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A Combination Method of Liquid Hot Water and Phosphotungstic Acid Pretreatment for Improving the Enzymatic Saccharification Efficiency of Rice Straw

Shengming Zhang [1,2,*], Tiehan Mei [1,3], Chonghao Zhu [1], Huimin Shang [1], Shushan Gao [1,3], Liyuan Qin [1] and Haitao Chen [1,4,*]

[1] College of Engineering, Northeast Agricultural University, Harbin 150030, China; a07180409@neau.edu.cn (T.M.); s210702023@neau.edu.cn (C.Z.); s190701020@neau.edu.cn (H.S.); a07190058@neau.edu.cn (S.G.); qinliyuan@neau.edu.cn (L.Q.)
[2] Agricultural Engineering Postdoctoral Research Station, Northeast Agricultural University, Harbin 150030, China
[3] Key Laboratory of Pig-Breeding Facilities Engineering, Ministry of Agriculture and Rural Affairs, Harbin 150030, China
[4] Heilongjiang Province Technology Innovation Center of Mechanization and Materialization of Major Crops Production, Harbin 150030, China
* Correspondence: zhangshengming@neau.edu.cn (S.Z.); htchen@neau.edu.cn (H.C.); Tel.: +86-451-55191606 (S.Z.); +86-451-55190081 (H.C.)

Abstract: Chemical pretreatment can significantly improve the enzymatic hydrolysis efficiency of lignocellulosic biomass, thereby improving the yield of sugar materials for the production of cellulosic ethanol, but commonly used acid–base catalysts are difficult to recover and reuse. In this work, a combination method of liquid hot water (LHW) and phosphotungstic acid (PTA) pretreatment was performed to improve the saccharification efficiency of rice straw, and we attempted to evaluate the reuse effect of PTA catalysts. The rice straw was first treated with LHW at 180 °C for 90 min, and then treated with 20 mM PTA at 130 °C for 60 min. After pretreatment, the cellulose hydrolysis efficiency and glucose recovery of the rice straw increased by 201.85% and 164.25%, respectively. Glucose accounted for 96.8% of the total reducing sugar in the final enzymatic hydrolysate. After each PTA pretreatment, approximately 70.8–73.2% of the PTA catalyst could be recycled. Moreover, the catalytic activity of the PTA catalyst that had been used five times did not decrease. The improved enzymatic saccharification efficiency was attributed to the removal of 89.24% hemicellulose and 21.33% lignin from the lignocellulosic substrate. The two-step LHW-PTA pretreatment could pretreat biomass in the field of cellulosic ethanol production.

Keywords: rice straw; pretreatment; liquid hot water; phosphotungstic acid; enzymatic hydrolysis

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1. Introduction

Crop straw is the largest byproduct of agricultural production. With the increasingly serious energy crisis and environmental problems, the development of crop straw resources is a research hotspot of widespread concern [1,2]. Converting biomass into bioethanol, which complements or replaces petroleum-based fuel, can reduce the transportation sector's dependence on petroleum resources. After a series of treatments such as saccharification, fermentation, and distillation, the crop straw can be converted into cellulosic ethanol, thereby realizing the high-value utilization of crop straw resources [3]. The recalcitrant nature of the lignocellulose is the main reason for limiting the conversion and utilization of the carbohydrate. Therefore, it is necessary to pretreat the crop straw to improve the accessibility of cellulase to the substrate, thereby improving the saccharification efficiency of straw lignocellulose [4,5].

Chemical pretreatment and comprehensive pretreatment methods are effective in improving the saccharification efficiency of the biomass [6,7]. Zahoor et al. [8] used KOH/urea to pretreat rice straw, which increased the cumulative sugar yield of the rice straw by 4.7-fold. However, the KOH and urea cannot be recycled and reused for pretreatment. Using sulfuric acid solution to pretreat lignocellulosic raw materials, even without enzymatic hydrolysis, a high total sugar yield can be obtained [9,10]. However, it is also difficult to recycle the sulfuric acid catalyst, and before the fermentation process, the acid hydrolysate needs to be neutralized and detoxified, resulting in an increase in production costs. Some pretreatment methods, such as the dilute sulfuric treatment [11,12] and acid- or alkali-combined steam explosion treatment [13,14], can be used in combination with the enzymatic hydrolysis processes to reduce the production of fermentation inhibitors. However, the acid or alkali catalysts still cannot be reused.

Heteropoly acids belong to the category of solid acids. Due to the advantages of high catalytic activity, good thermal stability, low equipment corrosion, and easy recycling, heteropoly acids show a trend of gradually replacing the liquid acid catalysts in various chemical production types [15,16]. In particular, some heteropoly acids showed better selectivity in degrading lignin of biomass than that of sulfuric acid. Zhang et al. [17] found that 20–97% of lignin in wood can be catalytically degraded by the $H_4[Si(W_3O_{10})_4]\cdot xH_2O$ catalyst using γ-valerolactone as a solvent. Shatalov [18] found that the lignin in eucalyptus can be degraded through oxidation pathways in $H_6[PMo_{8.9}V_{3.1}O_{40}]$ or $H_7[PMo_{7.9}V_{4.2}O_{40}]$ aqueous solution, and the degradation rate of lignin can reach from 11.1% to 37.1%. Therefore, heteropoly acid catalysts may improve the efficiency of biomass enzymatic hydrolysis by degrading lignin.

