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Integrated Bioprocess for Cellulosic Ethanol Production from Wheat Straw: New Ternary Deep-Eutectic-Solvent Pretreatment, Enzymatic Saccharification, and Fermentation

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Abstract: Wheat straw (WS) is an excellent raw material for biofuel ethanol production. However, the recalcitrance of WS prevents its efficient utilization. In this study, a novel ternary deep eutectic solvent (DES) was developed for enhancing component separation and enzymatic saccharification of WS. Without any detoxification and sterilization, the DES-treated WS hydrolysate was successfully used to produce ethanol. Overall, this research evaluated the effect of ternary DES pretreatment on WS at various temperatures and adjusted the enzyme load, substrate concentration, and fermentation method of treated WS. The results suggested that the cellulose recovery of treated WS after DES pretreatment (120 °C, 1 h) was 94.73 ± 0.22%, while the removal of xylan and lignin reached 89.53 ± 0.36% and 80.05 ± 0.62%, respectively. Importantly, at enzyme loading of 11.4 filter paper unit (FPU)/g WS with 16% fermentation substrate concentration, 91.15 ± 1.07% of cellulose was hydrolyzed, and the glucose yield was 71.58 ± 1.34%. The maximum ethanol yield of DES-treated WS was 81.40 ± 0.01%.

Keywords: pretreatment; lignocellulosic biomass; bioethanol; glucose

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1. Introduction

In recent decades, bioethanol has attracted much attention from researchers as it can be used in various applications, such as hydrogen production, pharmaceutics, and fuel [1–4]. Pretreatment, enzymatic saccharification, and fermentation are commonly used to produce cellulosic ethanol from lignocellulosic biomass as raw material [5,6]. However, the recalcitrant structure of lignocellulosic biomass limits enzyme accessibility, making it more difficult to convert into fermentable sugars and bioethanol [7–9]. China has many straw biomass resources that have been squandered and cannot be used properly [10]. Therefore, converting straw waste to cellulosic ethanol has significant practical implications.

Wheat straw (WS) is primarily composed of cellulose, hemicellulose, and lignin, which are wrapped around each other and provide WS with its structural and chemical stability [11]. As a result of its stubborn nature, WS is difficult to hydrolyze directly into fermentable sugars by enzymatic hydrolysis. Researchers have struggled to come up with a cost-effective, efficient, and environmentally friendly pretreatment technique to break up complicated lignocellulose and improve enzyme accessibility [12–14]. Physical, chemical, physico-chemical, and biological methods are currently the most prevalent pretreatment procedures [15–19]. When WS was treated with acetone at 180 °C for 40 min, the amount of lignin removed was 76% [20]. By pretreating WS with ammonium sulfite, the lignin content can be effectively reduced and the efficiency of enzymatic saccharification enhanced [21].

However, traditional chemical pretreatment methods, on the other hand, make it difficult to recycle chemical reagents and pollute the environment [22,23]. Therefore, researchers began to look for new pretreatment methods to solve the problems caused by traditional pretreatment methods. Ionic liquids (ILs) are considered as green replacements for organic solvents because of their strong polarity, good stability, good solvability, facile synthesis, and recovery [24,25]. However, ILs are difficult to use in industry due to their high cost and complicated preparation process [26].

Recently, deep eutectic solvents (DESs) have become a research hotspot due to their environmental friendliness, low cost, low toxicity, and ease of recycling and reuse [27,28]. DESs are eutectic mixture of two or three components with lower freezing points than the separate components in a specified molar ratio [29]. DES is now thought to be an effective pretreatment strategy for reducing the recalcitrance of lignocellulosic biomass [30,31]. As far as we know, most of the described investigations employed choline chloride (ChCl) and different carboxylic acids to produce DES [32,33]. Zhao pretreated WS at 70 °C for 9 h with ChCl/monoethanolamine-based DES [34]. After pretreatment, 71.4% of lignin was removed, leaving 93.7% of the cellulose. However, lignin solubility in these DESs is limited because ChCl is a non-aromatic-ring quaternary ammonium salt with poorer phase-transfer catalytic activity than aromatic-ring quaternary ammonium salts [35,36]. Glycerol (GL) is a natural chemical that is produced as an inexpensive by-product of the biodiesel industry [37]. It is a type of hydrogen-bond donor (HBD) that is employed in the production of DES [38]. However, glycerol-based DESs have been shown to be less successful in biomass pretreatment [39] because the pretreatment performance of DES without acid was so poor [40–42]. To improve the impact of pretreatment, Lewis acid was included into DES [42–45].

In our previous works, DES based on triethylbenzyl ammonium chloride/lactic acid (TEBAC/LA) showed a good pretreatment effect on lignocellulose, and enzymatic hydrolysis efficiency of lignocellulose was significantly improved after DES pretreatment. After pretreatment with TEBAC/LA (1:9)-based DES at 100 °C for 10 h, the lignin removal of WS could achieve 75.69% [36]. TEBAC/LA (1:7)-based DES at 120 °C for 90 min could remove 61.40% of the lignin from corn straw (CS) [12]. However, pretreatment duration and removal impact of TEBAC/LA-based DES cannot be adequately balanced. In light of this, the goal of this research was to design a simple, environmentally friendly, novel DES pretreatment technique that would shorten the pretreatment time and increase the pretreatment effect. Herein, ternary DES was made using TEBAC, GL, and aluminum chloride hexahydrate (ACH) in a 1:2:0.05 molar ratio. The effect of pretreatment temperature on the delignification of WS in a short period (60 min) at various temperatures was systematically investigated. Scanning electron microscopy (SEM), Fourier-transform infrared (FT-IR) spectroscopy, and X-ray diffraction (XRD) analyses were used to examine the structures of raw and treated WS. Furthermore, the effect of cellulase-loading on enzymatic hydrolysis was studied at the optimum pretreatment temperature. Finally, the effects of separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), and saccharification and co-fermentation (SSCF) methods on ethanol production from WS, as well as the effects of substrate concentration on ethanol production performance were investigated.

