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Enhanced Saccharification of Purple Alfalfa via Sequential Pretreatment with Acidified Ethylene Glycol and Urea/NaOH

Bo Fan †, Lin Zhang †, Zhengyu Tang †, Liang Chen and Yu-Cai He *

National-Local Joint Engineering Research Center of Biomass Refining and High-Quality Utilization, School of Pharmacy, Changzhou University, Changzhou 213164, China; fanboxiaoyu@cczu.edu.cn (B.F.); 16439112@smail.cczu.edu.cn (L.Z.); 18435417@smail.cczu.edu.cn (Z.T.); chenCCZU@outlook.com (L.C.)
* Correspondence: yucaihe@cczu.edu.cn or heyucai2001@163.com
† These authors contributed equally to this work.

Abstract: Purple Alfalfa is an inexpensive, abundant, readily available lignocellulosic material. This work was attempted to develop an efficient combination pretreatment by sequential $HClO_4$–ethyl glycol–H_2O (1.2:88.8:10, $w/w/w$) extraction at 130 °C in 0.5 h and urea/NaOH (urea 12 wt%, NaOH 7 wt%) soaking at −20 °C for 0.5 h for the pretreatment of purple alfalfa. The porosity, morphology, and crystallinity of pretreated purple alfalfa were characterized with SEM, FM, XRD, and FTIR. This combination pretreatment had a significant influence on hemicellulose removal and delignification. The above changes could enhance cellulose accessibility to enzymes and improve the enzymatic digestibility of cellulose. High yields of reducing sugars from pretreated purple alfalfa were obtained at 93.4%. In summary, this combination pretreatment has high potential application in the future.

Keywords: purple alfalfa; pretreatment; urea/NaOH; ethylene glycol; saccharification

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1. Introduction

With an expanding world population and increasing resource demand, lignocellulosic biomass has gained more and more attention due to its abundance and renewability, which has been transformed into biofuel molecules and value-added bio-based compounds [1]. Alfalfa (*Medicago sativa*) is known as a commercial crop cultivated domestically throughout the whole world with an output of over 0.1 billion tons/year. In China, alfalfa is planted in a large area, and its annual output is about 1.0 million tons. Alfalfa has been widely utilized as animal feed, and bioenergy crops [2,3], and its leaves and stems have been utilized for biofuel production [4,5]. Alfalfa is a kind of typical lignocellulosic material, which is mainly composed of lignin, hemicellulose, cellulose [6]. The rigid structure of untreated biomass is highly recalcitrant to bacteria or enzymatic digestion [7–11]. Pretreatment is a crucial prerequisite step for the biotransformation of lignocellulosic materials into liquid biofuels and chemicals [3,12–15]. Numerous pretreatment techniques, including chemical, physical, physical-chemical, biological strategies, and their combinations have been utilized to enhance the enzyme digestion of lignocellulose [16–21].

It is known that urea/NaOH (UN) can be utilized to dissolve cellulose in biomass at cold temperatures [22–24]. UN-based pretreatments have been used to disrupt some rigid intermolecular chemical bonds and dissolve lignin for facilitating polysaccharides to contact cellulases during enzymatic saccharification. During the pretreatment of cotton linter with UN, the increased NaOH (15 wt%) could suddenly change the transformation of cellulose I into the cellulose II polymorph [25]. A combination of urea (5 wt%) with NaOH (15 wt%) could significantly reduce the transformation magnitude. Lou et al. [26] used UN (8 wt% NaOH, 12 wt% urea) to treat *Miscanthus* at 21 °C in 0.5 h, the enzymatic saccharification rate of pretreated *Miscanthus* could reach 72% after 72 h. Dai et al. [27] combined *Sphingobacterium* sp. LD-1 pretreatment at 30 °C with UN (NaOH 4 wt%, urea 6 wt%) at −10 °C for enhancing the enzymatic digestion of rice straw, which resulted in a 1.4-fold increase of reducing sugar than that of sole UN treatment. Clearly, UN-based pretreatments had a great potential in the enhancement of the enzymatic saccharification of lignocellulosic materials.

