolysis of hemicelluloses to oligosaccharides or monosaccharides [53]. These results are in agreement with those obtained in the present study.

Liu et al. [54] evaluated different conditions of pressure (1.5, 2.5, and 3.5 MPa) and reaction time (0.16, 0.83, and 1.5 min), concluding that pressure was the main operational condition in the SE pretreatment of CS that determined the physical force for morphological changes (physical pretreatment) while a longer reaction time contributed to the thermochemical reactions (physical pretreatment). The longer time and higher pressure contributed to more efficient degradation of hemicelluloses to improve the glucose yield in enzymatic hydrolysis, indicating that SE reduces the biomass recalcitrance more effectively, even without complete degradation of hemicellulose.

The cellulose crystallinity has been considered an important factor in determining the hydrolysis rates of cellulosic biomass, even if only this parameter is not sufficient to explain the recalcitrance of lignocellulosic biomass [37]. XRD analysis (Figure 4) was carried out to evaluate the crystallinity of the lignocellulosic structure of CS in natura and the conditions of SE that resulted in higher enrichment in cellulose (SE/10/180 and SE/10/200).

Figure 4 shows that SE promoted a change in the structure of CS. The peak of cellulose (around 22°) became more prominent. In this study, the peak for cellulose was found at 21.68° (CS in natura), 22.34° (SE/10/180), and 22.43° (SE/10/200). The appearance of a new peak in the PCS occurred at 14.9° and 15.14° for conditions SE/10/180 and SE/10/200, respectively, indicating a pronounced presence of cellulose. Peaks around 15° and 16.5° correspond to the presence of holocellulose, micronized cellulose, and microcrystalline cellulose [55]. Chen et al. [50] reported peaks at approximately 15.804°, 22.081°, 26.464°, 44.662°, and 72.566° in PCS, which are associated with the cellulosic structure. However, in the current study, only the peaks around 15° and 22° were confirmed, as shown in Figure 4.

Figure 4 indicates that the amorphous cellulose was partially or completely depolymerized during the steam explosion, as evidenced by the increased crystallinity index of PCS, as well as the possible depolymerization of some lignin. This conclusion is supported by Table 1, which shows that the liquid fraction obtained from the steam explosion contains glucose and cellobiose, both derived from cellulose. The higher CrI was attributed to the removal of non-cellulosic components (as evidenced by the decreased hemicellulose content shown in Figure 3) and thus the increase in the proportion of cellulose and lignin. Hemicellulose and lignin are the amorphous components of lignocellulosic biomass, while intact cellulose is the crystalline component [49]. Li et al. [56] reported an increase in CrI of CS after SE.

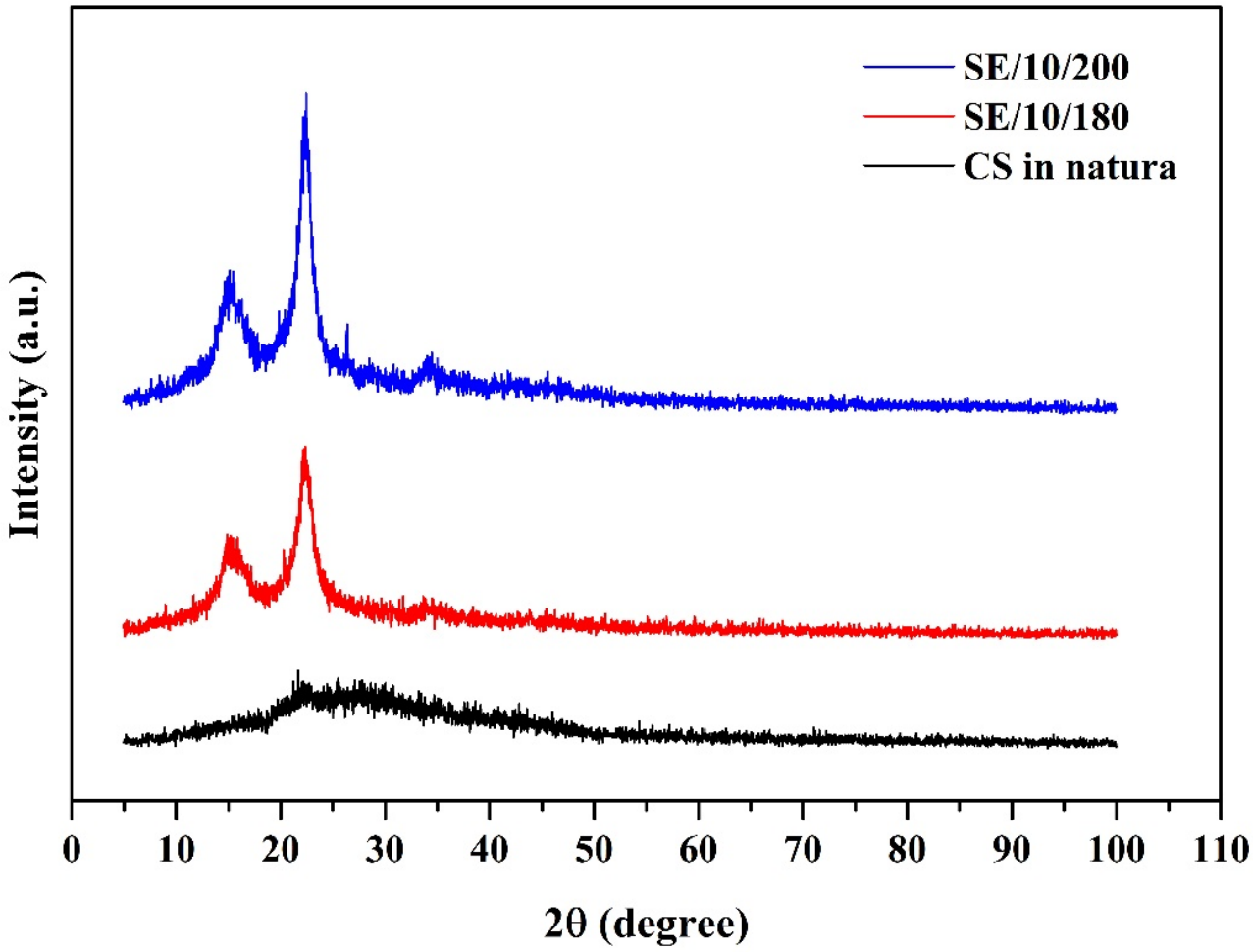


Figure 4. XRD of corn stover untreated (CS) and pretreated (PCS) by SE.

Table 1 presents the CrI of in natura and pretreated CS by steam explosion. Furthermore, Table 1 presents the method used to calculate the CrI, where it is possible to note that the majority of studies use the peak height method. Regardless of the method used, an increase in CrI in the CS occurred after SE pretreatment. Despite being more common, the peak height method overestimated the CrI value by 79–87% and corroborates the results from Park et al. [57]. However, CrI analysis using the peak height method is the more recurrent method found in the literature.