2. Materials and Methods

2.1. Materials

WS was collected from a field in Lianyungang city (Jiangsu province), cleaned with deionized water, dried, crushed, and sieved using a 60-mesh sieve. Celluclast 2.0 L (75 FPU/mL) was given by Novozymes (China) Investment Co., Ltd. (Beijing, China). for saccharification. The alcohol-producing active dry yeast was generously provided by Angel Yeast Co., Ltd. (Hubei, China). Analytical grade triethylbenzyl ammonium chloride (TEBAC), glycerol (GL), and aluminum chloride hexahydrate (ACH) were obtained from Shanghai Macklin Biochemical Technology Co., Ltd. (Shanghai, China). and used

without further purification. Anhydrous ethanol was purchased from GHTECH Co., Ltd. (Shantou, China).

2.2. DES Pretreatment of WS

DES was made by mixing TEBAC, GL, and ACH in a 1:2:0.05 molar ratio and stirring for 1 h at 100 °C until the mixture was clear and translucent. The TEBAC/GL/ACH (1:2:0.05)-based DES was put in a dry, clean vessel after the reaction. The WS and DES were added in a three-neck bottle with a solid–liquid mass ratio of 1:15 and pretreated for 1 h at 100, 120, and 140 °C, respectively. After the reaction, 60–80 mL anhydrous ethanol was added into the three-neck bottle and swirled for 1 h at 60 °C. The cellulose-rich residue was then collected using vacuum-pumping filtration and washed with anhydrous ethanol and hot deionized water to remove the DES adhering to the surface of residue.

2.3. Enzymatic Saccharification and Fermentation

The enzyme-loading-optimization experiments were performed in a 50 mL Erlenmeyer flask containing 2 g treated WS (120 °C, 1 h) and 25 mL citrate buffer (50 mM, pH = 4.8). Cellulase was employed at concentrations of 2.3, 6.9, 11.4, and 16.0 filter paper unit (FPU)/g WS, respectively.

The substrate content optimization experiments were performed at various substrate concentrations (5, 6.25, 8.3, and 12.5 mL citrate buffer (50 mM, pH = 4.8)/g WS) in a 50 mL Erlenmeyer flask containing a particular quantity of untreated or pretreated WS. The enzymatic saccharification process was performed at 50 °C in a constant temperature incubator shaker (ZQZY-80BS, Shanghai, China) with a 150 rpm shaking speed.

The fermentation-method-optimization experiments were carried out as follows: SHF was carried out by inoculating an 18 h culture of Angel Yeast (10% *v/v*, inoculum volume) into a reducing sugar-rich saccharified broth obtained from enzymatic saccharification test. SSF was performed by inoculating an 18 h culture of Angel Yeast (10% *v/v*, inoculum volume) in a reducing sugar-rich saccharified broth that had been pre-enzymolized for 36 h, while SSCF was performed in a 100 mL flask with a specific amount of treated WS and 25 mL of citrate buffer (50 mM, pH = 4.8) according to different substrate concentrations, inoculating an 18 h culture of Angel Yeast (10% *v/v*, inoculum volume). The fermentation process was carried out at 40 °C in a ZQZY-80BS constant-temperature incubator shaker with a 120 rpm shaking speed.

2.4. Analytical Methods

The composition of WS (cellulose, hemicellulose, and lignin) before and after DES pretreatment were determined by National Renewable Energy Laboratory (NREL)-defined analytical techniques [46]. High performance liquid chromatography (HPLC, Agilent Technologies 1260 Infinity II, Santa Clara, CA, USA) with an Aminex@HPX-87H Column (300 × 7.8 nm, Bio-Rad Corp., Santa Clara, CA, USA) and a refractive-index detector (RID) was used to determine the contents of fermentable sugars and ethanol at 55 °C using 5 mM H_2SO_4, purchased from Guangzhou Chemical Reagent Factory (Guangzhou, China), as mobile phase. The flow rate was set as 0.5 mL/min. Prior to analysis, all samples were particle-removed using a 0.45 μm pore diameter filter.

The following equations were used to compute the cellulose conversion, glucose yield, and xylose conversion from the enzymatic hydrolysis process:

$$\text{cellulose conversion}(\%) = \frac{c_1 \times V \times 0.9 + c_2 \times V \times 0.95}{m_1} \times 100\% \quad (1)$$

$$\text{glucose recovery}(\%) = \frac{c_1 \times V}{m_2 \times 1.111} \times 100\% \quad (2)$$

$$\text{xylose conversion}(\%) = \frac{c_3 \times V \times 0.88}{m_3} \times 100\% \quad (3)$$

where c_1, c_2, and c_3 (g/L) are the glucose, cellobiose, and xylose concentration in the enzymatic hydrolysate, respectively; V is the hydrolysate volume (L); m_1 is the cellulose mass of substrate (g); m_2 is the cellulose mass of raw material (g); and m_3 is the xylan mass of substrate (g).

SEM was used to examine the morphology and structure of the WS samples before and after DES treatments. FT-IR analyses with a resolution of 4 cm^{-1} were used to detect the WS samples before and after DES processing. The sample were scanned 32 times with the range of 500–4000 cm^{-1}. XRD was used to determine the crystallinity of the WS samples before and after DES pretreatment. The radiation source of XRD was Cu Ka with accelerating voltage of 40 kV and a power of 40 mA. The diffraction pattern, 2θ, was obtained with intensities ranging from 5 to 50° at a rotation speed of 80 rpm. The crystallinity index (CrI) of cellulose was determined using the equation below [47]:

$$\mathrm{CrI} = \frac{\mathrm{I_{002}} - \mathrm{I_{am}}}{\mathrm{I_{002}}} \times 100\% \quad (4)$$

where I_{002} is the diffraction intensity from the 002-crystal plane at about $2\theta \approx 22.5°$ and I_{am} is the amorphous diffraction intensity at $2\theta \approx 18.2°$.