In the past few years, organosolv pretreatment has been utilized to pretreat different biomasses [13,28,29]. Although many pretreatment processes have been performed concerning organosolv pretreatment using low boiling-point solvents (e.g., ethanol, acetone, methanol, etc.) [30], these solvents have major disadvantages because of their high volatility and flammability under high-pressure conditions. However, high-boiling solvents (e.g., glycerol, boiling-point 290 °C; ethylene glycol (EG), boiling-point 197.6 °C) might be conducted without highly flammable and volatile solvents [31–33]. Related to glycerol, EG has a lower viscosity [34]. In biomass liquefaction, the acidified EG could pretreat cellulose to form EG-glucosides [35]. Acidified EG was used for liquefying bagasse at 190 °C [36]. $HClO_4$–EG–H_2O (1.2:88.8:10, *w*/*w*/*w*) was utilized to treat corn stover via microwave (200 W) at 100 °C in 5 min [37].

In this study, UN and acidified EG were used to pretreat purple alfalfa (PAF). Furthermore, scanning electron microscopy (SEM), Fourier transformed IR (FTIR), fluorescent Microscope (FM), and X-Ray diffraction (XRD) were used to characterize untreated and pretreated PAF. Finally, an efficient pretreatment was developed and systematically investigated by combining EG with UN pretreatment, which resulted in a noticeable increase of enzymatic saccharification of PAF.

2. Materials and Methods

2.1. Materials

Raw purple alfalfa (PAF) was collected from a village in Luan city (Anhui, China). Spezyme CP cellulase (105 FPU/mL) and Novozyme 188 (665 CBU/mL) were obtained from Sigma (St. Louis, MO, USA). Bull serum albumin (BSA), NaOH, ethylene glycol (EG), $HClO_4$, urea (U), and other reagents were bought from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2. Pretreatment of PAF

Pretreatment with urea/NaOH (UN): untreated PAF (UT-PAF) or inorganic acid–EG–H_2O-treated PAF (diameter < 3.0 mm) (seen below pretreatment with inorganic acid–EG–H_2O) was immersed in UN (NaOH 7 wt%, urea 12 wt%) at the solid-to-liquid ratio of 1:20 (*w*/*w*). To examine the performance temperature on the effects of enzymatic saccharification, PAFs were pretreated at −20–20 °C in UN for 10–50 min. To examine pretreatment time on the effects of enzymatic saccharification, PAFs were pretreated at −20 °C for 10–50 min in UN. The solid PAFs were collected by filtration and further washed with distilled water until colorless. The collected PAF was oven-dried at 60 °C.

Pretreatment with inorganic acid–EG–H_2O: UN-PAF or UT-PAF (diameter < 3.0 mm) was mixed with inorganic acid–EG–H_2O at a solid-to-liquid ratio of 1:20 (*w*/*w*). To examine inorganic acid type on the effects of enzymatic saccharification, PAFs were pretreated with inorganic acid–EG–H_2O containing H_2SO_4, $HClO_4$, or HCl) (1.2 wt%) at 130 °C for 0.5 h. To examine $HClO_4$ dosage on the effects of enzymatic saccharification, PAFs were pretreated with $HClO_4$–EG–H_2O containing $HClO_4$ (0–2.4 wt%) and EG (88.8 wt%) at 130 °C for 0.5 h. To examine EG loading on the effects of enzymatic saccharification, PAFs were pretreated with $HClO_4$–EG–H_2O containing $HClO_4$ (1.2 wt%) and EG (58.8–98.8 wt%) at 130 °C for 0.5 h. To examine pretreatment temperature on the effects of enzymatic saccharification, PAFs were pretreated with $HClO_4$–EG–H_2O containing $HClO_4$ (1.2:88.8:10, *w*/*w*/*w*) at 110–150 °C for 0.5 h. To examine pretreatment time on the effects of enzymatic saccharification, PAFs were pretreated with $HClO_4$–EG–H_2O (1.2:88.8:10, *w*/*w*/*w*) at 130 °C for 15–90 min.