Hemicellulose is more prone to hydrolysis in acidic solutions than lignin is [19,20]. If the crop straw is directly pretreated with heteropoly acid, pentose sugars degraded by hemicellulose, low molecular weight acid-soluble lignin, and heteropoly acid may be mixed, which is unfavorable for the separate utilization of biomass components and catalyst recovery. Liquid hot water (LHW) pretreatment is an efficient method to extract hemicellulose from biomass [21]. The surface area and porosity of the pretreated biomass can be increased after LHW pretreatment, which is beneficial to improve the accessibility of cellulose [22]. However, the lignin cannot be effectively degraded with LHW pretreatment. Therefore, it is often performed in combination with other pretreatment methods to achieve a better effect [23,24]. Xia et al. [25] used a combination of LHW and Na_2CO_3–O_2 methods for reed pretreatment, thereby avoiding the loss of carbohydrates and increasing total sugar yield. Araújoa et al. [26] found that lignin in corncobs can be effectively removed after the combined pretreatment of LHW, NaOH, and ionic liquid [Bmim]Cl, thus obtaining a high-quality lignocellulose raw material for the preparation of membrane materials. However, in the above methods, whether or how to reuse the catalysts such as sodium carbonate, sodium hydroxide, and ionic liquids needs to be further studied.

Therefore, to improve the saccharification efficiency of rice straw and provide more possibilities for the value-added utilization of cellulose and hemicellulose, a combined LHW PTA pretreatment was performed on the rice straw. In the initial LHW pretreatment, the effects of reaction time, reaction temperature, and solid/liquid ratio on the chemical composition of rice straw were studied. In the subsequent PTA pretreatment, the effects of reaction time, reaction temperature, and PTA concentration on the chemical composition and enzymatic saccharification efficiency of rice straw were investigated. After that, the structure of rice straw before and after pretreatment was analyzed with an X-ray diffraction spectrometer (XRD) and Fourier transform infrared spectroscopy (FT-IR). Lastly, the reuse performance of the recovered PTA catalyst was also evaluated. In this study, a catalyst-recyclable biomass pretreatment method was developed that can save catalyst costs and reduce environmental pressure, thereby providing a green and efficient pretreatment method for cellulosic ethanol production.

2. Materials and Methods

2.1. Materials and Reagents

Air-dried rice straw (containing 37.8% cellulose, 18.4% hemicellulose, 14.6% lignin, 11.3% ethanol extracts, 14.2% ash and 3.7% moisture) was collected from Northeast China, 44°55′12″ N, 127°9′ E. It was milled using an FW-100 mini plant cell crusher (Tianjin Taisite Instrument Co., Tianjin, China) to collect the 40-to-60-mesh powder. Then, the powder was subjected to Soxhlet extraction with absolute ethanol for 48 h and vacuum-dried at 45 °C to a constant weight to obtain the raw material (control containing 44.47% cellulose, 21.64% hemicellulose, 17.18% lignin, and 16.71% ash). The control was used for further pretreatment and analysis. The phosphotungstic acid and cellulose enzyme (cellulase enzyme blend Cellic CTec2) were purchased from Sigma-Aldrich Co. LLC. (Shanghai, China).

2.2. Pretreatment Method

Two-step LHW-PTA pretreatment tests were performed in a 100 mL hydrothermal microreactor (Beijing Huotong Experimental Instrument Co., Ltd., Beijing, China). For the initial LHW pretreatment method, the control and distilled water were added into a hydrothermal microreactor with a polytetrafluoroethylene liner. The mixture was carefully stirred, and the reactor lid was screwed on tightly. After that, the microreactor was transferred to the oven to start the reaction. The effect of the reaction temperature was studied at 150, 160, 170, 180, 190, and 200 °C; the effect of the reaction time was investigated at 30, 60, 90, 120, and 180 min; and the effect of solid/liquid ratio (w/v, g/mL) of the control to distilled water was studied at 1:10, 1:20, 1:30, and 1:40. After that, the mixture was filtered under reduced pressure while washing the solid residue with 50 mL of hot distilled water. The filtrate was collected and stored in the screw-top glass bottle for further analysis and use if necessary. The solid residue was dried at 105 °C for 4 h and further treated with the PTA.

Then, 3 g of LHW-treated solid residue and 60 mL of PTA solution were added into the hydrothermal microreactor to start the reaction. The reaction temperature was 120, 130, 140, and 150 °C; the reaction time was 30, 60, 90, and 120 min; and the concentration of PTA solution was investigated at a range of 10 to 50 mM. After the reaction had been completed, the mixture was filtered under reduced pressure, while the solid residue was washed to neutrality with about 200 mL of distilled water. The PTA-treated solid residue was dried at 105 °C for 4 h for further analysis and enzymatic hydrolysis.

2.3. Catalyst Recycling Method

Approximately 260 mL of filtrate from the PTA pretreatment was concentrated to about 15 mL by a rotary evaporator. The concentrate was mixed with 30 mL of ether and transferred to a separatory funnel to stand for 10 min. The bottom-layer solution was collected and mixed with 20 mL of ether. After centrifugation for 5 min, the supernatant was transferred to a Petri dish and purged with air at room temperature in the dark. After all the ether had been evaporated, the recovered PTA was obtained that could be reused for the pretreatment of rice straw.

2.4. Enzymatic Saccharification Method

The enzymatic hydrolysis experiment referred to the method that Xia et al. [25] described with some modifications. Briefly, it was performed in 20 mL of sodium acetate buffer solution (pH 4.8) at a substrate concentration of 2% (w/v). The active load of the Cellic CTec2 enzyme was approximately 20 FPU/g of the substrate. The parameters of the shaker were set as follows: the temperature was 50 °C, enzymatic hydrolysis time was 72 h, and rotation speed was 100 rpm. After the enzymatic hydrolysis experiment had been completed, the mixture was centrifuged for 5 min. The sugar content was then determined.