3. Results and Discussion

3.1. The Properties of DES

The measured pH of TEBAC/GL/ACH (1:2:0.05)-based DES was 0.45 and the viscosity of TEBAC/GL/ACH (1:2:0.05)-based DES was 4982.5 mPa·s (25 °C). The FT-IR spectra of TEBAC/GL/ACH (1:2:0.05)-based DES, TEBAC, GL and ACH are shown in Figure 1.

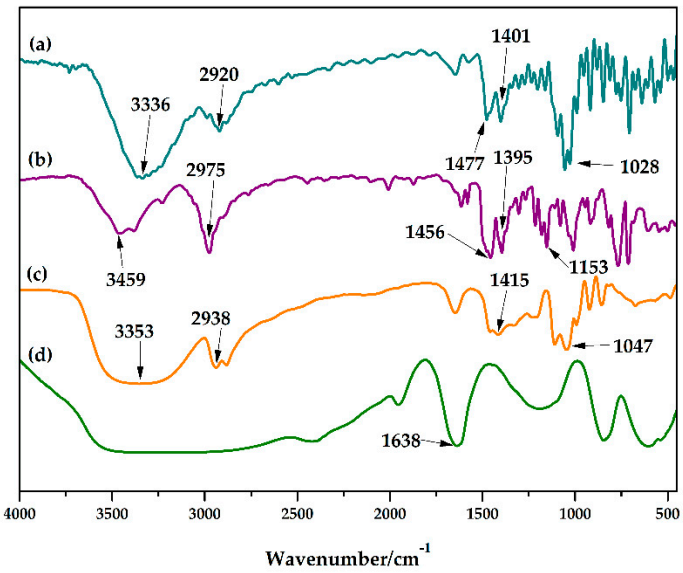

Figure 1. FT-IR spectra for (**a**) TEBAC/GL/ACH (1:2:0.05)-based DES, (**b**) TEBAC, (**c**) GL, and (**d**) ACH.

It is evident that for TEBAC/GL/ACH (1:2:0.05)-based DES, the stretching vibration of –OH was shifted from 3353 cm^{-1} to 3336 cm^{-1} compared with GL. Because TEBAC contained a small amount of water, the –OH group stretching appeared at 3459 cm^{-1}. In addition, the C-N group from TEBAC disappeared at 1153 cm^{-1} in the TEBAC/GL/ACH (1:2:0.05)-based DES sample, indicating that the hydrogen bond was formed between –OH group from GL and C-N group from TEBAC. In TEBAC/GL/ACH (1:2:0.05)-based DES, the peak of ACH at 1638 cm^{-1} also disappeared. Owing to ACH carrying three electronegative atoms of chlorine, it makes contributions to the formation of TEBAC/GL/ACH (1:2:0.05)-based DES [48]. The peaks at 2920 cm^{-1}, 1477 cm^{-1}, 1401 cm^{-1}, and 1028 cm^{-1} represented

-CH, -CH_2, -CH_3, and C=O, respectively [49]. Compared with TEBAC, GL, and ACH, the peaks of the above functional groups in TEBAC/GL/ACH (1:2:0.05)-based DES all shifted.

3.2. DES Pretreatment of WS at Different Temperatures

The effects of different pretreatment temperatures (100, 120, and 140 °C) on cellulose recovery, lignin removal, and enzymatic saccharification efficiency of WS by TEBAC/GL/ACH (1:2:0.05)-based DES were investigated at a short reaction time (1 h). Tables 1 and 2 summarize the pretreated solid yields, compositions, cellulose reservation, removal of lignin and hemicelluloses, and enzymatic conversion of WS under various pretreatment temperatures for 1 h at a ratio of 1:15 (solid to liquid).

Table 1. Effects of different pretreatment temperatures on component contents of WS.

Pretreatment	Solid Recovery (%)	Content (%)		
		Cellulose	Xylan	Lignin
Untreated WS	100	33.02 ± 0.27	17.26 ± 0.23	18.58 ± 0.45
100 °C	55.09 ± 0.15 [a, *]	57.29 ± 0.44 *	10.35 ± 0.70 *	9.94 ± 0.21 *
120 °C	45.80 ± 0.38 *	68.29 ± 0.40 *	3.94 ± 0.07 *	8.09 ± 0.46 *
140 °C	41.11 ± 0.20 *	60.94 ± 0.34 *	1.37 ± 0.32 *	7.67 ± 0.46 *

[a] Data: means ± standard deviations. * Data: means compared with untreated WS; effects of different pretreatment temperature have significant difference ($p < 0.05$), shown in Table S1.

Table 2. Effects of different pretreatment temperatures on recovery, removal, and enzymatic hydrolysis of WS.

Pretreatment	Cellulose Recovery (%)	Xylan Removal (%)	Lignin Removal (%)	Cellulose Conversion [b] (%)	Glucose Recovery [c] (%)
Untreated WS	100	0	0	22.48 ± 0.38	22.48 ± 0.38
100 °C	95.61 ± 0.42 [a, *]	66.97 ± 0.20 *	70.53 ± 0.32 *	77.65 ± 1.53 *	58.61 ± 0.27 *
120 °C	94.73 ± 0.22 *	89.53 ± 0.36 *	80.05 ± 0.62 *	91.15 ± 1.07 *	71.58 ± 1.34 *
140 °C	75.86 ± 0.59 *	96.74 ± 0.37 *	83.02 ± 0.68 *	97.49 ± 0.80 *	57.98 ± 1.70 *

[a] Data: means ± standard deviations. [b] Based on treated WS. [c] Based on untreated WS. * Data: means compared with untreated WS; effects of different pretreatment temperature have significant difference ($p < 0.05$), shown in Table S1.