Under agitation at 110–150 °C for 15–90 min, PAF regeneration, PAF recovery, and EG recovery were conducted as the previous procedures [1]. The collected PAF was oven-dried at 60 °C.

The involving pretreatments were known as urea/NaOH soaking (UN), EG–$HClO_4$–H_2O extraction (EG), sequential urea/NaOH soaking and EG–$HClO_4$–H_2O extraction (UN–EG), and sequential EG–$HClO_4$–H_2O extraction and urea/NaOH soaking (EG–UN). The used biomass samples were known as untreated PAF (UT-PAF), urea/NaOH soaked PAF (UN-PAF), EG–$HClO_4$–H_2O-extracted PAF (EG-PAF), EG–$HClO_4$–H_2O-extracted urea/NaOH-soaked PAF (UN–EG-PAF), and urea/NaOH-soaked EG–$HClO_4$–H_2O-extracted PAF (EG–UN-PAF).

2.3. Saccharification of PAF

Non-catalytic proteins (e.g., bovine serum albumin (BSA)) have been added to saccharification media for the reduction of unproductive cellulase adsorption to lignin, which would enhance the availability of enzymes to substrates during enzymatic hydrolysis. Saccharification of PAFs (20 g/L) was performed in 15 mL of sodium acetate buffer (50 mM, pH 4.8) containing 40 µL of tetracycline hydrochloride (10 mg/mL), BSA (0.10 g/mL), and a cocktail of enzymes (30 FPU/g PAF and 60 CBU/g PAF). Incubation was conducted at 50 °C with continuous shaking (180 rpm) for 1–72 h.

2.4. Analytical Method

The chemical compositions (e.g., glucan, xylan, and lignin) of PAFs were measured as the National Renewable Energy Laboratory (NREL) procedures [38]. Reducing sugars were measured using the dinitrosalicylic acid (DNS) method [39]. PAFs were recorded by JSM-6360LA scanning electron microscopy (SEM) (JEOL, Tokyo, Japan) at 15 kV, Nikon Eclipse Ti-S Fluorescent Microscope (FM) at 100×, NICOLET PROTÉGÉ 460 Fourier transformed IR (FTIR) spectra (Thermo Electron Co., Waltham, MA, USA), and D/max 2500 PC X-ray diffraction (XRD) (Rigaku Co., Akishima-shi, Japan) in the 2θ range between 5° and 80° in steps of 0.02°.

The yield of reducing sugars from PAFs was defined as the following:

$$\text{Yield of reducing sugars\%} = [\text{Reducing sugars released}] \times 0.9/[\text{Glucan and Xylan in PAF}] \times 100\%$$

where 0.9 is the conversion factor for glucose to glucan.

The crystallinity index (CrI) was calculated as below:

$$CrI\% = (I_{002} - I_{am})/I_{002} \times 100\%$$

where I_{002} is the intensity for the crystalline portion of cellulose at ~$2\theta = 22.0°$ and I_{am} is the amorphous portion at ~$2\theta = 16.3°$.

3. Results and Discussion

3.1. Pretreatment with Organic Acid–EG–H_2O

High-boiling solvents (HBSs) can be efficiently used for pretreating biomass [40,41]. In addition, inorganic acids might assist solvent pretreatment for the enhancement of enzymatic saccharification [37,42]. In this study, three inorganic acids (H_2SO_4, $HClO_4$, and HCl) were separately applied as additives in EG–H_2O to evaluate their synergistic effects. After the pretreatment with inorganic acid–EG–H_2O (1.2:88.8:10, $w/w/w$) at 130 °C for 0.5 h, PAFs were enzymatically hydrolyzed for 72 h. It was found that HCl, $HClO_4$, and H_2SO_4 could enhance the pretreatment efficiency, thus increasing the enzymatic saccharification of PAFs (Figure 1a). The yields of reducing sugars were 66.4%, 75.2%, and 68.6%, respectively. In EG–H_2O (88.8:10, w/w) without inorganic acid at 130 °C for 0.5 h, the yield of reducing sugars reached 55.1%, which was slightly higher than that of UT-PAF (49.0%). Clearly, $HClO_4$ could be chosen as a good inorganic acid additive for enhancing the enzymatic digestion of PAF.