Table 1 shows that the CrI of CS in natura can vary between 27 and 57%, while the CrI of CS treated by SE can vary between 32 and 80%. Pretreatment promotes the opening of the biomass structure, but the increase in crystallinity may indicate greater recalcitrance of the cellulose remaining in the solid after the steam explosion. This fact is due to the removal or reduction of more easily available amorphous cellulose during SE. The analysis of liquid fraction (prehydrolyzed) and recovered prehydrolyzed showed the formation of sugars derived from cellulose (glucose and cellobiose). The effect of SE caused hydrolysis both in crystalline and amorphous zones and a slower conversion of crystalline cellulose compared with amorphous cellulose that would increase the crystallinity percentage of the prehydrolyzed biomass. In addition, the removal of hemicellulose increases the mean pore size of the substrate and therefore increases the accessibility and the probability of the cellulose becoming hydrolyzed [45].

Table 1. Crystallinity index for untreated CS and pretreated by SE at different conditions of temperature, pressure, and time.

Condition	Crystallinity Index (CrI)		Reference
	Method	%	
CS in natura	Peak height	27.80	[40]
		37.10	[56]
		49.70	[54]
		51.20	[58]
		55.00	[49]
		56.50	[50]
		49.30	This study
Corn stalk in natura	Amorphous subtraction	30.00	[41]
CS in natura		28.30	This study
CS pretreated by SE	**Crystallinity Index (CrI)**		**Reference**
	Method	**%**	
P: 3.5 MPa; t: 1.5 min	Peak height	32.20	[54]
P: 1.0 MPa; T: 184 °C; t: 15 min		38.80	[40]
P: 1.6 MPa; T: 200 °C; t: 5 min		48.80	[58]
P: 10 MPa; T: 250 °C; t: 10 min		50.20	[56]
P: 1.5 MPa; T: 200 °C; t: 3 min		58.00	[49]
T: 150 °C; t: 3 min		65.19	[50]
P: 5.0 MPa; T: 180 °C; t: 10 min		75.82	This study
P: 5.0 MPa; T: 200 °C; t: 10 min		79.87	
P: 5.0 MPa; T: 180 °C; t: 10 min	Amorphous subtraction	42.44	
P: 5.0 MPa; T: 200 °C; t: 10 min		42.61	

In general, higher SE temperatures led to a higher CrI. Similarly, higher pressures and reaction times also increased CrI. The shapeless cellulose regions may have been degraded in the pretreatment process and the regions of crystalline remained largely unchanged [59,60]. The solid fraction from SE was a product rich in crystalline cellulose while the liquid fraction was rich in products from hemicellulose (xylose and arabinose) and amorphous cellulose degradation (glucose and cellobiose). In addition, the recovery of cellulosic and hemicellulosic sugar during pretreatment was crucial for maximizing fermentable sugar production. In the present study, alongside the primary liquid fraction obtained from steam explosion (prehydrolyzed), the sugars retained in the pretreated solid fraction after washing (recovered prehydrolyzed) were also considered. The evaluation of the recovered prehydrolyzed is not commonly addressed in most studies.

The chemical compositions of corn stover (CS) after steam explosion (SE) pretreatment are presented in Table 2. The influence of SE pretreatment can be partially assessed by analyzing the composition of the liquid phase, which is mainly composed of xylose, arabinose, cellobiose, glucose, organic acids, and inhibitors. This liquid fraction was divided into prehydrolyzed and recovered prehydrolyzed, and both were characterized in terms of cellobiose, glucose, xylose, and arabinose (fermentable sugars); acetic acid, formic acid, and levulinic acid (organic acids); and furfural and 5-hydroxymethylfurfural [HMF] (inhibitors).

Table 2. Liquid fraction compositions from corn stover after pretreatment by SE at different conditions.

Component (g/100 g of CS)	**SE/2.5/160**		**SE/2.5/180**		**SE/2.5/200**		**SE/10/160**		**SE/10/180**		**SE/10/200**	
	P	**RP**	**P**	**RP**	**P**	**RP**	**P**	**RP**	**P**	**RP**	**P**	**RP**
Xylose	0.77 ± 0.05	0.30 ± 0.02	1.15 ± 0.15	0.28 ± 0.18	1.08 ± 0.19	0.41 ± 0.13	1.71 ± 0.65	0.38 ± 0.24	1.42 ± 0.36	1.16 ± 0.00	1.35 ± 0.12	0.13 ± 0.01
Arabinose	0.87 ± 0.01	0.17 ± 0.02	1.27 ± 0.04	0.17 ± 0.01	0.36 ± 0.13	0.37 ± 0.04	0.80 ± 0.20	0.28 ± 0.09	1.03 ± 0.22	0.31 ± 0.01	0.68 ± 0.17	0.20 ± 0.05
Cellobiose	0.16 ± 0.03	2.78 ± 0.19	0.43 ± 0.07	0.17 ± 0.03	0.50 ± 0.05	0.16 ± 0.02	1.10 ± 0.27	0.25 ± 0.05	0.63 ± 0.08	0.31 ± 0.01	1.04 ± 0.02	0.19 ± 0.03
Glucose	0.06 ± 0.00	0.18 ± 0.16	0.06 ± 0.02	0.22 ± 0.05	0.36 ± 0.15	0.27 ± 0.15	1.08 ± 0.19	0.16 ± 0.09	0.13 ± 0.06	0.31 ± 0.16	0.36 ± 0.07	0.30 ± 0.14
Total sugars	**1.86 ± 0.03**	**3.43 ± 0.39**	**2.91 ± 0.07**	**0.84 ± 0.18**	**2.30 ± 0.52**	**1.21 ± 0.34**	**4.69 ± 1.30**	**1.07 ± 0.29**	**3.21 ± 0.71**	**2.09 ± 0.17**	**3.43 ± 0.38**	**0.82 ± 0.13**
Acetic acid	0.37 ± 0.11	0.18 ± 0.01	0.57 ± 0.08	0.18 ± 0.14	2.04 ± 0.13	2.19 ± 0.76	2.26 ± 0.26	0.46 ± 0.24	0.81 ± 0.14	0.79 ± 0.06	1.58 ± 0.16	0.91 ± 0.30
Formic acid	0.12 ± 0.02	0.03 ± 0.00	0.14 ± 0.02	0.20 ± 0.27	0.40 ± 0.01	0.42 ± 0.10	0.13 ± 0.11	0.32 ± 0.45	0.36 ± 0.12	0.35 ± 0.48	0.27 ± 0.02	0.44 ± 0.26
Levulinic acid	0.02 ± 0.00	0.06 ± 0.00	0.04 ± 0.03	0.04 ± 0.06	0.24 ± 0.14	0.50 ± 0.19	0.24 ± 0.31	0.13 ± 0.07	0.06 ± 0.01	0.20 ± 0.02	0.20 ± 0.03	0.19 ± 0.07
Total acids	**0.51 ± 0.08**	**0.27 ± 0.01**	**0.75 ± 0.13**	**0.42 ± 0.47**	**2.68 ± 0.26**	**3.11 ± 1.05**	**2.63 ± 0.69**	**0.91 ± 0.77**	**1.23 ± 0.24**	**1.34 ± 0.40**	**2.05 ± 0.21**	**1.54 ± 0.63**
Furfural	0.03 ± 0.00	0.04 ± 0.00	0.07 ± 0.01	0.08 ± 0.03	0.35 ± 0.30	1.05 ± 0.04	0.82 ± 0.11	0.39 ± 0.48	0.10 ± 0.02	0.19 ± 0.00	0.44 ± 0.22	0.65 ± 0.06
HMF	0.09 ± 0.01	0.05 ± 0.01	0.23 ± 0.21	0.10 ± 0.07	0.70 ± 0.18	0.71 ± 0.45	0.98 ± 0.23	0.19 ± 0.14	0.13 ± 0.02	0.66 ± 0.34	0.57 ± 0.12	0.11 ± 0.02
Total inhibitors	**0.12 ± 0.02**	**0.09 ± 0.01**	**0.30 ± 0.22**	**0.18 ± 0.10**	**1.05 ± 0.48**	**1.76 ± 0.41**	**1.80 ± 0.34**	**0.58 ± 0.62**	**0.23 ± 0.00**	**0.85 ± 0.33**	**1.01 ± 0.34**	**0.76 ± 0.08**
$Y_{SE,C}$	3.18 ± 0.27		0.90 ± 0.05		1.29 ± 0.02		2.68 ± 0.28		1.38 ± 0.02		1.90 ± 0.19	
$Y_{SE,H}$	2.12 ± 0.02		2.87 ± 0.22		2.23 ± 0.11		3.18 ± 0.37		3.41 ± 0.90		2.37 ± 0.17	
$Y_{SE,T}$	**5.30 ± 0.25**		**3.76 ± 0.17**		**3.51 ± 0.13**		**5.77 ± 0.72**		**5.30 ± 0.38**		**4.25 ± 0.36**	