As shown in Table 1, the percentage of residue recovered declined with increasing temperature, from 100% (untreated WS) to 41.11 ± 0.20% after 140 °C, suggesting significant loss of various components in WS. Fang et al. reported that no significant removal of lignin was observed when using ChCl/GL-based DES to pretreat WS [41]. This is due to the weak H-bond-accepting by the occupied-site anions and deficient-proton/inactive-acid sites [39]. In addition, the delignification ratio of ChCl/GL-based DES with $AlCl_3$ could reach 67.88% [42]. In this study, the removal of lignin improved steadily as the temperature was raised, from 70.53 ± 0.32% to 83.02 ± 0.68% by using TEBAC/GL/ACH (1:2:0.05)-based DES. Furthermore, xylan removal was enhanced progressively from 66.97 ± 0.20% to 96.74 ± 0.37%. These findings indicate that TEBAC/GL/ACH (1:2:0.05)-based DES has a better removal effect on lignin and hemicellulose, and high temperature can increase bonds breaking between cellulose, xylan, and lignin. The conversion of residual cellulose produced after enzymatic saccharification was 97.49 ± 0.80% when the pretreatment temperature increased to 140 °C. The fact that the cellulose conversion was lower than glucose recovery was due to some cellulose in WS being lost after pretreatment. Zhao et al. reported ChCl/monoethanolamine-based DES could remove 71.4% lignin and reserve 93.7% cellulose at 70 °C for 9 h and 89.8% cellulose was converted to glucose by enzymatic saccharification [34]. This means that TEBAC/GL/ACH (1:2:0.05)-based DES has better pretreatment ability and can convert more cellulose to glucose. Regrettably, the recovery of glucose was 57.98 ± 1.70% at 140 °C and the yield of glucose at 140 °C was lower than that at 120 °C. This was because some of the cellulose was lost when the lignin and hemicellulose

were removed as the temperature rose, and the enzymatic hydrolysis of glucose yield was poor. As can be seen from Table 3, using TEBAC/GL/ACH (1:2:0.05)-based DES to pretreat WS had the benefit of having a high lignin removal and high cellulose recovery in a short time. TEBAC/GL/ACH (1:2:0.05)-based DES-treated WS performed best at 120 °C, which was identified as the ideal reaction temperature for the pretreatment procedure, in terms of cellulose recovery, cellulose conversion, and glucose yield. Moreover, the recycling of DES is the key to its industrialization. We then examined the recycling performance of DES. The water was rotary-evaporated from the DES–water mixture at the end of each test, and the DES was dried as before, then reused in a further pretreatment reaction. After pretreating WS at 120 °C for 1 h, the recovery of DES was 98.9%. By using the recycled DES to pretreat WS, the cellulose recovery reached 97.69% and the lignin removal was 69.98% (see Table S3), showing the good recycling capability of DES. The reason for the decrease in properties of DES after recycling was because of the carboxylic acids and phenolic compounds produced by cellulose, hemicellulose, and lignin decomposition during the DES pretreatment.

Table 3. The pretreatment effects of different DESs and pretreatment conditions on WS.

DES	Molar Ratio	DES Pretreatment Condition		Recovery of Glucan (%)	Biopolymer Removal (%)		Reference
		Temperature (°C)	Time (h)		Lignin	Hemicellulose	
ChCl/MEA	1:6	70	9	93.7	71.4	42.1	[34]
ChCl/MEA [a]	1:6	90	12	90.8	81.0	47.3	[34]
TEBAC/LA	1:9	100	10	-	79.7	-	[36]
Alanine/LA	1:9	60	24	-	23.7	-	[50]
ChCl/BA/PEG-200 [b]	1:1:1.5	120	4	-	88.4	84.4	[43]
TEBAC/GL/ACH	1: 2: 0.05	120	1	94.7	80.1	89.5	This study

[a] MEA: monoethanolamine. [b] PEG-200: polyethylene glycol-200.

3.3. Optimization of Enzyme Loading

Figure 2 shows the enzymatic saccharification results of DES-pretreated WS at various enzyme loadings. The glucose concentration and cellulose conversion content in the four groups showed an increasing trend in the first 96 h of enzymatic saccharification, reaching a maximum value at 96 h, as shown in Figure 2A,B.

Obviously, the higher the cellulase loading, the higher the cellulose conversion during the first 72 h of enzymatic saccharification. Because most of the hemicellulose was removed by DES, the fluctuation in xylan conversion and xylose content was not noticeable as the enzyme loading increased, as illustrated in Figure 2C,D. As shown in Table S4, cellulase loading has significance influence of glucose concentration and cellulose conversion ($p < 0.05$). Wu et al. reported that after 78.4% lignin removed, 97.9% cellulose conversion was achieved by using complexed cellulases (30.0 FPU/g raw material and 60.0 CBU/g raw material) [51]. In addition, the cellulase loading at 27 FPU/g raw material achieved 88.61% glucose conversion [52]. In this study, when the enzyme loading was 11.4 FPU/g WS, the cellulose conversion, glucose content, xylan conversion, and xylose content were 91.92 ± 0.75%, 48.05 ± 0.38 g/L, 126 ± 18.0%, and 2.26 ± 0.32 g/L, respectively. After pretreatment by TEBAC/GL/ACH (1:2:0.05)-based DES, cellulose conversion can achieve more than 90% at low cellulase loading. However, the increase of cellulase loading can improve glucose yield to a certain extent, but the cost will increase as well. The high cost of cellulase is one of the barriers to the industrialization of cellulosic ethanol [53]. As a result of the angle of impact and economy, 11.4 FPU/g WS was determined to be the best enzyme loading.