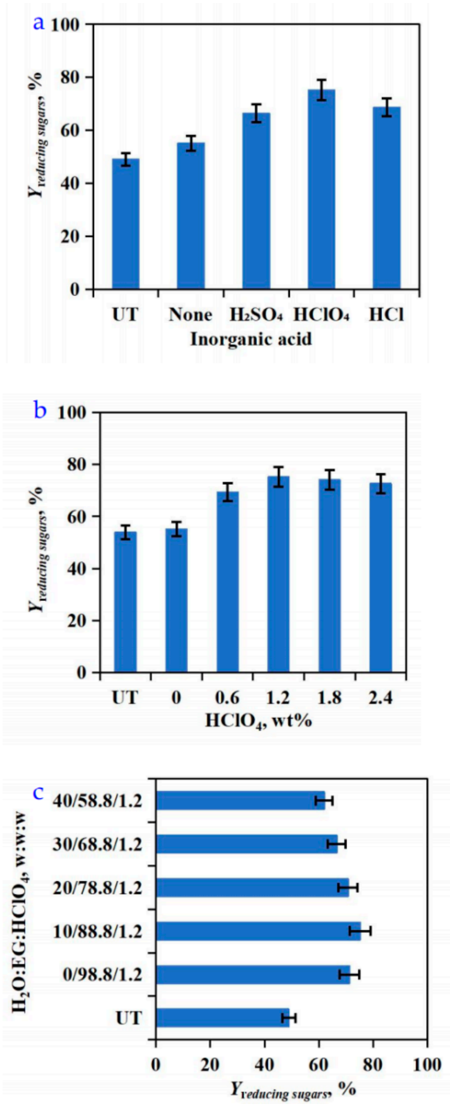

Figure 1. Effects of inorganic acid type (H_2SO_4, $HClO_4$, or HCl) (1.2 wt%) on the yield of reducing sugars [EG 88.8 wt%, H_2O 10 wt%, 130 °C, 0.5 h] (**a**). Effects of $HClO_4$ dosage (0–2.4 wt%) on the yield of reducing sugars [130 °C, 0.5 h, EG 88.8 wt%] (**b**). Effects of EG loading (58.8–98.8 wt%) on the yield of reducing sugars [$HClO_4$ 1.2 wt%, 130 °C in 0.5 h] (**c**).

Furthermore, the effects of $HClO_4$ dosage in the pretreatment media (EG–$HClO_4$–water) were examined on the enhancement of enzymatic saccharification (Figure 1b). By increasing the $HClO_4$ dosage from 0 to 1.2 wt% in EG–$HClO_4$–H_2O containing 88.8 wt% EG at 130 °C in 0.5 h, the yields of reducing sugars increased from 55.1% to 75.2%. Over 1.2 wt%, the saccharification decreased slightly. Thus, the optimum $HClO_4$ dose was 1.2 wt%. Moreover, the effects of EG dosage in the pretreatment media (EG-$HClO_4$-water) were examined on the enhancement of enzymatic saccharification. Upon raising the EG dosage from 58.8 wt% to 88.8 wt% in EG–$HClO_4$–H_2O containing 1.2 wt% of $HClO_4$ at 130 °C for 0.5 h, the yields of reducing sugars increased from 61.9% to 75.2% (Figure 1c). By raising EG dosage from 88.8 wt% to 98.8 wt%, the saccharification dropped from 75.2% to 71.3%. Clearly, the optimum EG loading was 88.8 wt% in EG–$HClO_4$–H_2O at 130 °C for 0.5 h. Performance temperature and time had profound effects on the enzymatic saccharification [37,39]. Upon raising the performance temperature from 110 to 130 °C for 0.5 h, the yield of reducing sugars increased significantly. Over 130 °C, a slight decrease of saccharification was observed. By increasing the performance time from 15 to 30 min at 130 °C, the yield of reducing sugars rose. Prolonging the performance time from 30 min to 90 min, the enzymatic saccharification had no significant change. It was found that the optimum performance time and temperature were 0.5 h and 130 °C, respectively (Figure 2a,b). Therefore, EG-$HClO_4$-water (88.8:1.2:10, *w*/*w*/*w*) as pretreatment media was utilized to pretreat PAFs at 130 °C for 0.5 h.