2.5. Analytical Methods

2.5.1. Quantitative Analysis of Chemical Composition

The chemical composition of rice straw before and after the pretreatment was determined according to the NREL/TP-510-42618 method [27]. Briefly, the content of lignin was determined by a two-step hydrolysis method with sulfuric acid in which acid-insoluble lignin was analyzed with a Persee T6 UV spectroscope (Beijing persee General Instruments Co., Ltd., Beijing, China). The reducing sugars were analyzed with an Agilent 1260 HPLC (high-performance liquid chromatography) apparatus (Agilent Technologies Inc., Santa Clara, CA, USA) with a refractive index detector. Chromatographic columns were the Bio-Rad HPX-87H column and Micro-Guard Cation H guard column, the flow rate of 0.005 M sulfuric acid solution was 0.5 mL/min, the sample volume was 50 µL, and the column temperature was 55 °C. The sugar content in the enzymatic hydrolysis test was also determined with the HPLC method. All experiments were performed in triplicate. Data were statistically analyzed with ANOVA using software SPSS 17.0 and presented as mean ± standard deviation.

Solid recovery, cellulose retention, hemicellulose removal, lignin removal, sugar yield, enzymatic hydrolysis efficiency, and sugar recovery were calculated on the basis of the following equations:

$$\text{Solid recovery } (\%) = \frac{\text{mass of sample after treatment}}{\text{mass of sample before treatment}} \times 100 \quad (1)$$

$$\text{Cellulose retention } (\%) = \frac{\text{cellulose mass of pretreated sample}}{\text{cellulose mass of sample before pretreatment}} \times 100 \quad (2)$$

where pretreated samples (same as Equation (3) below) were Samples 1 to 22.

$$X \text{ removal } (\%) = \frac{X \text{ mass of sample before pretreatment} - X \text{ mass of pretreated sample}}{X \text{ mass of sample before pretreatment}} \times 100 \quad (3)$$

where X is lignin or hemicellulose.

$$\text{Sugar yield of straw sample } (\text{mg/g}) = \frac{\text{mass of sugar in enzyme hydrolysate}}{\text{mass of straw sample before enzymatic hydrolysis}} \quad (4)$$

where straw samples (same as Equations (5) and (6) below) are the control or Samples 1 to 22; sugar is glucose, pentose, or reducing sugar; pentose is the sum of xylose and arabinose; and the reducing sugar is the sum of glucose and pentose.

$$\text{Enzymatic hydrolysis efficiency of } Y \text{ of straw sample}(\%) = \frac{\text{mass of sugar in enzyme hydrolysate}}{\text{theoretical sugar mass of straw sample before enzymatic hydrolysis}} \times 100 \quad (5)$$

where Y is cellulose, hemicellulose, or the straw sample itself; the sugar corresponding to cellulose is the glucose; the sugar corresponding to hemicellulose is pentose; and the sugar corresponding to the straw sample itself is the reducing sugar.

$$\text{Glucose (or pentose) recovery } (\%) = \frac{\text{mass of glucose (or pentose) in enzyme hydrolysate}}{\text{theoretical glucose (or pentose) mass of the corresponding control}} \times 100 \quad (6)$$

where corresponding control is the treated sample mass divided by the solid recovery.

2.5.2. Relative Crystallinity of Straw Cellulose

The X-ray diffraction spectra of the untreated and treated rice straw were recorded on ab Empyrean intelligent X-ray diffractometer (Malvern Panalytical Ltd., Almelo, The Netherlands). The scanning range was from 10° to 50° at a scanning speed of 2° per minute. The relative crystallinity of the cellulose of the rice straw samples was calculated using the Segal formula [26,28]:

$$CrI = \frac{I_{002} - I_{am}}{I_{002}} \times 100 \quad (7)$$

where *CrI* is the relative crystallinity of the cellulose, I_{002} (comprising the contribution of both crystalline and amorphous regions) is represented by the maximal diffraction intensity scanned in the range of $2\theta = 22°–23°$, and I_{am} (comprising only the amorphous contribution) is represented by the minimal diffraction intensity scanned in the range of $2\theta = 18°–19°$.

2.5.3. FT-IR Analysis

The FT-IR spectra of rice straw before and after the pretreatment were recorded on a Nicolet iS50 spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) in the range of 4000–500 cm^{-1}. The resolution and number of scans were set to 2 cm^{-1} and 32 times, respectively.

3. Results and Discussion

3.1. Effect of Reaction Conditions of Two-Step Pretreatment on Chemical Composition

3.1.1. Initial LHW Pretreatment

The chemical composition of the control and LHW-pretreated rice straw samples is shown in Table 2. The effect of reaction temperature increasing from 160 to 200 °C was observed using a solid/liquid ratio of 1:20 (rice straw/distilled water, *w*/*v*) and a reaction time of 90 min. A hemicellulose removal rate of 73.71% was obtained at the reaction temperature of 180 °C (run 3). Meanwhile, the lignin removal rate and cellulose retention rate were 4.15% and 95.54%, respectively. Increasing reaction temperature led to an increase in lignin removal and a decrease in cellulose retention, but no significant change in hemicellulose removal. The effect of reaction time increasing from 30 to 180 min was investigated using a reaction temperature of 180 °C and a solid/liquid ratio of 1:20. When the reaction time was over 90 min, the main effects were reflected in the improvement of lignin removal and the reduction in cellulose retention, while there was no significant change in hemicellulose removal. The effect of the solid/liquid ratio was studied using a reaction temperature of 180 °C and a reaction time of 90 min. The hemicellulose removal rate of 73.71% was obtained at a solid/liquid ratio of 1:20. Increasing the solid/liquid ratio led to a decrease in cellulose retention, while the hemicellulose removal rate did not significantly change. In all experiments, the maximal removal rate of lignin was approximately 7.36–8.81%.