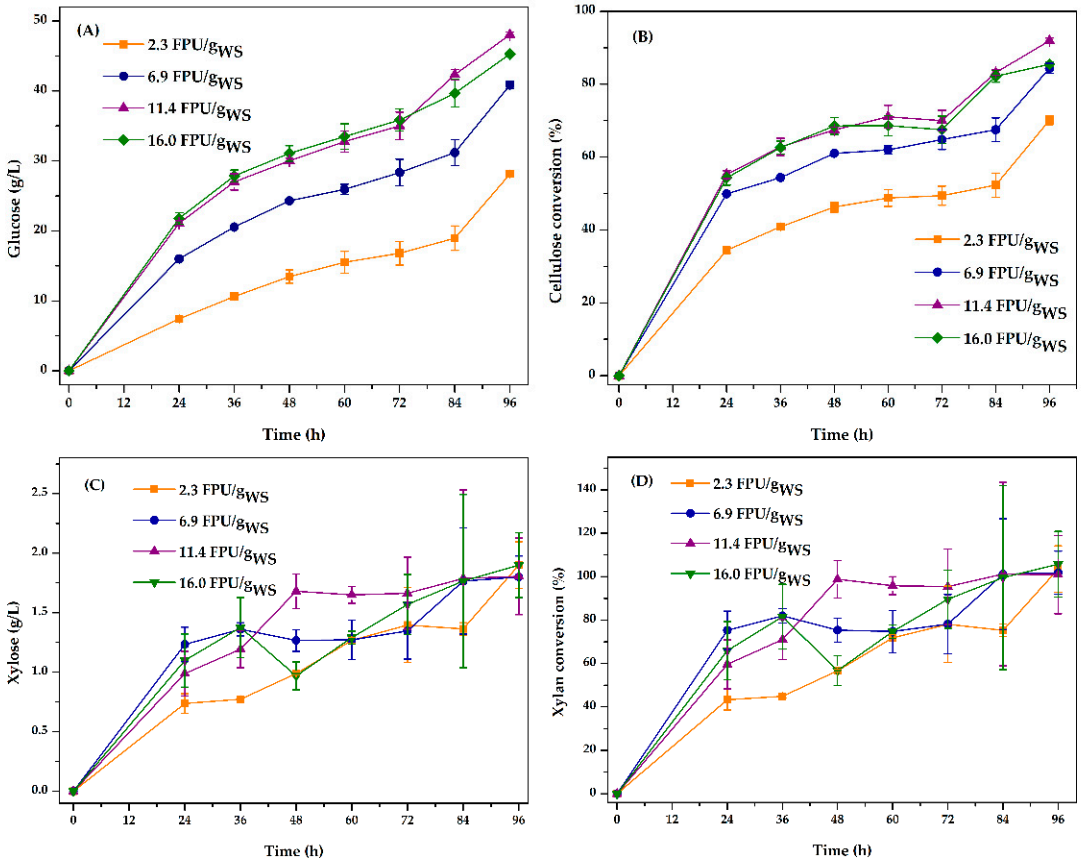

Figure 2. Enzymatic saccharification of DES pretreated WS under different enzyme loadings at a solids content of 8% (*w/v*) in citric buffer solution for 96 h. (**A**) Glucose concentration, (**B**) cellulose conversion, (**C**) xylose concentration, and (**D**) xylan conversion.

3.4. Optimization Analysis of Ethanol Fermentation Process

3.4.1. Optimization of Fermentation Method

In order to investigate the effects of different fermentation methods on ethanol yield, Figure 3 depicts the ethanol growth and glucose consumption of three types of fermentation.

The concentration of glucose declined as SHF and SSCF progressed, but the concentration of ethanol rose. At the end of fermentation, the highest ethanol concentration was 15.42 ± 0.31 g/L in SHF. However, SSCF devoured all the glucose in 24 h and had a maximum ethanol concentration of 12.56 ± 0.36 g/L. Furthermore, glucose concentration and ethanol concentration both increased throughout the first 6 h of SSF. The slope of the glucose concentration curve was greater than that of ethanol concentration curve, due to the rate of enzymatic saccharification being greater than that of fermentation. After 6 h of reaction, the glucose content dropped and the ethanol concentration rose, suggesting that enzymatic saccharification was complete or that the rate of enzymatic saccharification was slower than fermentation. Obviously, the ethanol concentration of SSF was lower than SHF and SSCF and the maximum ethanol content of SSF was 8.58 ± 0.14 g/L. The reason of this phenomenon was the lower efficiency of hydrolysis, which should be kept at low temperature for enzymatic saccharification in order to be compatible with fermentation [53]. In conclusion, the ideal fermentation process, with the maximum ethanol production, was determined to be SHF.