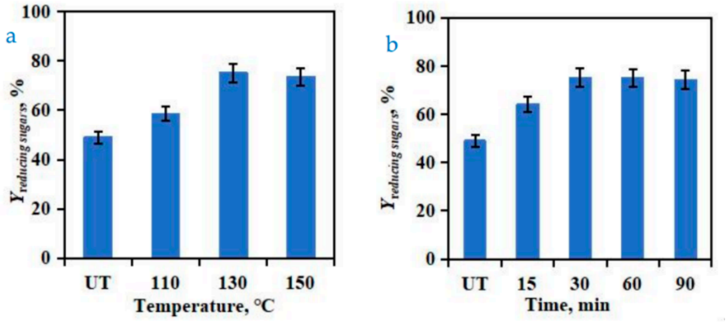

Figure 2. Effects of performance temperature (110–130 °C) for 30 min in EG-$HClO_4$-water (88.8:1.2:10, *w*/*w*/*w*) on the yield of reducing sugars (**a**). Effects of pretreatment time (15–90 min) at 130 °C in EG-$HClO_4$-water (88.8:1.2:10, *w*/*w*/*w*) on the yield of reducing sugars (**b**).

3.2. Pretreatment with UN

UN-based pretreatment has been utilized to improve the enzymatic saccharification of lignocellulosic biomass [43,44]. In this study, aqueous UN containing NaOH (7 wt%) and urea (12 wt%) was used to pretreat PAF. After different performance temperatures (−20 °C, −10 °C, 0 °C, 10 °C, and 20 °C) for 0.5 h, PAFs were enzymatically hydrolyzed for 72 h. It was found that pretreatment temperature had a significant influence on the yields of reducing sugars. As depicted in Figure 3a, the enzymatic saccharifications of pretreated PAFs (20 g/L) were improved greatly compared with that of the untreated one. After 72 h, the yields of reducing sugars from untreated and treated samples at −20 °C, −10 °C, 0 °C, 10 °C, and 20 °C were 69.0%, 63.9%, 63.3%, 62.4%, and 61.8%. Above all, the PAFs pretreated with UN at cold conditions could make them more widely able to be accessed and easy to be enzymatically digested, and thus enhanced the enzymatic saccharification efficiency [26,44]. Obviously, the highest yield of reducing sugars was found at −20 °C. Therefore, −20 °C was chosen as the optimal temperature for UN pretreatment.

To further improve the performance of pretreatment, the effects of pretreatment time (10–50 min) were also examined at −20 °C. As illustrated in Figure 3b, pretreatment time had a significant influence on the enzymatic saccharification. The saccharifications of pretreated PAFs (20 g/L) were improved greatly compared to that of UT-PAF. After 72 h, the yields of reducing sugars from PAF pretreated for 10 min, 20 min, 30 min, 40 min, 50 min, and 60 min were 63.4%, 64.5%, 69.0%, 67.0%, and 65.3%, respectively. Obviously, the highest yield of reducing sugars was observed at 0.5 h. Thus, 0.5 h was chosen as the optimal time for UN pretreatment at −20 °C.