Previous research found that the removal of hemicellulose or lignin from lignocellulosic substrates can increase the accessibility of cellulase to the substrate, which is beneficial in improving the enzymatic hydrolysis efficiency of lignocellulosic substrates [3,29]. Furthermore, low cellulose retention means less cellulose that can be saccharified. The enzymatic hydrolysis efficiency of LHW-pretreated rice straw samples was evaluated in subsequent tests (data in Supplementary Table S1). Results show that the enzymatic hydrolysis efficiency and glucose recovery rate of Sample 3 were 48.58% and 47.92%, respectively. These values were the highest in all LHW-pretreated samples. Moreover, hemicellulose is the third most abundant component in biomass, which can be processed into chemicals such as sugar, acid, and furfural [30,31]. Compared with the reaction conditions of run 3, the removal rate of hemicellulose could not be further improved by increasing the reaction temperature, prolonging the reaction time, or increasing the solid/liquid ratio. The enzymatic hydrolysis efficiency of hemicellulose of Sample 3 was 37.07%. Therefore, the optimal reaction conditions for LHW pretreatment were determined as follows: the solid/liquid ratio of rice straw to distilled water was 1:20, and the system was heated at 180 °C for 90 min.

3.1.2. Second Pretreatment with PTA

Sample 3 was further treated with phosphotungstic acid as a catalyst. PTA pretreatment conditions and the effects on the chemical composition are shown in Table 1. The hemicellulose in the rice straw was further removed by PTA pretreatment with a removal rate of about 87.97–90.8%. Since most of the hemicellulose (approximately 73.71%) of the rice straw had been removed during the LHW pretreatment, it could not be judged whether the PTA pretreatment conditions had a significant effect on the removal of hemicellulose. The effect of PTA pretreatment is mainly reflected in the removal of lignin. Under the reaction conditions of run 14, the lignin removal rate reached 21.33%. After that, by increasing the reaction temperature, prolonging the reaction time, or increasing the concentration of PTA, the lignin removal rate did not change significantly, but the cellulose retention rate showed a downward trend. Previous research found that 14.3–89.7% of hemicellulose in biomass can be removed by pretreatment with phosphotungstic acid as a catalyst, and the removal rate of hemicellulose is negatively correlated with the retention rate of cellulose [20,32,33]. The results of this study are consistent with previous studies. To assess which sample had the best saccharification effect, the enzymatic hydrolysis test was further performed.

3.2. Enzymatic Hydrolysis Efficiency

The glucose and reducing sugar yields of straw samples before and after the LHW and LHW-PTA pretreatment are shown in Figure 1 (data in Supplementary Table S1). In all samples, the highest glucose yield (465.55 ± 5.15 mg/g of the substrate) and reducing sugar yield (481.16 ± 4.08 mg/g of the substrate) were obtained in Sample 14, which were 3.79- and 2.72-fold, respectively, greater than those of the control. They were also 1.54- and 1.45-fold greater than those of sample 3, indicating that the followed PTA pretreatment could further improve the saccharification efficiency of the rice straw. Therefore, the reaction conditions of run 3 were determined to be the optimal PTA pretreatment conditions: the solid/liquid ratio was 3 g of Sample 3 per 60 mL of 20 mM phosphotungstic acid solution, and the system was heated at 130 °C for 60 min.

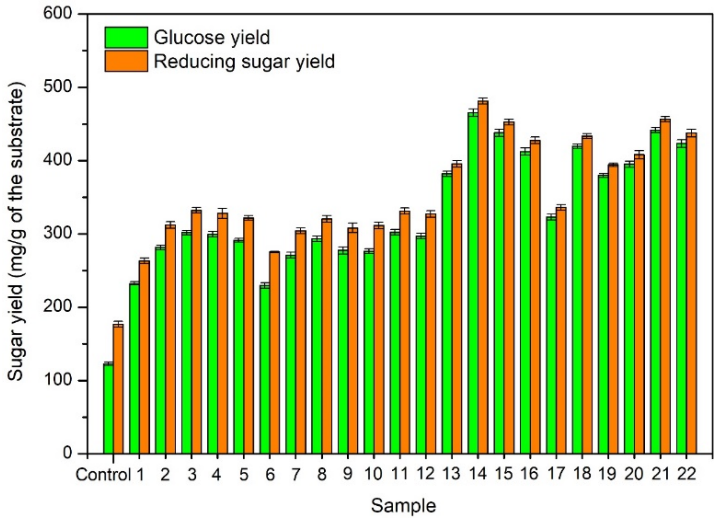

Figure 1. Glucose and reducing sugar yields of straw samples before and after LHW and LHW-PTA pretreatment.

Table 1. Changes in chemical components of rice straw samples before and after PTA pretreatment.