P—prehydrolyzed. RP—recovered prehydrolyzed.

Figure 5 shows the SEM of CS and PCS. Figure 5A,B refer to CS in natura, while Figure 5C–F refer to PCS under SE/2.5/160 and SE/10/200 conditions. The topographic structure in CS was flat and uniform, with some protuberances inherent to corn stover. After SE/2.5/160 and SE/10/200 pretreatment, changes were observed on the surface of the corn stover, which were more intense with increasing SE conditions. The milder SE/2.5/160 condition resulted in noticeable changes, resulting in a more porous and irregular structure, while, in the SE/10/200 condition, the formation of spherical structures was observed on the surface of the biomass. Similar results were obtained by Azani et al. [61] and Besserer et al. [62]. According to these authors, lignin linked to cellulose and hemicellulose undergoes cleavage due to the thermal and mechanical effects of steam explosion (SE), forming spheres of pseudolignin and lignin. These spheres condense after fragmentation, confirming lignin depolymerization. Furthermore, in Figure 5E,F, the biomass surface appeared rough and melted, attributed to the high temperature, indicating that the SE/10/200 condition significantly altered the lignocellulosic structure.

Table 2 shows that monosaccharides represented the main components of the liquid phase fraction. In particular, the prehydrolyzed contained a higher concentration of sugars than the recovered prehydrolyzed, which constituted the primary liquid fraction produced by SE. SE pretreatment led to a greater formation of sugars, followed by organic acids and inhibitors, in both the prehydrolyzed and the recovered prehydrolyzed—except under the SE/2.5/200 condition, where acid formation, especially acetic acid, exceeded that of sugars.

However, the formation of inhibitors remained satisfactorily low in comparison with acids and sugars under all conditions, except for SE/10/200, where the sugar concentration was similar to that of the inhibitors. Among the sugars, hemicellulose-derived monosaccharides (xylose and arabinose) were produced in higher concentrations due to the extensive hydrolysis of hemicellulose, also evidenced by its reduced content in the pretreated corn stover (Figure 3). The highest concentrations of xylose and arabinose—considering both liquid fractions (prehydrolyzed + recovered prehydrolyzed)—were 3.90 g/100 g under SE/10/180, while the highest combined concentration of cellobiose and glucose was 3.18 g/100 g under SE/2.5/160. The highest total sugar yields were 5.76 g/100 g and 5.30 g/100 g under SE/10/160 and SE/10/180, respectively.

In general, an increase in the production of organic acids and inhibitors was observed with rising temperature and reaction time. Among the acids, acetic acid was the most predominant, resulting from the degradation of hemicellulosic components. The production of inhibitors, on the other hand, remained relatively constant. Therefore, longer reaction times and higher temperatures may promote the conversion of sugars into acids and inhibitors, while very low temperatures may be insufficient to reduce biomass recalcitrance and may limit the hydrolysis of holocellulose, particularly hemicellulose. Overall, there was an increase in the yield (Y) of fermentable sugars derived from hemicellulose (xylose and arabinose) compared with those from cellulose (glucose and cellobiose). The concentration of total sugars (including both dimers and monomers) released from CS (Table 2) serves as an important indicator of the effectiveness of the SE pretreatment. Given the satisfactory sugar production and the high residual cellulose content in PCS (Figure 3), the PCS obtained under the SE/10/200 condition was selected for enzymatic hydrolysis (EH).

The combined results presented in Section 3.2 provide the following conclusions: (i) observed increase in cellulose content and reduction in hemicellulose after steam explosion pretreatment (SE) can be attributed to the hydrolytic cleavage of glycosidic bonds in hemicellulosic polymers; (ii) in some cases, partial lignin depolymerization under high-temperature and pressure conditions translates to a reduction in lignin content; however, compared with the initial percentage, results in an increase in cellulose and lignin content due to the depolymerization of hemicellulose; (iii) the autohydrolysis promoted by acetic

acid released from acetylated hemicellulose facilitates the removal of amorphous hemicellulosic fractions, enhancing cellulose exposure and accessibility to enzymatic attack; (iv) the increase in the crystallinity index (CrI) after SE indicates that amorphous cellulose and hemicellulose were preferentially degraded, leaving a solid fraction enriched in crystalline cellulose; (v) the SEM analysis corroborates this structural transformation, revealing defibrillation, pore formation, and lignin reallocation in the form of spherical droplets (pseudolignin). These structural modifications collectively reduce biomass recalcitrance and explain the higher enzymatic digestibility and sugar yields observed for pretreated corn stover (PCS) compared with untreated biomass, as observed in Section 3.3.