3.3. Combination Pretreatment with UN and EG

Furthermore, EG–UN (sequential EG–$HClO_4$–water and urea/NaOH) and UN–EG pretreatment were attempted to pretreat UT-PAF. After the pretreatment, the saccharification rate of EG-UN-PAF (61.2 wt% glucan, 8.2 wt% xylan, 18.3 wt% lignin) was higher than those of UT-PAF (29.0 wt% glucan, 14.9 wt% xylan, 11.5 wt% lignin), UN-PAF (37.0 wt% glucan, 14.6 wt%, 17.5 wt% lignin), EG-PAF (54.6 wt% glucan, 10.7 wt% xylan, 19.5 wt% lignin) and UN–EG-PAF (57.6 wt% glucan, 9.2 wt% xylan, 18.8 wt% lignin). During the enzymatic digestion, the yields of reducing sugars from EG–UN-PAF appeared to be ~two-folds higher than that from UT-PAF. The yield of reducing sugars of UT-PAF, UN-PAF, EG-PAF, UN–EG-PAF, and EG–UN-PAF were 49.0%, 69.0%, 75.2%, 88.1%, and 93.4% (Figure 3c), respectively. The combination pretreatment UN-EG and EG-UN could facilitate the enzymatic hydrolysis of PAFs. Probably, lignin and xylan in UN–EG-PAF, and EG–UN-PAF did not reach the amount to inhibit and/or deactivate cellulases [1,2]. Notably, the EG–UN-PAF had a higher yield of reducing sugars and glucose than that of UN–EG-PAF. Thus, EG–UN (sequential EG–$HClO_4$–water and urea/NaOH) pretreatment was more suitable to pretreat PAF than UN–EG pretreatment.

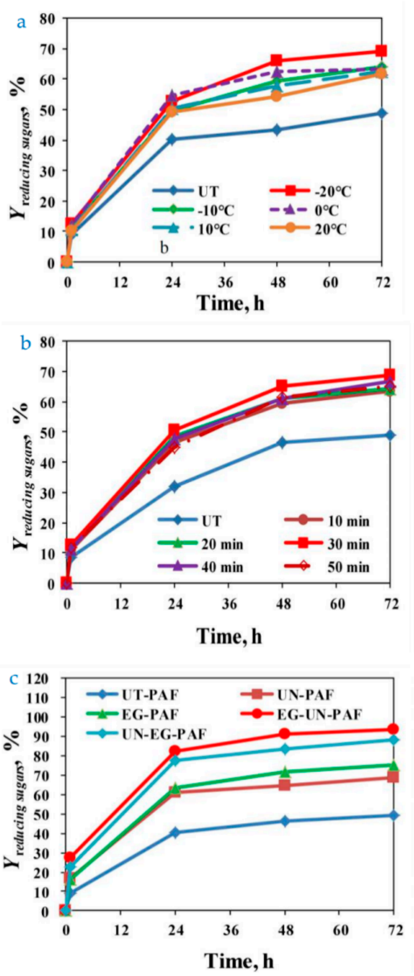

Figure 3. Effects of performance temperature (−20 °C, −10 °C, 0 °C, 10 °C, or 20 °C) for 30 min on the yield of reducing sugars (**a**). Effects of pretreatment time (10–50 min) at −20 °C on the yield of reducing sugars (**b**). Effects of pretreatment method at −20 °C for 30 min on the yield of reducing sugars (**c**).

3.4. Characteristics of Untreated and Pretreated PAFs

The structural features (e.g., porosity, morphology, and crystallinity) of PAFs were characterized with SEM. Figure 4 illustrates the morphology difference of UN-PAF and pretreated PAFs. UT-PAF had a smooth surface. While UN-PAF, EG-PAF, UN–EG-PAF, and EG–UN-PAF had significant cracks and rougher surfaces compared to UT-PAF. These results indicated that more cellulose was exposed on the surface after the pretreatment, which was consistent with the results of the chemical composition analysis of pretreated PAFs. Overall, these pretreatments showed an obvious effect on hemicellulose removal and delignification, which would result in the enhancement of enzymatic saccharification [34,37].