Run	Sample	Pretreatment Condition			Solid Recovery [1] (%)	Chemical Composition (%)			Composition Changes (%)		
		Temp. (°C)	Time (min)	Concentration (mM)		Cellulose	Hemicellulose	Lignin	Hemicellulose Removal	Lignin Removal	Cellulose Retention
3	3	—	—	—	—	54.15 ± 0.30	7.25 ± 0.14	20.99 ± 0.18	73.71 ± 0.47 f [2]	4.15 ± 0.94 e	95.54 ± 0.35 a
13	13	120	60	20	91.76 ± 0.15	55.78 ± 0.22	3.43 ± 0.07	20.29 ± 0.24	88.58 ± 0.24 de	14.96 ± 0.82 b	90.30 ± 0.42 c
14	14	130	60	20	88.79 ± 0.16	55.88 ± 0.25	3.44 ± 0.05	19.40 ± 0.36	89.24 ± 0.16 cd	21.33 ± 1.16 a	87.54 ± 0.36 d
15	15	140	60	20	82.64 ± 0.18	52.75 ± 0.13	3.39 ± 0.05	20.78 ± 0.30	89.85 ± 0.12 bc	21.59 ± 0.87 a	76.92 ± 0.30 f
16	16	150	60	20	78.48 ± 0.33	50.12 ± 0.11	3.52 ± 0.17	21.85 ± 0.17	89.98 ± 0.44 b	21.69 ± 0.37 a	69.40 ± 0.40 i
17	17	130	30	20	95.70 ± 0.19	55.80 ± 0.08	3.47 ± 0.10	20.47 ± 0.32	87.97 ± 0.33 e	10.55 ± 1.14 d	94.22 ± 0.12 b
18	18	130	90	20	79.25 ± 0.28	50.76 ± 0.19	3.38 ± 0.17	21.72 ± 0.17	90.28 ± 0.42 ab	21.41 ± 0.76 a	70.97 ± 0.21 h
19	19	130	120	20	72.93 ± 0.33	46.43 ± 0.05	3.48 ± 0.16	23.53 ± 0.38	90.80 ± 0.38 a	21.61 ± 1.51 a	59.75 ± 0.27 j
20	20	130	60	10	90.54 ± 0.18	54.67 ± 0.17	3.46 ± 0.20	21.04 ± 0.31	88.63 ± 0.61 de	13.02 ± 1.23 c	87.33 ± 0.21 d
21	21	130	60	30	84.87 ± 0.12	53.77 ± 0.19	3.50 ± 0.18	20.31 ± 0.21	89.22 ± 0.48 cd	21.30 ± 0.89 a	80.50 ± 0.19 e
22	22	130	60	40	80.65 ± 0.16	51.60 ± 0.27	3.42 ± 0.07	21.24 ± 0.16	90.00 ± 0.21 b	21.76 ± 0.49 a	73.42 ± 0.25 g

[1] Solid recovery was calculated on the basis of Sample 3; [2] values followed by different letters indicate significant difference at $p \leq 0.05$.

Table 2. Changes in chemical components of rice straw samples before and after the LHW pretreatment.

Run	Sample	Pretreatment Condition			Solid Recovery [1] (%)	Chemical Composition (%)			Composition Changes (%)		
		Temp. (°C)	Time (min)	Solid/Liquid Ratio (g/mL)		Cellulose	Hemicellulose	Lignin	Hemicellulose Removal	Lignin Removal	Cellulose Retention
—	Control	—	—	—	—	44.47 ± 0.03	21.64 ± 0.31	17.18 ± 0.20	—	—	—
1	1	160	90	1:20	83.12 ± 0.17	52.95 ± 0.36	9.74 ± 0.26	19.97 ± 0.21	62.57 ± 0.97 d [2]	3.37 ± 1.04 d	98.96 ± 0.83 a
2	2	170	90	1:20	81.78 ± 0.44	53.70 ± 0.21	7.67 ± 0.20	20.29 ± 0.14	71.01 ± 0.79 b	3.43 ± 1.10 d	98.75 ± 0.77 a
3	3	180	90	1:20	78.46 ± 0.16	54.15 ± 0.30	7.25 ± 0.14	20.99 ± 0.18	73.71 ± 0.47 a	4.15 ± 0.94 d	95.54 ± 0.35 c
4	4	190	90	1:20	76.12 ± 0.27	54.83 ± 0.30	7.57 ± 0.33	20.91 ± 0.52	73.36 ± 1.10 a	7.36 ± 2.01 abc	93.85 ± 0.59 d
5	5	200	90	1:20	72.60 ± 0.34	52.51 ± 0.13	7.85 ± 0.30	21.65 ± 0.33	73.65 ± 1.02 a	8.51 ± 1.16 ab	85.72 ± 0.17 g
6	6	180	30	1:20	87.68 ± 0.25	50.09 ± 0.18	13.84 ± 0.26	18.77 ± 0.47	43.90 ± 1.07 e	4.22 ± 2.47 d	98.76 ± 0.27 a
7	7	180	60	1:20	82.65 ± 0.31	53.19 ± 0.46	8.37 ± 0.20	19.93 ± 0.41	68.04 ± 0.59 c	4.11 ± 1.93 d	98.86 ± 0.47 a
8	8	180	120	1:20	73.21 ± 0.47	53.18 ± 0.23	7.49 ± 0.45	22.08 ± 0.18	74.65 ± 1.51 a	5.92 ± 0.92 bcd	87.56 ± 0.75 f
9	9	180	180	1:20	72.26 ± 0.20	51.85 ± 0.08	7.85 ± 0.30	22.57 ± 0.37	73.79 ± 0.82 a	5.09 ± 1.22 cd	84.25 ± 0.19 h
10	10	180	90	1:10	81.17 ± 0.16	53.00 ± 0.33	9.77 ± 0.26	20.13 ± 0.26	63.36 ± 0.82 d	4.90 ± 0.99 cd	96.75 ± 0.35 b
11	11	180	90	1:30	75.21 ± 0.26	55.09 ± 0.23	7.48 ± 0.24	20.90 ± 0.40	74.01 ± 0.69 a	8.41 ± 1.74 ab	93.17 ± 0.53 d
12	12	180	90	1:40	74.34 ± 0.20	54.58 ± 0.14	7.67 ± 0.42	21.08 ± 0.30	73.88 ± 1.67 a	8.81 ± 1.14 a	91.24 ± 0.40 e

[1] Solid recovery was calculated on the basis of control; [2] value followed by different letters indicate a significantly difference at $p \leq 0.05$.