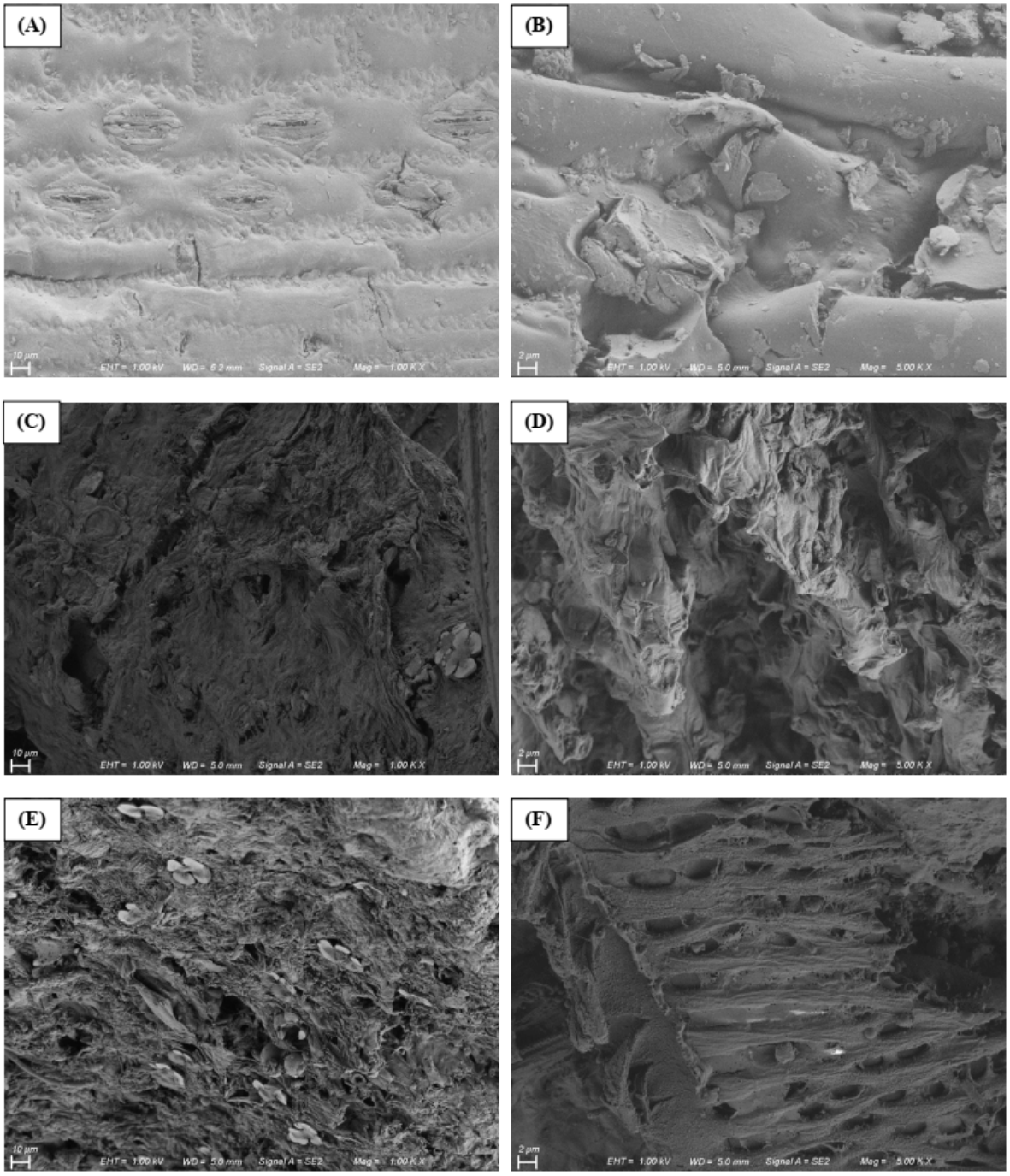


Figure 5. SEM of CS (**A**,**B**), PCS at SE/2.5/160 (**C**,**D**), and PCS at SE/10/200 (**E**,**F**), at a magnitude and resolution of 1000× and 10 µm, and 5000× and 2 µm.

3.3. Enzymatic Hydrolysis (EH) Results

The results of EH are presented in Table 3. Experimental conditions were represented as "enzymatic hydrolysis/FPU/% load". The results show higher sugar production using PCS compared with CS in natura of all conditions. As expected, SE promoted a decrease in the corn stover's recalcitrance and facilitated the action of enzymes. The results also show that there was no marked increase in total sugar production related to the variation in FPU and biomass load. The highest yields of total sugars were 41.57 ± 1.43 g/100 g PCS and 41.33 ± 6.03 g/100 g PCS for EH/30 FPU/2% PCS and EH/15 FPU/1% PCS, respectively.

Table 3. Composition of hydrolyzed obtained from CS and PCS by EH.

Component (g/100 g of CS)	**Experimental Conditions**							
	15 FPU 1% CS	**15 FPU 1% PCS**	**15 FPU 2% CS**	**15 FPU 2% PCS**	**30 FPU 1% CS**	**30 FPU 1% PCS**	**30 FPU 2% CS**	**30 FPU 2% PCS**
Xylose	3.34 ± 0.14	12.30 ± 7.12	3.59 ± 0.04	15.43 ± 0.01	2.73 ± 0.37	17.69 ± 1.08	3.64 ± 0.20	11.68 ± 2.54
Cellobiose	0.79 ± 0.00	1.02 ± 0.40	1.01 ± 0.01	0.82 ± 0.07	9.42 ± 3.03	0.88 ± 0.29	1.08 ± 0.05	0.84 ± 0.01
Glucose	13.62 ± 0.41	28.00 ± 1.49	13.52 ± 0.01	24.02 ± 0.72	9.93 ± 2.32	20.27 ± 1.87	13.79 ± 0.21	29.05 ± 1.10
Total sugars	**17.75 ± 0.27**	**41.33 ± 6.03**	**18.12 ± 0.06**	**40.26 ± 0.66**	**22.08 ± 0.34**	**38.84 ± 3.23**	**18.51 ± 0.06**	**41.57 ± 1.43**
$E_{EH,C}$	51.87 ± 1.47	39.19 ± 1.48	52.29 ± 0.06	33.53 ± 0.88	69.66 ± 2.55	28.55 ± 2.91	53.53 ± 0.92	40.36 ± 1.51
$E_{EH,H}$	19.13 ± 0.82	-	20.57 ± 0.22	-	15.66 ± 2.10	-	20.87 ± 1.12	-
$E_{EH,T}$	**39.24 ± 0.59**	**53.92 ± 7.86**	**40.05 ± 0.13**	**52.53 ± 0.87**	**48.83 ± 0.75**	**50.67 ± 4.22**	**40.93 ± 1.00**	**54.23 ± 1.86**
$Y_{EH,H}$	3.34 ± 0.14	12.30 ± 7.12	3.59 ± 0.04	15.43 ± 0.01	2.73 ± 0.37	17.69 ± 1.08	3.64 ± 0.20	11.68 ± 2.54
$Y_{EH,C}$	14.41 ± 6.42	29.02 ± 13.49	14.53 ± 6.25	24.84 ± 0.72	19.35 ± 0.25	21.15 ± 9,70	14.87 ± 6.35	29.89 ± 14.11
$Y_{EH,T}$	**17.75 ± 0.27**	**41.33 ± 6.03**	**18.12 ± 0.06**	**40.26 ± 0.66**	**22.08 ± 0.34**	**38.84 ± 3.23**	**18.51 ± 0.45**	**41.57 ± 1.43**

An important issue in pretreatment is the mode of operation, and few studies address this issue. Zimbardi et al. [63] investigated the EH of corn stover following sulfuric acid impregnation and sequential SE pretreatment, achieving a sugar yield of 57.80 g/100 g (a 1.89-fold increase). In the present work, steam explosion alone was applied, and the highest yield obtained was 45.82 g/100 g (a 2.5-fold increase). It is worth noting, however, that, despite the high yields associated with acid and alkaline pretreatments, these methods are less environmentally favorable due to the use of chemical reagents and present operational drawbacks such as equipment corrosion and the formation of inhibitory compounds [64–67].