The PAF samples before and after pretreatment were also observed using FM. Compared to UT-PAF, the pretreated PAFs, especially EG–UN-PAF, had swollen fibers and short microfiber bundles (Figure 5). The crystallinity degree of cellulose has been considered an important factor in resisting enzymatic hydrolysis. Therefore, XRD was used to examine the crystal properties of untreated and pretreated PAFs (Figure 6a). The main diffraction peaks of 16.3° and 22.0° were attributed to the typical crystalline structures of cellulose I 101 and 002, respectively. The increasing order of the crystallinity index was $CrI_{(EG\text{-}PAF)}$ = 45.0 >

$CrI_{(UN–EG-PAF)} = 43.6 > CrI_{(EG–UN-PAF)} = 37.5 > CrI_{(UN-PAF)} = 29.9 > CrI_{(UT-PAF)} = 29.4$. After EG, UN, EG–UN, and UN–EG pretreatments, the *CrI* values of pretreated PAFs increased because of the removal of the amorphous components in PAFs and the increase of the glucan contents.

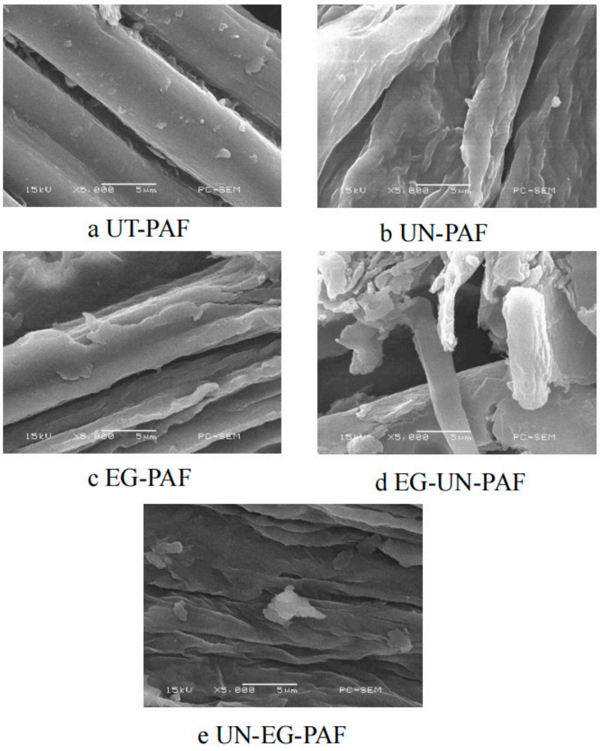

Figure 4. SEM images of UT-PAFs (**a**), UN-PAF (**b**), EG-PAF (**c**), EG-UN-PAF (**d**), and UN-EG-PAF (**e**).

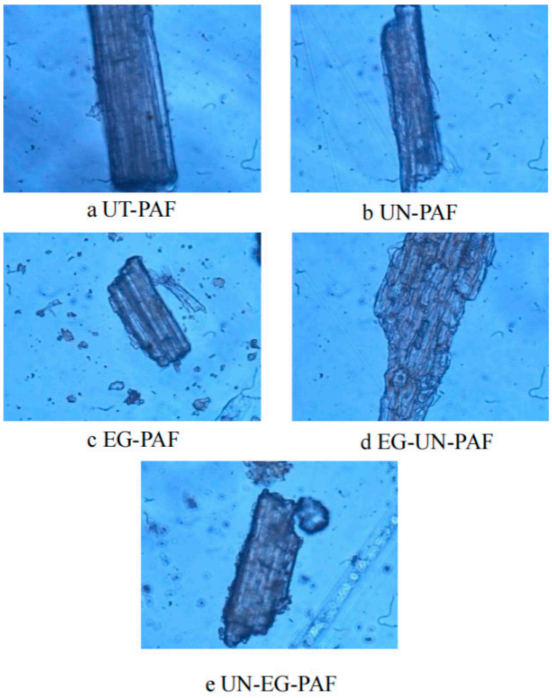

Figure 5. FM images of UT-PAFs (**a**), UN-PAF (**b**), EG-PAF (**c**), EG-UN-PAF (**d**), and UN-EG-PAF (**e**).