The enzymatic hydrolysis efficiency of cellulose, enzymatic hydrolysis efficiency of straw samples, and glucose recovery of straw samples before and after pretreatment are shown in Figure 2 (data in Supplementary Table S1). Results show that the enzymatic hydrolysis efficiency of cellulose and the hydrolysis efficiency of rice straw were significantly improved after LHW and LHW-PTA pretreatment. Moreover, the effect of two-step LHW-PTA pretreatment was better than that of only LHW pretreatment. In all samples, the enzymatic hydrolysis efficiency of cellulose of Sample 14 (74.98 ± 0.53%), Sample 15 (74.72 ± 0.70%), Sample 16 (74.06 ± 0.78%), Sample 18 (74.4 ± 0.6%), Sample 19 (73.62 ± 0.45%), Sample 21 (73.93 ± 0.27%) and Sample 22 (73.83 ± 0.72%) was significantly higher because the lignin removal of these samples (about 21.30–21.76%) was higher than that of the other samples (lower than 13.02%). The highest glucose recovery of 65.64% was obtained in Sample 14 because its cellulose retention was the highest compared to other PTA-treated samples with good lignin removal. As a result, compared with the control, the enzymatic hydrolysis efficiency of cellulose and glucose recovery of Sample 14 were increased by 201.85% and 164.25%, respectively; in Sample 3, they increased by 49.48% and 36.98%, respectively.

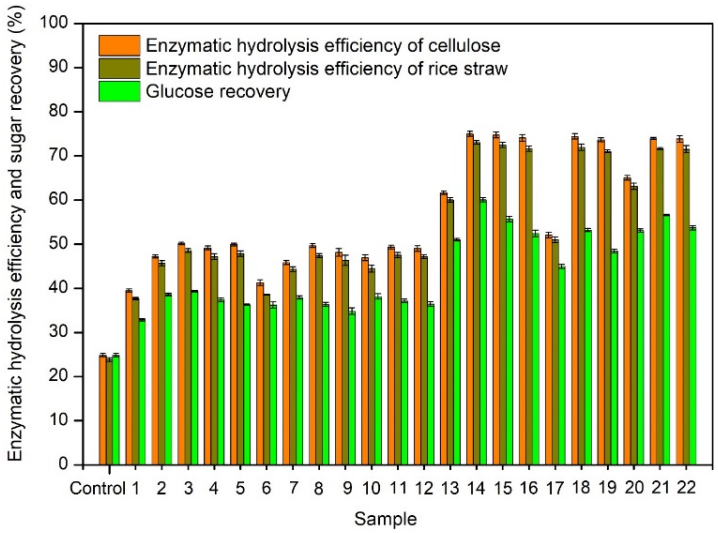

Figure 2. Enzymatic hydrolysis efficiency of cellulose, enzymatic hydrolysis efficiency of straw samples, and glucose recovery of straw samples before and after LHW and LHW-PTA pretreatment.

The pentose sugars released by the hydrolysis of hemicellulose cannot be used in traditional *Saccharomyces cerevisiae* fermentation for ethanol production [4]. Although new industrial strains can be used to convert pentose sugars to ethanol, these strains are relatively expensive to develop and use [34,35]. In this study, the glucose in the final enzymatic hydrolysate accounted for 96.8% of the total reducing sugar. Due to the low contents of xylose and arabinose in the substrate, the conversion of pentoses to ethanol is not necessarily a consideration during fermentation, which means that a more cost-effective strain can be selected for fermentative sugar production. Moreover, approximately 89.24% of hemicellulose in the rice straw substrate was removed by the LHW-PTA pretreatment, indicating that the content of available pentose in the lignocellulosic substrate was very low. Therefore, in the enzymatic hydrolysis process, it could replace the cellulose composite enzyme with a lower-cost cellulase. In addition, approximately 73.71% of hemicellulose was removed from the rice straw by the first-step LHW pretreatment. If necessary, the degradation products of hemicellulose can be further separated and utilized. Therefore, the two-step LHW-PTA pretreatment can provide more possibilities for the utilization of cellulose and hemicellulose.

3.3. XRD Analysis

Figure 3 shows the XRD results of different samples. The calculated *CrI* values of the control, and Samples 3 and 14 were 16.41, 21.86, and 30.13, respectively. Compared with the control, the *CrI* values of Samples 3 and 14 increased by 33.21% and 83.61%, respectively. The *CrI* value represents the proportion of crystalline regions in the sum of crystalline and amorphous regions. Theoretically, the reduction of cellulose crystalline area was beneficial to the enzymatic hydrolysis of cellulose [29]. However, most studies on the pretreatment of biomass with LHW showed that the *CrI* (measured by XRD) of treated biomass becomes larger as well as this study [36,37]. Li et al. [38] suggested that the increase in *CrI* was mainly due to the removal of hemicellulose rather than the change in the cellulose crystal structure. Dimitrelos et al. [39] also used ATR-IR analysis to prove that the crystalline area of cellulose was reduced after treatment with LHW. In addition, there is no consensus on the effect of PTA treatment on the *CrI* value of biomass. Chen et al. [32] found that the *CrI* of PTA-treated corn stover was 16.7% lower than that before pretreatment. In contrast, Xie et al. [33] found that the *CrI* of corn straw treated with PTA increased by 48.6–64.9%. On the basis of the above analysis, the elevated *CrI* values of Samples 3 and 14 are attributed to the removal of hemicellulose, while the increased *CrI* value did not adversely affect the enzymatic hydrolysis efficiency of the rice straw samples.