The cellulose conversion efficiency was higher than that of hemicellulose when analyzing the use of untreated corn stover (CS). This is because CS in natura contains more cellulose than hemicellulose (Figure 3), and enzymes are more effective at degrading cellulose, resulting in a higher yield of cellulose-derived sugars. Although the pretreated corn stover (PCS) had a higher crystallinity index (CrI) than untreated CS, its degradation proceeded more rapidly, as demonstrated by the enzymatic hydrolysis (EH). This is due to the fact that steam explosion (SE) reduces lignin polymerization and breaks the bonds between lignin and cellulose, thereby improving enzyme accessibility to cellulose [56].

In contrast, the cellulose conversion efficiency was lower than that of hemicellulose when analyzing the use of PCS. Although PCS still contained more cellulose than hemicellulose (Figure 3), it was enriched in crystalline cellulose, which is more resistant to enzymatic attack. Nonetheless, the total sugar yield from cellulose was higher than from hemicellulose, since SE significantly reduced the hemicellulose content. The hemicellulose conversion efficiency is not included in Table 3 because the hemicellulose content in PCS was only 2.5%.

The total efficiency and total sugar yield were higher for PCS, ranging between 50–55 g/100 PCS and 38–42 g/100 PCS, respectively, while for CS it varied between 40–48 g/100 g CS and 17–23 g/100 g CS, respectively. The highest total sugar conversion

efficiency and total sugar yield were obtained in the condition 30 FPU 2% PCS, resulting in 54.23 ± 1.86 g/100 g and 41.57 ± 1.43 g/100, respectively.

The effect of enzyme dosage and solid loading on the kinetic yield is shown in Figure 6. The total fermentable sugar yield using PCS was higher than CS (Figure 6A) in all conditions of EH. Fermentable sugar yield profiles presented the maximum hydrolysis yield rate on the first 6 h varying between 1.21 g/100 g.h CS—1.78 g/100 g. h CS and 3.11 g/100 g.h PCS—4.97 g/100 g.h PCS. After 6 h, the hydrolysis yield rate reduced to 0.35 g/100 g.h CS—0.18 g/100 g.h CS and 0.54 g/100 g.h PCS—0.21 g/100 g.h PCS.

The behavior of cellulose and hemicellulose digestibility of PCS is shown in Figure 6B and 6C, respectively. For glucose and cellobiose, the maximum hydrolysis yield rate was 3.39 g/100 g.h cellulose in the first 6 h of the reaction at EH/30 FPU/1% PCS condition (Figure 6B). The hydrolysis yield rate between 6 and 48 h was 0.02 g/100 g.h cellulose. The hydrolysis rate of hemicellulose was very similar in the first 6 h at EH/15 and 30 FPU/1% PCS (1.61 g/100 g.h hemicellulose and 1.59 g/100 g.h hemicellulose, respectively). The hydrolysis yield rate between 6 and 48 h was 0.19 g/100 g.h hemicellulose and 0.06 g/100 g.h hemicellulose, respectively (Figure 6C).

The effect of enzyme dosage and solid loading on the kinetic yield is shown in Figure 6. Across all EH conditions, PCS produced a greater total fermentable sugar yield compared with CS (Figure 6A). Fermentable sugar yield profiles presented the maximum hydrolysis yield rate in the first 6 h, varying between 1.21 g/100 g.h CS—1.78 g/100 g. h CS and 3.11 g/100 g.h PCS—4.97 g/100 g.h PCS. After 6 h, the hydrolysis yield rate reduced to 0.35 g/100 g.h CS—0.18 g/100 g.h CS and 0.54 g/100 g.h PCS—0.21 g/100 g.h PCS.