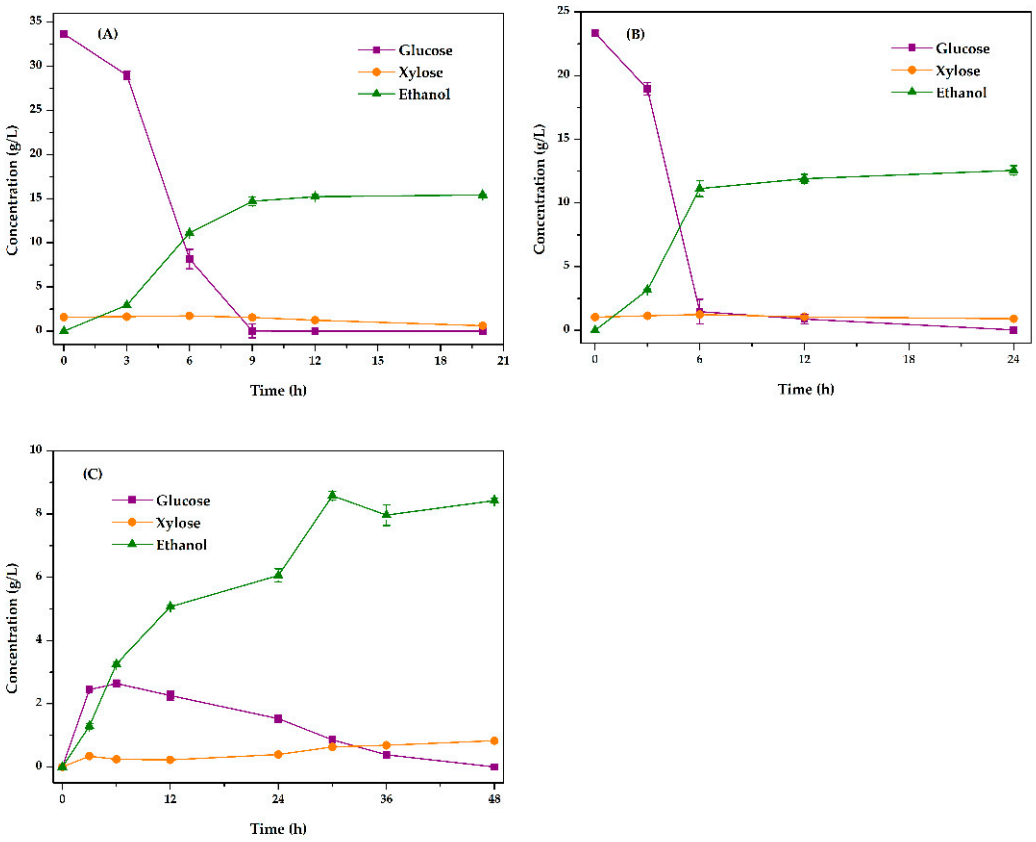

Figure 3. The curves of ethanol growth and fermentable sugars consumption by different fermentation methods. (**A**) SHF, (**B**) SSCF, and (**C**) SSF.

3.4.2. Optimization of Fermentation Substrate Content Conditions

When the process of enzymatic hydrolysis and fermentation was carried out in high substrate concentrations, high concentration of sugar and ethanol could be produced. That could improve the cost-effectiveness of biomass conversion [54,55]. However, with an increase in substrate content, the viscosity of the slurry would increase and the mass transfer would be limited. That might affect the conversion of enzymatic saccharification and the yield of ethanol [56]. Hence, to choose a suitable substrate content could effectively improve the biomass conversion. Figure 4 depicts the outcomes of enzymatic saccharification and fermentation by SHF under various fermentation substrate concentrations.

As illustrated in Figure 4, the glucose concentration of different substrate concentrations rose steadily over time, reaching equilibrium after 84 h. As can be seen in Figure 5, the concentration of glucose produced by enzymatic saccharification increased as the fermentation substrate increased.

However, the conversion of cellulose was inversely associated with the fermentation substrate and was trending downward. This is because when the quantity of the fermentation substrate increases, so does the glucose concentration achieved by enzymatic saccharification, which suggests that having a lot of fermentation substrate is good for the fermentation. This demonstrates that substrate concentration showed significant influence of fermentable sugar and ethanol concentration (see Table S2, $p < 0.05$). A high quantity of fermentation substrate, however, causes agitation resistance, which hinders the coupling of cellulase with lignocellulose, resulting in lower cellulose conversion [55]. In Figure 6, it is clearly shown that glucose was consumed in the first 12 h in the fermentation group with 8–16% fermentation substrate concentration, whereas the fermentation group with 20% fermentation substrate content was finished in 18 h. Overall, the production of ethanol increased as the fermentation substrate concentration increased, although the fermentation duration increased.