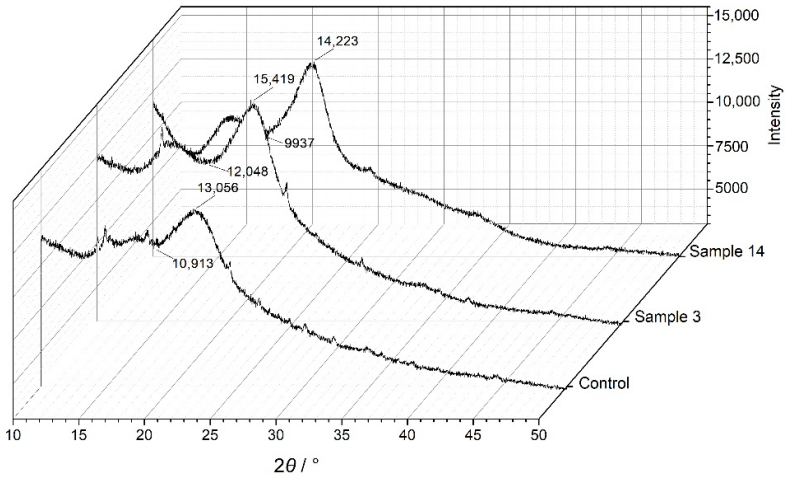

Figure 3. XRD patterns of rice straw samples before and after LHW and LHW-PTA pretreatment.

3.4. FT-IR Analysis

Figure 4 shows the FT-IR spectra of different samples. In all spectra, absorption at 1114 and 897 cm^{-1} was attributed to the C–OH skeletal vibration [26] and β-1,4 glycosidic bond of cellulose [32,40]. Compared with spectrum a (control), the intensity of the above peaks was significantly enhanced in spectra b (Sample 3) and c (Sample 14), which was related to the increased cellulose content in the treated samples. Peaks at 1056, 1164, 1318, and 1374 cm^{-1} were assigned to the C–O–C pyranose ring skeletal vibration, asymmetric C–O–C stretching, CH_2 wagging, and C–H asymmetric deformation of cellulose, respectively [26]. The intensity of these peaks did not change significantly. Absorption at 1730 and 1247 cm^{-1} was attributed to the C=O stretching and C–O stretching of the acetyl group in hemicellulose [13]. Compared with spectrum a, the intensity of the above peaks was significantly weaker in spectra b and c because the hemicellulose content of the pretreated samples was significantly reduced compared to the control. Moreover, the $–OCH_3$ of lignin was observed at 1425 and 1458 cm^{-1} [13,32] in all spectra. After pretreatment, the intensity of the above peaks for Samples 3 and 14 did not change significantly. However, in spectra b and c, the intensity of the peak at 1515 and 1605 cm^{-1} (aromatic skeletal stretching of lignin) [41] was stronger than that of spectra a because 4.15% and 21.33% of lignin could be removed from the control with the LHW and LHW-PTA pretreatments, respectively, but

the relative content of lignin in Samples 3 and 14 was increased. The result is consistent with previous chemical analyses.

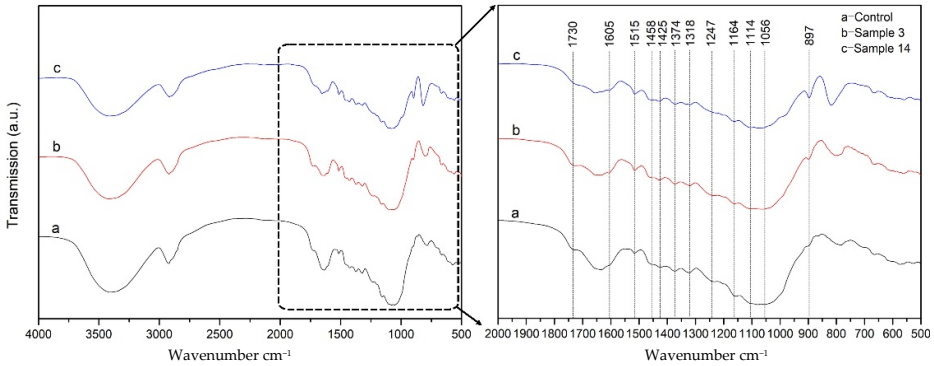

Figure 4. FT-IR spectra of rice straw samples before and after LHW and LHW-PTA pretreatment.

3.5. Reuse Performance of PTA Catalyst

Sample 3 was used as the raw material, and the experiment was performed according to the treatment method of run 14. A fresh PTA catalyst was used for the first test. The PTA recovered from the previous experiment was used to catalyze the next reaction in sequence, and the evaluation experiments were performed five times in sequence. To collect enough recovered PTA catalysts for the next reaction, multiple reactions can be performed under the same experimental conditions. Results are shown in Table 3. Compared with the sample obtained from the first reaction, the solid recovery, glucose yield, and enzymatic hydrolysis efficiency of samples obtained from the five consecutive experiments did not change significantly, indicating that the catalytic activity of the PTA catalyst recovered for the fifth time was not reduced.

Table 3. Recovery rate and reuse effect of PTA catalyst.

Run	Solid Recovery (%)	Catalyst Recovery (%)	Glucose Yield (mg/g)	Enzymatic Hydrolysis Efficiency of Cellulose (%)
First test	88.79 ± 0.16 a [1]	73.24 ± 2.30 a	465.55 ± 5.15 a	74.98 ± 0.53 a
Sequence 1	89.13 ± 0.55 a	70.81 ± 4.33 a	469.09 ± 8.24 a	75.55 ± 1.33 a
Sequence 2	88.44 ± 0.45 a	71.02 ± 2.32 a	461.59 ± 3.61 a	74.34 ± 0.58 a
Sequence 3	88.92 ± 0.42 a	72.98 ± 3.07 a	462.06 ± 6.12 a	74.42 ± 0.99 a
Sequence 4	88.84 ± 1.20 a	71.62 ± 3.50 a	466.58 ± 5.55 a	74.38 ± 1.04 a
Sequence 5	88.41 ± 0.54 a	73.06 ± 2.08 a	464.80 ± 6.48 a	74.86 ± 1.04 a

[1] Values followed by different letters indicate a significantly difference at $p \leq 0.05$.