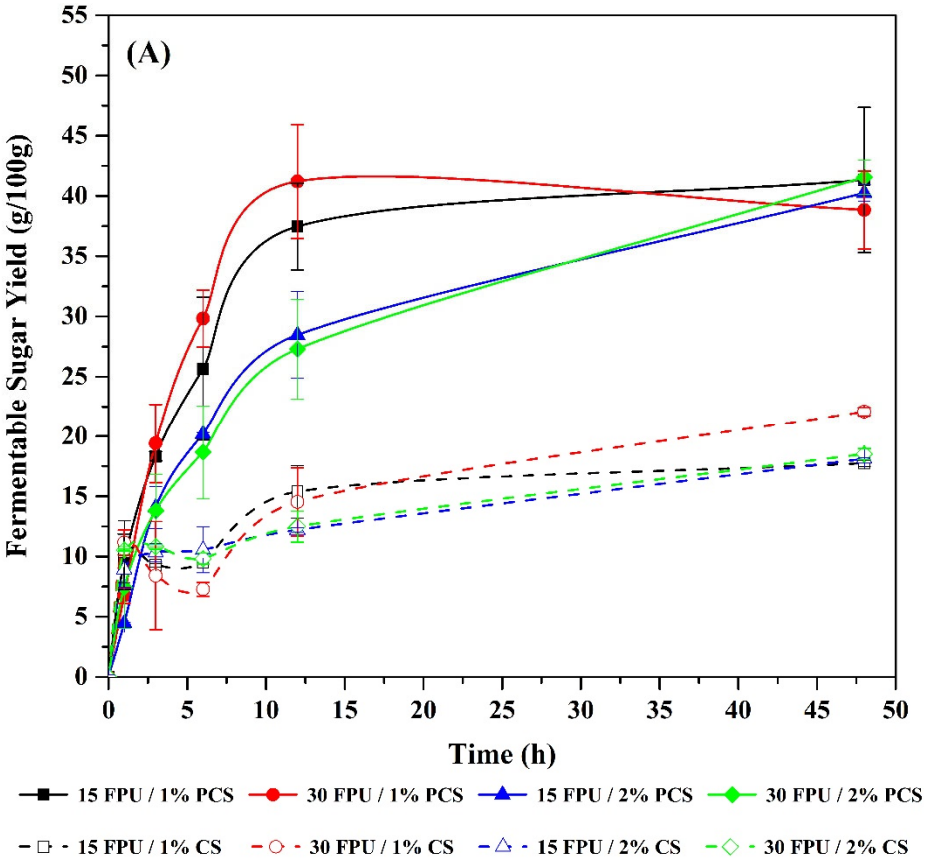


Figure 6. *Cont.*

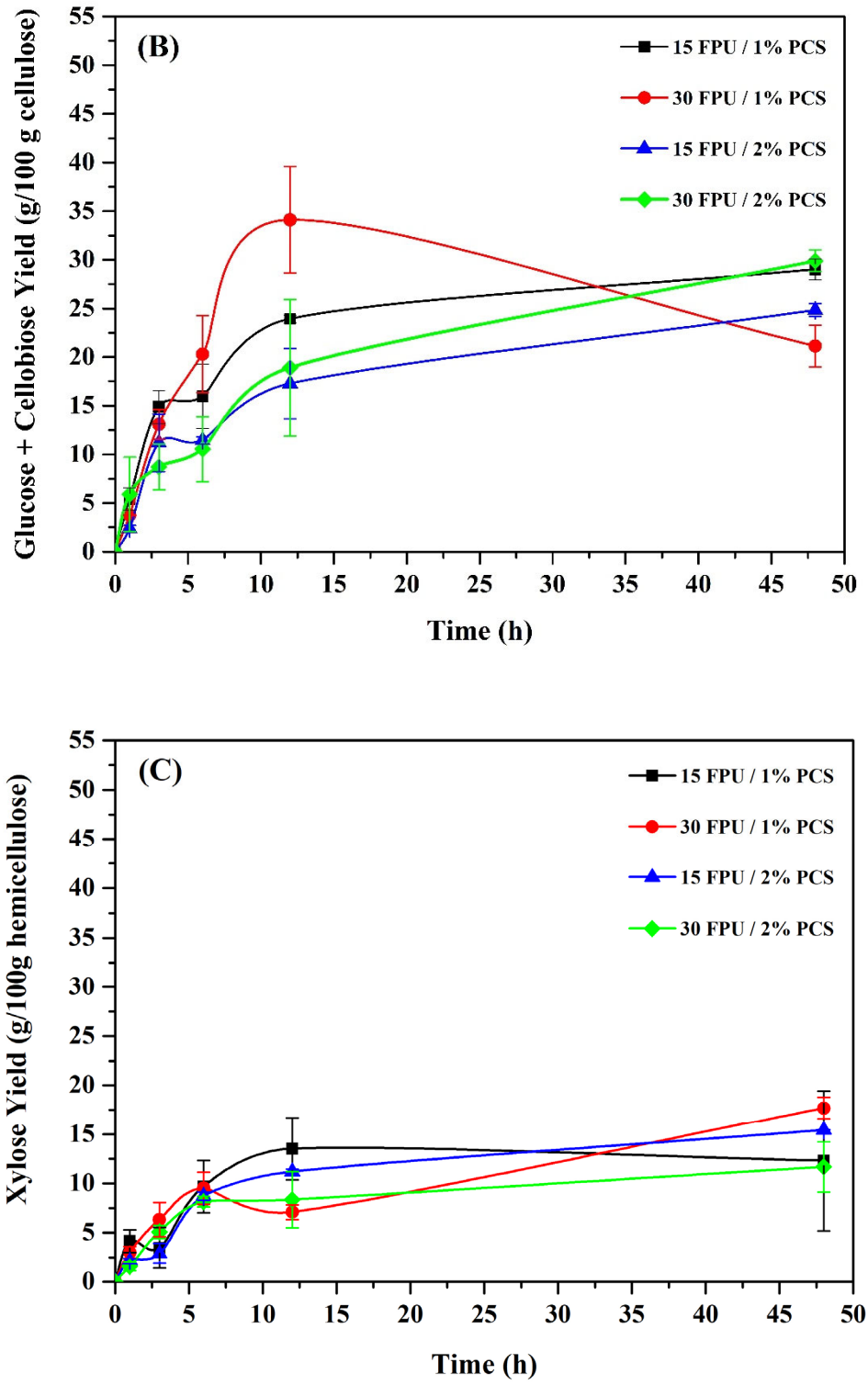


Figure 6. EH kinetics: (**A**) total sugar using CS and PCS, (**B**) cellulose-derived sugar using PCS, and (**C**) hemicellulose-derived sugar using PCS.

The behavior of cellulose and hemicellulose digestibility of PCS is presented in Figures 6B and 6C, respectively. The maximum hydrolysis yield rate for glucose and cellobiose was 3.39 g/100 g.h cellulose in the first 6 h of the reaction at EH/30 FPU/1% PCS condition (Figure 6B). The hydrolysis yield rate between 6 and 48 h was 0.02 g/100 g.h cellulose. The hemicellulose hydrolysis rate during the first 6 h was similar for EH/15 and 30 FPU at 1% PCS, reaching 1.61 and 1.59 g/100 g·h of hemicellulose, respectively. The hy-

drolysis yield rate between 6 and 48 h was 0.19 g/100 g.h hemicellulose and 0.06 g/100 g.h hemicellulose, respectively (Figure 6C).

Zhang et al. [68] investigated the effects of using urea combined with steam explosion on the enzymatic hydrolysis of corn stover. The highest sugar yield was 35.01 g/100 g PCS (an increase of 116.42%). This value is lower than the maximum yield found in this study (Figure 7), but it corroborates the positive effect of SE in increasing sugar production by EH. Figure 7 shows that SE considerably improved the EH process, increasing sugar yield 2.5 times compared with CS in natura.