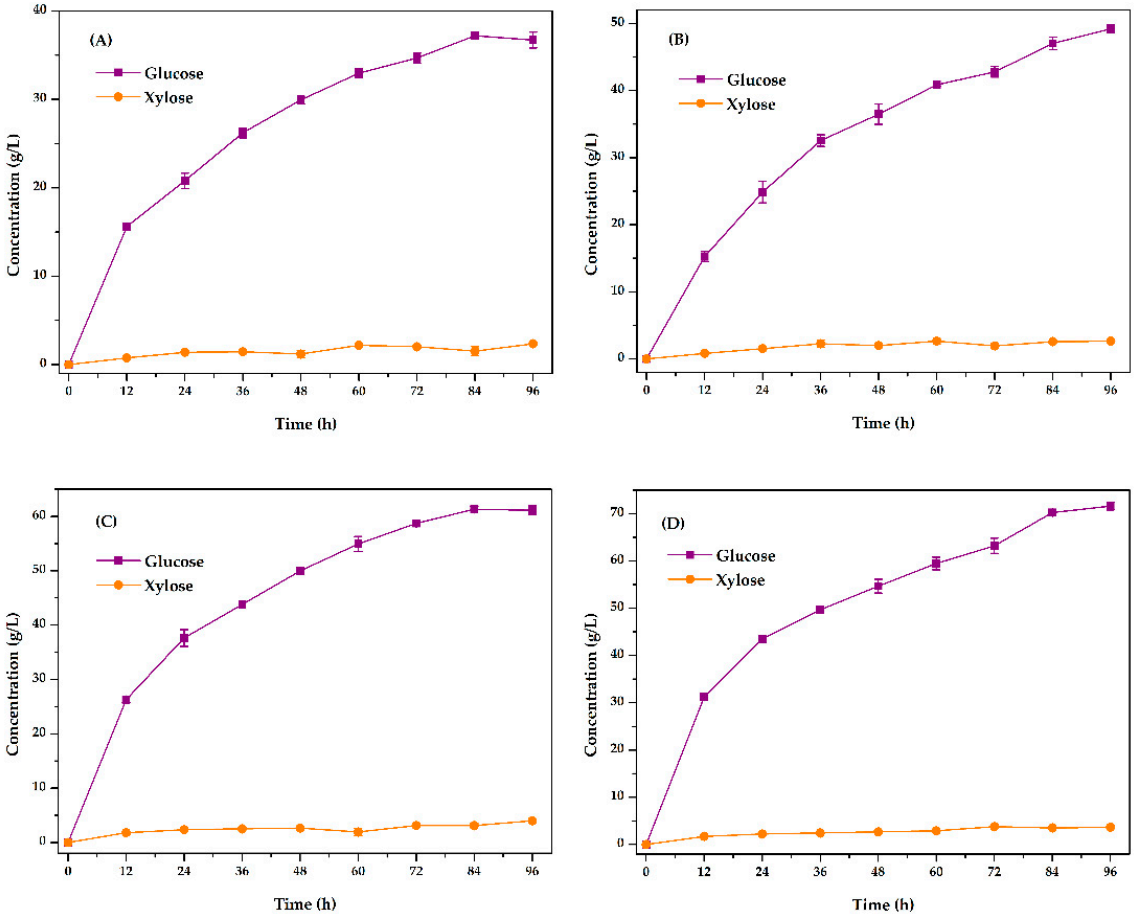

Figure 4. The results of enzymatic saccharification under different fermentation substrate concentrations. (**A**) Solids content of 8% (*w*/*v*), (**B**) solids content of 12%, (**C**) solids content of 16%, and (**D**) solids content of 20%.

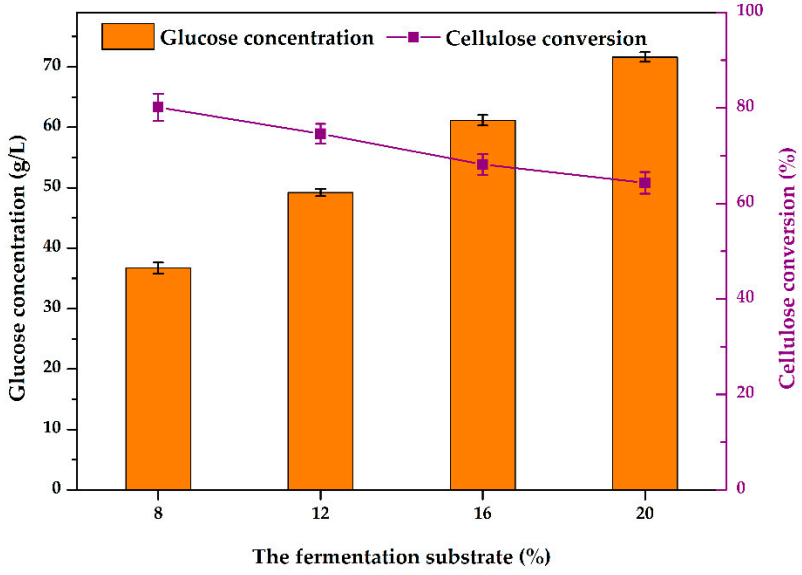

Figure 5. Comparison of enzymatic saccharification results with different substrate concentrations.

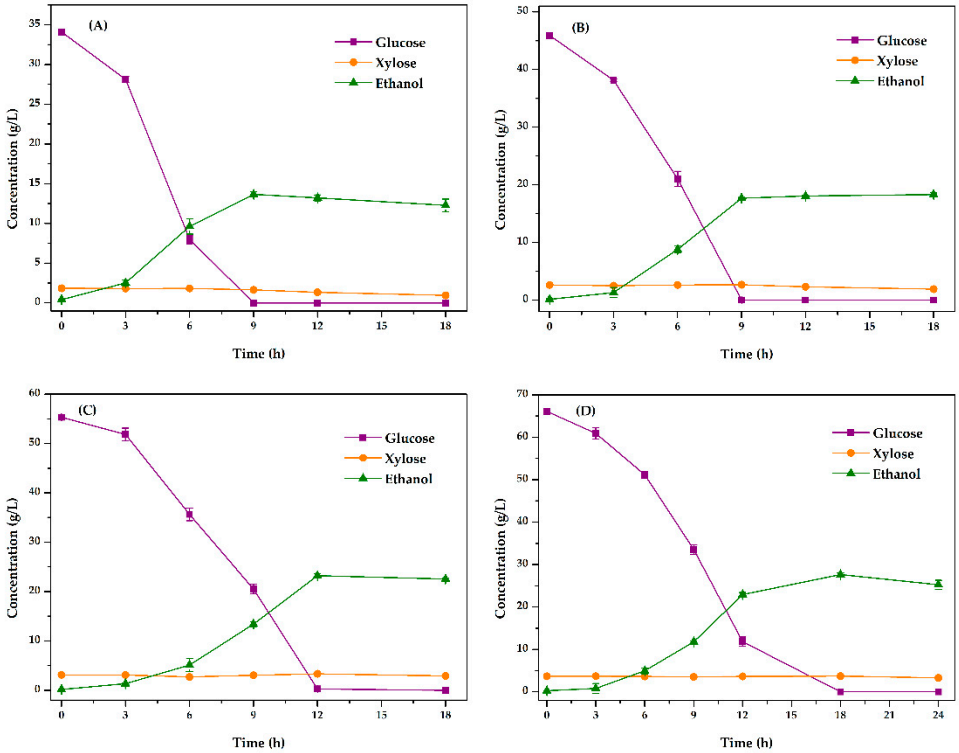

Figure 6. The results of SHF under different fermentation substrate content. (**A**) Solids content of 8% (*w*/*v*), (**B**) solids content of 12%, (**C**) solids content of 16%, and (**D**) solids content of 20%.

3.5. Characterization of WS before and after Pretreatment

FT-IR spectra were used to identify chemical structural changes in untreated and DES-treated WS at various pretreatment temperatures (see Figure 7).