Tsegaye et al. [42] found that 515 mg/g reducing sugar yield and 62.09% reducing sugar recovery can be obtained from the rice straw by the combined treatment of acetic acid/formic acid and microbial hydrolysis. An et al. [43] demonstrated that 63.2% of glucose recovery was obtained from rice straw by a combination of chemical and microbial pretreatment. In this study, glucose yield was 465.55 mg/g, the enzymatic hydrolysis efficiency of cellulose was 74.98%, and glucose recovery was 65.64%. This good effect was achieved on the basis of 70.8–73.2% of the PTA catalyst being recycled and reused. Sorn et al. [44] found that an ionic liquid catalyst used in the pretreatment of rice straw can be recovered almost in full, but the catalytic activity of the catalyst was reduced. When the catalyst was reused for the third time, enzymatic hydrolysis efficiency decreased from 79.34% in the first use to 55.23%. Xie et al. [33] demonstrated that the PTA/deep eutectic solvent (DES) catalytic system could be recycled in corn stover pretreatment, but glucose yield decreased with the number of cycles. Therefore, although a small fraction of PTA

was not recovered in this study, the two-step LHW-PTA pretreatment was conducive to reducing production costs and improving environmental benefits.

3.6. Mass Balance

The mass balance of two-step LHW-PTA pretreatment based on 100 g of rice straw is shown in Figure 5. In total, 100 g of air-dried rice straw contains 37.8 g of cellulose, 18.39 g of hemicellulose, 14.6 g of lignin, 11.3 g of ethanol extracts, 14.2 g of ash, and 3.7 g of moisture. It was successively used for LHW and PTA pretreatments under optimal conditions. The unpretreated and pretreated rice straw samples were saccharified using cellulase at 20 FPU/g of the substrate. After LHW pretreatment, 66.69 g of solid residue was recovered, which contained 36.11 g of cellulose, 4.84 g of hemicellulose, and 14.0 g of lignin; 20.1 g of glucose can be recovered from the enzymatic hydrolysate of LHW pretreated solid residue. Compared with the control that recovered 10.43 g of glucose, there was an increase of 92.71%. After the PTA pretreatment, 59.21 g of solid residue was recovered, which contained 33.09 g of cellulose, 2.04 g of hemicellulose, and 11.49 g of lignin. Then, 27.57 g of glucose could be recovered after enzymatic hydrolysis, and 0.92 g of pentose could be obtained by the LHW-PTA-pretreated sample after enzymatic hydrolysis, which was lower than that of the unpretreated sample. Because the pretreatment removed most of the hemicellulose in the rice straw. The degradation products from 13.55 g hemicellulose were present in the LHW treatment solution, which can be further processed and utilized. Results show that the two-step LHW-PTA pretreatment is a method that could significantly improve the recovery of glucose from rice straw, and the glucose in the enzymatic hydrolysate accounted for 96.8% of the total reducing sugar.

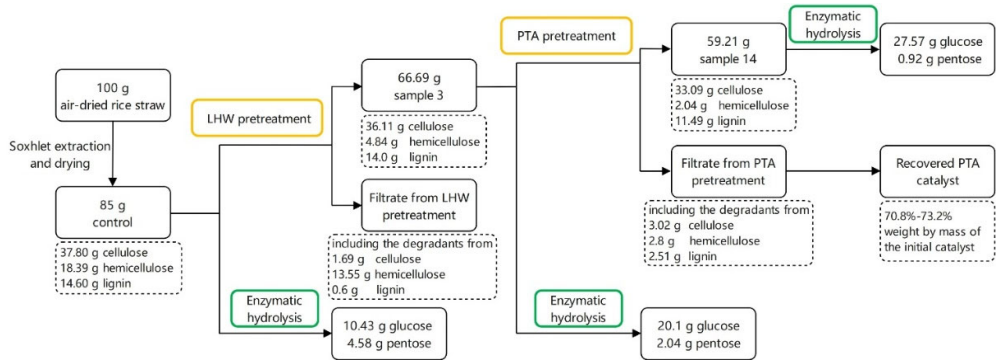

Figure 5. Mass balance diagram of rice straw before and after LHW-PTA pretreatment.

4. Conclusions

The two-step LHW-PTA pretreatment is an environmentally friendly biomass-processing strategy that can improve the saccharification efficiency of rice straw, providing more possibilities for the value-added utilization of cellulose and hemicellulose. The main effect of the first LHW pretreatment was reflected in the removal of 73.71% of hemicellulose; 21.33% of lignin could be removed by PTA pretreatment, thereby further improving the saccharification efficiency of rice straw. After enzymatic hydrolysis, glucose recovery of 65.64% was obtained, which was 36.98% and 164.25% higher than that of only LHW pretreatment and no pretreatment, respectively. About 70.8–73.2% of the PTA catalyst could be recycled, and the catalytic activity of the PTA catalyst that was used five times did not decrease. Future work could further analyze of loss mechanism of PTA catalysts to improve the recovery rate of catalysts, thereby further improving the economics of the combined pretreatment.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/en15103636/s1. Table S1: The sugar yield, enzymatic hydrolysis efficiency and sugar recovery of rice straw samples before and after the LHW and LHW-PTA pretreatment.

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