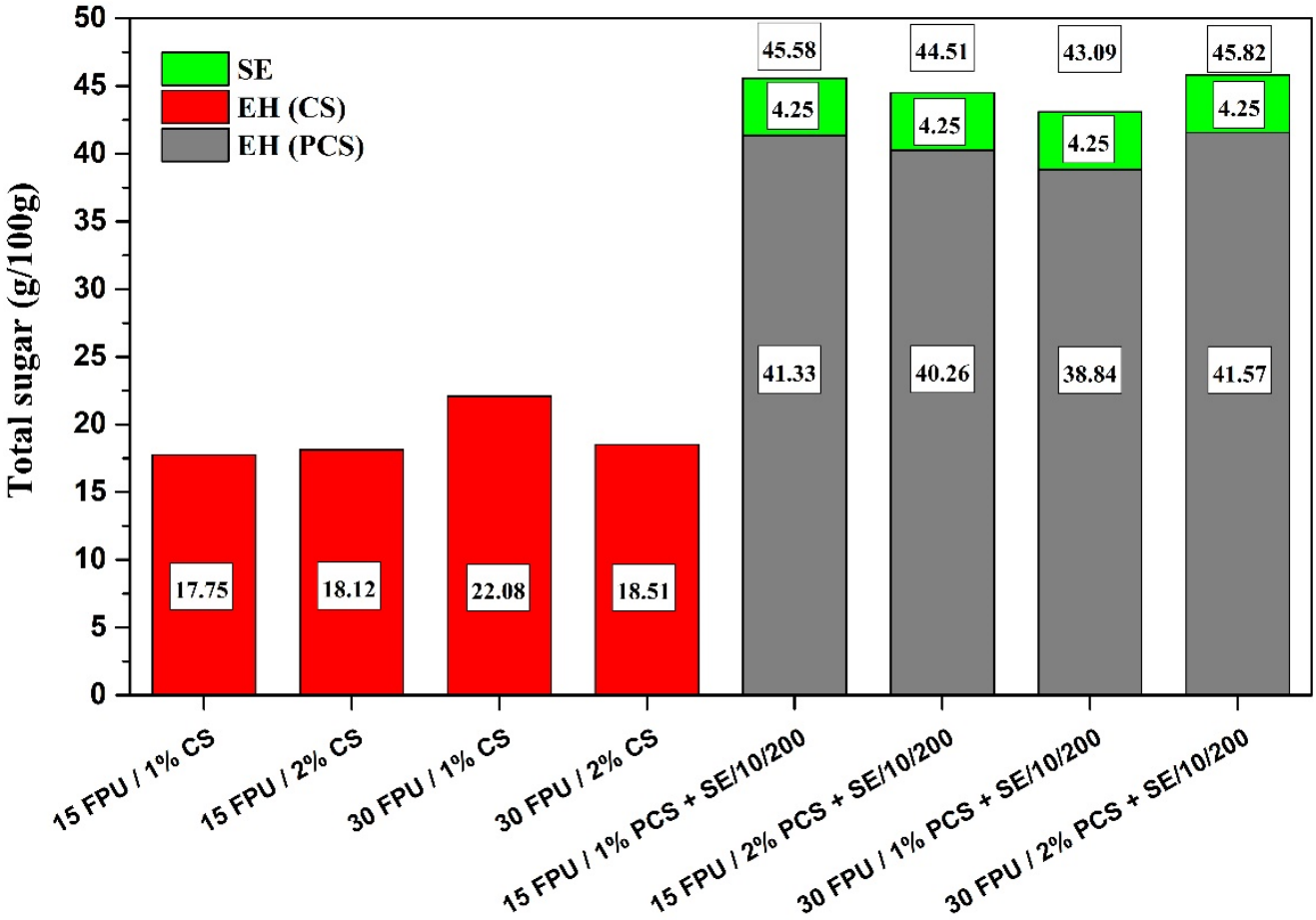


Figure 7. Total production of fermentable sugar in EH of CS, EH of PCS, and SE.

3.4. Alcoholic Fermentation Results

Figure 8 shows the results of alcoholic fermentation of hydrolyzed from enzymatic hydrolysis using 15 and 30 FPU. In both cases, glucose was entirely consumed in 12 h of fermentation, leading to a high ethanol production at a high rate. Maximum production rates (R_{et}) were obtained at 6 h, reaching 0.80 and 1.30 g/L.h for 15 and 30 FPU, respectively. After 12 h of fermentation, the ethanol production rate began to decline significantly due to the previously rapid consumption of glucose. The concentrations of other sugars present in the enzymatic hydrolyzed (EH) were low compared with glucose and thus did not significantly influence the fermentation.

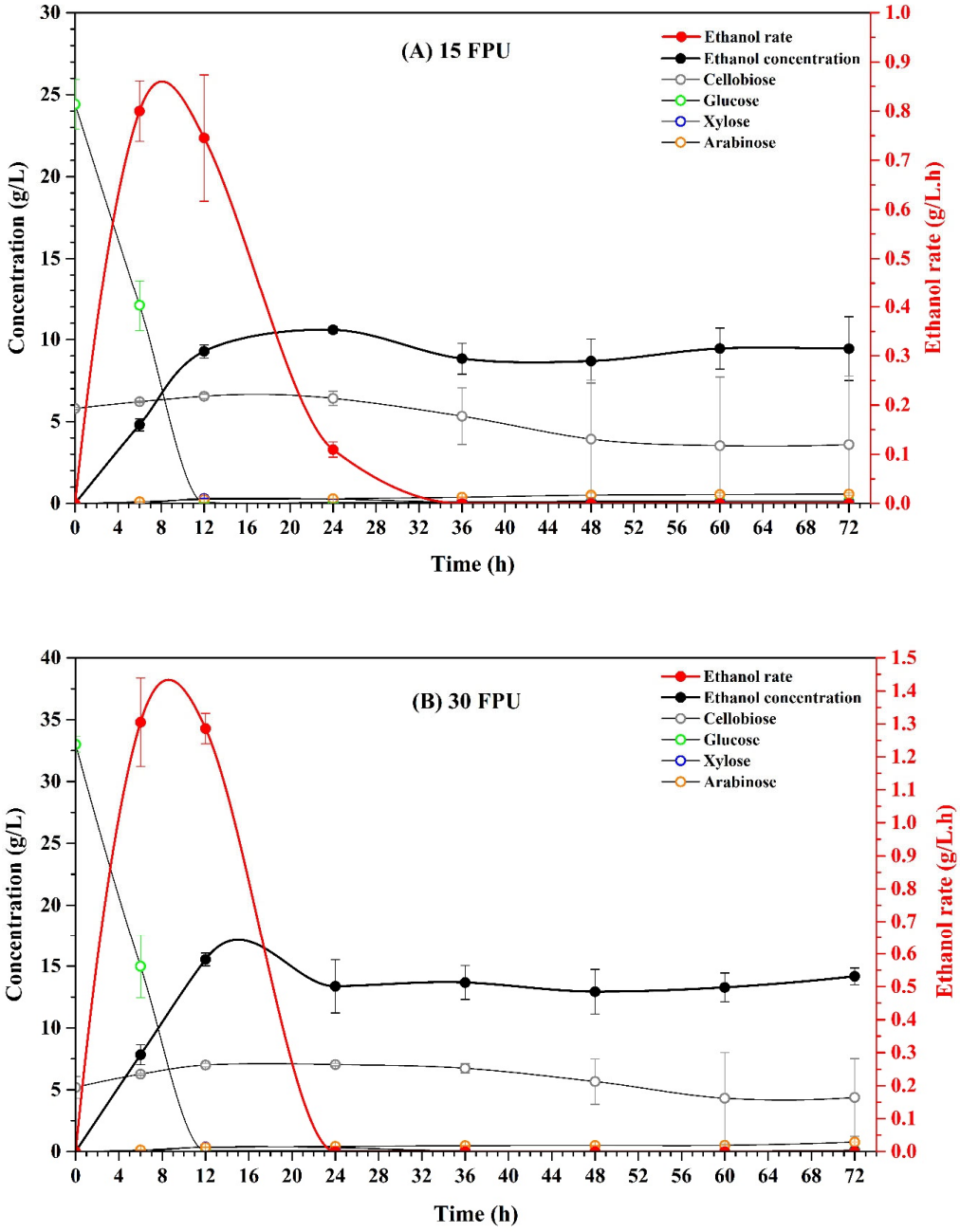


Figure 8. Ethanol concentration, ethanol rate, fermentable sugars, and platform chemicals in the fermentation of hydrolyzed from EH: (**A**) 15 FPU and (**B**) 30 FPU. The thicker continuous lines represent the product of interest (ethanol), shown in terms of concentration (black line) and production rate (red line). The solid faint black lines indicate the consumption of fermentable sugars, while the dashed faint black lines represent the formation of chemical platform compounds.