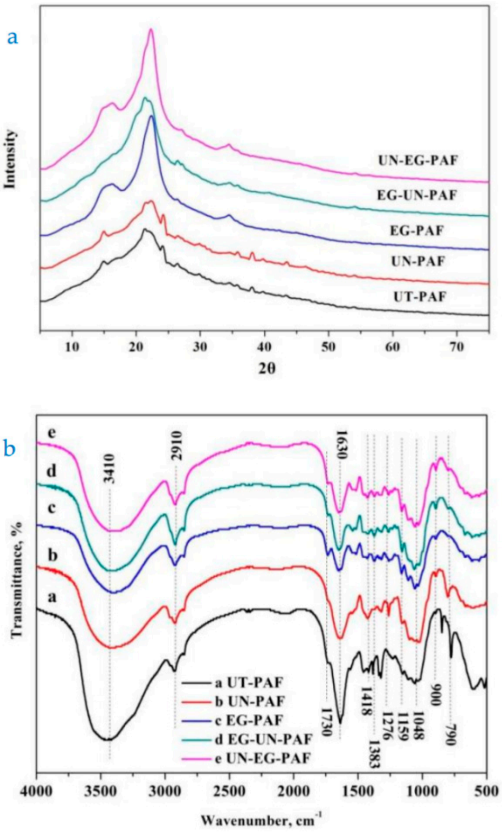

Figure 6. XRD (**a**) and FTIR (**b**) images of untreated and pretreated PAFs.

The FTIR spectra of PAF samples before and after pretreatment were recorded in Figure 6b. The peak near 900 cm^{-1} is related to the β-glycosidic bond among carbohydrates [45]. The peak near 1048 cm^{-1} is associated with C-O stretching [46]. The enhancement of absorption spectra at 1000–1200 cm^{-1} for the pretreated one indicated the increase in cellulose recovery in the solid residue. The peak near 1276 cm^{-1} is associated with C-O stretching vibration. The peak near 1383 cm^{-1} is ascribed to C-H bending [47]. The peak near 1418 cm^{-1} is attributed to the CH_2 bending [48]. The peak near 1630 cm^{-1} is associated with the absorbed water bending vibrations [49]. UN-PAF, EG-PAF, EG–UN-PAF, and UN–EG-PAF separated lignin from the fibers. A decrease was found in the absorbance of the 1730 cm^{-1} band assigned to the carbonyl functional groups from hemicellulose for UN-PAF relative to UT-PAF. The absorption peak at 2900 cm^{-1} is assigned to CH_2 stretching vibration [50]. The peak near 3400 cm^{-1} is attributed to the O-H stretching of the hydrogen bonds [51].

4. Conclusions

This work aimed at evaluating the feasibility of using combination pretreatment to enhance the enzymatic saccharification of PAF. After the optimization, an efficient combination pretreatment of PAF was developed via sequential pretreatment with EG–$HClO_4$–water (88.8:1.2:10, $w/w/w$) at 130 °C for 0.5 h and urea/NaOH (EG–UN) (urea 12 wt%, NaOH 7 wt%) at −20 °C for 0.5 h. SEM, FTIR, FM, and XRD indicated that EG–UN pretreatment could efficiently remove amorphous hemicelluloses and celluloses, incurring a partial increase of *CrI*. EG–UN-PAF could be effectively saccharified into reducing sugars with a high yield (93.4%). In summary, this established combination pretreatment would be helpful for enhancing the enzymatic hydrolysis of lignocellulosic materials for the production of biofuels and biobased chemicals.

Author Contributions: Conceptualization, methodology, and writing original draft, B.F. and L.Z.; data curation, Z.T.; software, L.C.; supervision, review, and revising manuscript, Y.-C.H. All authors have read and agreed to the published version of the manuscript.

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