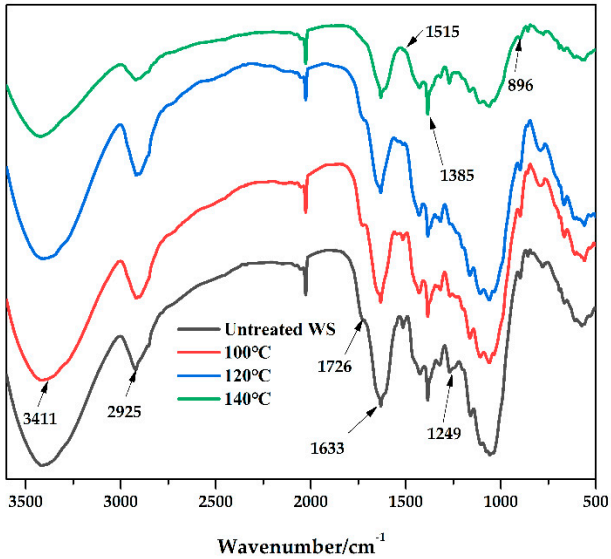

Figure 7. FT-IR spectrum of untreated and treated WS by DES at different pretreatment temperatures.

As the pretreatment temperature increased, the prominent peak at 1726 cm^{-1} progressively faded, suggesting that the hemicellulose was gradually eliminated [36]. Moreover, compared with the untreated WS, the peaks at 1511 cm^{-1} and 1249 cm^{-1} were significantly weakened, indicating that the β-ester bond between lignin polymers was broken and lignin was removed [57]. Because of the above FT-IR spectra data, DES broke the chemical

connections between lignocellulose to remove lignin, and the removal effect enhanced as the temperature was increased. The change in crystallinity of the cellulose could be shown in the XRD patterns (see Figure 8).

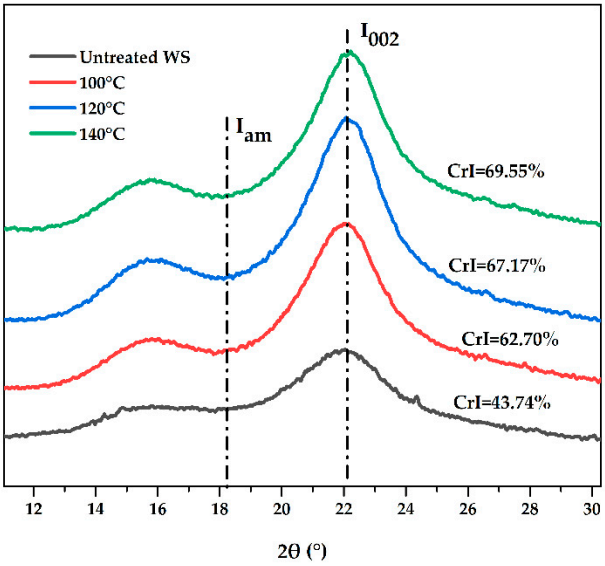

Figure 8. XRD patterns of untreated and treated WS by DES at different pretreatment temperatures.

After pretreatment, the crystallinity index of the cellulose (CrI) changed. Clearly, compared with the untreated WS (43.74%), the value of CrI increased to 69.55% with the increase in temperature. This demonstrated that most lignin and hemicellulose was removed by DES pretreatment, resulting in a drop in the amorphous components in WS and a rise in crystallization area, increasing the crystallinity index. The removal of lignin and hemicellulose may improve cellulase accessibility, increasing the efficiency of enzymatic saccharification. SEM was used to examine the morphological changes in WS before and after DES pretreatment at 120 °C (see Figure 9).

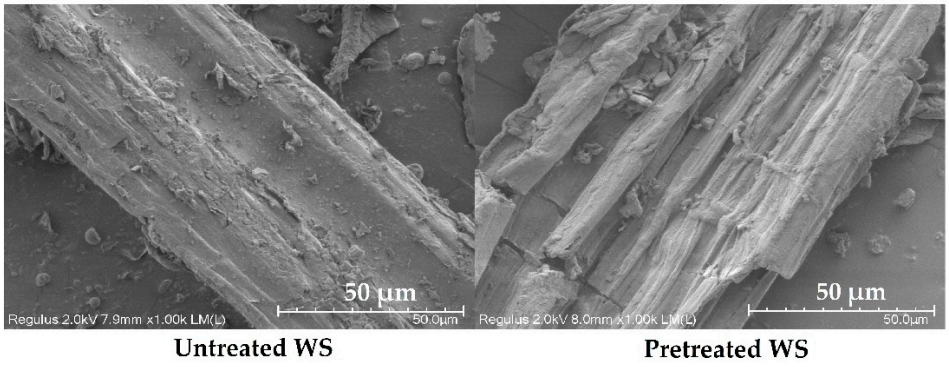

Figure 9. SEM pictures of untreated WS and pretreated WS using DES at 120 °C.

The untreated WS surface had a smooth and dense outer-layer structure. After DES pretreatment, there were numerous fractures and holes on the surface of WS, and the treated WS structure was fragmented into smaller structures. That is because lignin and hemicellulose were removed by DES, and cellulose was exposed outside the surface. As a result, the loose and porous WS afforded many binding sites for cellulase during enzymatic saccharification, resulting in a significant increase in enzyme digestibility.

4. Conclusions

In this study, TEBAC/GL/ACH (1:2:0.05) DES was used to pretreat WS, and a thorough investigation of the pretreatment temperature, enzyme loading, fermentation method, and fermentation substrate was made. This pretreatment technique improved the WS's ability to be saccharified by enzymes. The maximum ethanol yield of TEBAC/GL/ACH (1:2:0.05) DES-treated WS was 81.40 ± 0.01% (pretreatment conditions: 120 °C, 1 h; enzymatic saccharification conditions: solids content of 16%, 11.4 FPU/g WS of Celluclast 2.0 L; and fermentation method: SHF). Novel ternary DES exhibits significant application potential for the manufacture of second-generation fuel from biomass lignocellulose.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation8080371/s1, Table S1: ANOVA analysis and principal effects for temperature (T); Table S2: ANOVA analysis and principal effects for concentration of substrate (S); Table S3: Components content from recycled DES pretreated WS; Table S4: ANOVA analysis and principal effects for cellulase loadings (E).

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