Furthermore, cellobiose consumption was slightly reduced, as it is a disaccharide resulting from the incomplete depolymerization of cellulose during steam explosion and enzymatic hydrolysis. Nevertheless, its concentration remained low, as did that of the monosaccharides xylose and arabinose. Xylose and arabinose concentrations rise during fermentation because the enzyme utilized continues to hydrolyze hemicellulose and they are not consumed by Saccharomyces cerevisiae.

The initial glucose concentration (C_g) at the beginning of fermentation was 24.42 ± 1.51 at 15 FPU (Figure 8A) and 33.01 ± 0.61 g/L at 30 FPU (Figure 8B). The ethanol concentration (C_{et}) reached its maximum of 10.59 ± 0.22 g/L at 24 h with 15 FPU and 15.54 ± 0.53 g/L at 12 h with 30 FPU, respectively. Under the fermentation conditions evaluated in this study,

it was possible to achieve conversion efficiencies of 85.00% and 92.33% for 15 and 30 FPU, respectively. The ethanol yield ($Y_{et,g}$) was 0.43 and 0.47 g/g for 15 and 30 FPU, respectively. From 5.0 g of PCS utilized in EH, the maximum theoretical glucose yield is 4.107 g [44]. Therefore, in 0.1 L of fermentation medium, it would be possible to obtain 41.07 g/L of glucose. Assuming ideal conditions for both EH and fermentation, and considering a theoretical conversion yield of 0.51 g of ethanol per g of glucose [43], the maximum theoretical ethanol concentration ($C_{et,max}$) is 20.94 g/L. Thus, 50.54% (10.59 ± 0.22 g/L of ethanol) and 74.20% (15.54 ± 0.53 g/L of ethanol) of the maximum theoretical ethanol yield (20.94 g/L) from 5.0 g of PCS were achieved using enzyme loadings of 15 and 30 FPU, respectively.

Similar results have been reported for the EH of corn stover following pretreatment with urea and steam explosion, achieving a glucose-to-ethanol conversion of up to 48.3 g/g [68]. For sugarcane bagasse subjected to NaOH pretreatment, a conversion efficiency of up to 93% was observed [69]. Likewise, steam-exploded sugarcane achieved a glucose-to-ethanol conversion efficiency of 92% [70]. These results indicate that the sugar-to-ethanol conversion efficiencies obtained in this study are comparable to those reported in previous research. However, most studies do not account for the total amount of glucose that can be hydrolyzed from biomass when calculating ethanol yields in relation to hydrolyzable glucose. In contrast, this study highlights the remaining potential for improving access to residual cellulose that is not converted into glucose and, consequently, into ethanol. Specifically, between 49.46 and 25.80% of the theoretically accessible glucose in the PCS was not converted into ethanol. In 5.0 g of PCS, the maximum theoretical yield of glucose is 4.107 g, while the highest amount actually obtained was 3.30 g. Additionally, a portion of cellulose remained as cellobiose (0.4–0.7 g), indicating incomplete hydrolysis. Therefore, the enzymatic hydrolysis process could be optimized by increasing enzyme loading or extending the hydrolysis time beyond 72 h. Furthermore, some cellulose fractions remained in a crystalline state despite steam explosion pretreatment, which may have limited hydrolysis efficiency [71,72].

During enzymatic hydrolysis (EH), the improved accessibility of cellulose microfibrils allowed cellulase enzymes to act more efficiently despite the higher crystallinity of PCS. However, the persistence of crystalline cellulose regions and residual lignin likely restricted complete conversion, as reflected in the gradual plateau of sugar release. In the subsequent fermentation step, the high glucose concentration obtained from PCS hydrolysates supported rapid ethanol production by Saccharomyces cerevisiae, achieving up to 92.3% of the theoretical yield. This efficiency demonstrates that the SE + EH integration not only improved sugar liberation but also minimized the formation of inhibitory compounds, resulting in an optimal balance between pretreatment severity and fermentability. Overall, these findings confirm that the physicochemical disruption of lignocellulosic matrices through SE enhances downstream bioconversion, emphasizing its potential as a sustainable strategy for second-generation bioethanol production.

The use of CS has proven to be a viable alternative for the valorization of a widely available renewable agricultural residue, enabling the production of an alternative biofuel to fossil fuels. Moreover, the fermentation process is conducted without removing the CS or enzymes after EH, thereby eliminating additional separation steps. Finally, these findings demonstrate that the combination of steam explosion, enzymatic hydrolysis, and fermentation using *Saccharomyces cerevisiae* can efficiently produce second-generation ethanol under the evaluated conditions.

4. Conclusions

This study demonstrated the substantial reduction in recalcitrance achieved through steam explosion, confirming that the pretreatment markedly enhanced enzymatic hydrolysis and subsequent ethanol production. Steam explosion at 200 °C for 10 min significantly removed hemicellulose, increased porosity, and facilitated the exposure of cellulose, enabling higher enzymatic accessibility. As a result, the combined steam explosion + enzymatic hydrolysis process yielded up to 45.82 g of fermentable sugars per 100 g of pretreated corn stover, approximately 2.5 times higher than untreated corn stover (in natura). This improvement directly influenced fermentation performance, where glucose was fully consumed within 12 h, achieving an ethanol concentration of 15.54 g/L, a production rate of 1.30 g/L·h, and a conversion efficiency of 92.33%, resulting in an ethanol yield of 0.47 g/g of pretreated corn stover.

These findings highlight steam explosion as an effective pretreatment for enhancing the fermentability of lignocellulosic biomass, particularly when aiming to maximize second-generation ethanol production. The integrated steam explosion–enzymatic hydrolysis–fermentation route reduces operational steps and enables the valorization of an abundant agricultural residue such as corn stover, contributing to sustainable biofuel pathways.

Future work should explore optimized combinations of enzyme dosages and substrate loads, and strategies to further access crystalline cellulose regions that remain unconverted. Additionally, the development of integrated biorefinery schemes—such as recovering organic acids, inhibitors, or lignin-rich solids—could further improve the economic feasibility and environmental benefits of lignocellulosic biomass valorization. Applying this approach to other types of agricultural residues may broaden the applicability of steam explosion as a robust pretreatment for renewable biofuel production.

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