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Two-Stage Pretreatment of Jerusalem Artichoke Stalks with Wastewater Recycling and Lignin Recovery for the Biorefinery of Lignocellulosic Biomass

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Abstract: Jerusalem artichoke (*Helianthus tuberosus* L.) is emerging as one of the energy plants considered for biofuel production. Alkali and alkali-involved pretreatment methods have been widely used for the bioconversion of cellulosic materials due to their high sugar yield and low inhibitor release. However, the recovery and treatment of wastewater (black liquor) have been poorly studied. Here, we present a novel two-stage pretreatment process design for recycling black liquor. Jerusalem artichoke stalk (JAS) was first treated with 2% (*w*/*v*) NaOH, after which lignin was recovered by H_2SO_4 at pH 2.0 from the black liquor. The recycled solutions were subsequently used to treat the NaOH-pretreated JAS for the second time to dissolve hemicellulose. CO-pretreated JAS, hydrolysates, and acid-insoluble lignin were obtained after the above-mentioned two-stage pretreatment. A reducing sugar yield of 809.98 mg/g Co-pretreated JAS was achieved after 48 h at 5% substrate concentration using a cellulase dosage of 25 FPU/g substrate. In addition, hydrolysates containing xylose and acid-insoluble lignin were obtained as byproducts. The pretreatment strategy described here using alkali and acid combined with wastewater recycling provides an alternative approach for cellulosic biorefinery.

Keywords: Jerusalem artichoke stalk (JAS); alkali pretreatment; diluted acid pretreatment; lignin recovery; wastewater recycling; biorefinery

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1. Introduction

Lignocellulosic biomass has been widely recognized as a sustainable source for the production of biofuels and biochemicals to replace fossil oil. The pretreatment of lignocellulosic biomass promotes enzymatic hydrolysis and improves the yield of glucose from cellulose by removing hemicellulose or lignin [1,2]. However, the major bottleneck for lignocellulosic biorefinery is its high cost, and the pretreatment cost is an important factor to evaluate the feasibility of economic production from lignocellulosic feedstocks. To date, various pretreatment methods have been developed using alkali, dilute acid, liquid hot water, steam explosion, ionic liquids, and so on [3]. Among these pretreatment methods, alkali pretreatment is the most effective for removing lignin and improving cellulose contents [4–6]. For example, pretreatment with sodium hydroxide/urea can remove 10.9 g of lignin from 100 g of rice straw, and 91.54% of the cellulose and hemicellulose is well preserved [7]. However, the high cost and difficulty in alkali recycling have hindered its industrialization, not to mention the concern for environmental pollution by wastewater (black liquor) discharge. Therefore, the reuse of black liquor from thermo-alkaline pretreatment is expected to increase economic efficiency by reducing the cost of pretreatment and the amount of wastewater [8]. For example, the results indicated that instead of disposing

of alkali black liquor, it can be reused for at least five consecutive cycles for the pretreatment of rice straw [9,10]. Meanwhile, the extraction of lignin from black liquor using acid precipitation is another method [11]. Lignins produced from black liquor have been extracted through the fractionation of sugarcane bagasse with soda, while these lignins have different physico-chemical and thermal properties relative to lignin extracted with organic solvents [12]. If the acidic water remaining after lignin extraction pretreats the biomass again, it is expected to further improve the saccharification efficiency of the biomass.

The main objective of acid pretreatments is to solubilize the hemicellulosic fraction of the biomass and to make the cellulose more accessible to enzymes [13], and biodetoxification fungi have demonstrated their excellent capacity to degrade lignocellulose-derived inhibitors [14]. At the same time, many studies have focused on combined acid/alkali pretreatment processes. A semicontinuous sequential process of acid/alkali pretreatment has been successfully applied for the pretreatment of empty palm fruit bunch fibers, and 82% cellulose, less than 1% hemicellulose, and 30% lignin were obtained [15]. The combined pretreatment with HNO_3 and NaOH of Jerusalem artichoke stalks resulted in a glucose conversion of approximately 90.6% of the theoretical maximum value [16]. However, the generated wastewater is often neglected in pretreatment designs, and only limited studies have focused on the reuse of the pretreatment solution [10,17,18]. In addition to environmental concerns, wastewater also contains unused resources, including lignin and fermentable sugars, and the utilization of these byproducts is attractive for biorefinery.

Jerusalem artichoke (*Helianthus tuberosus* L.) can grow in poor soil and is resistant to pests and common plant diseases. Compared with most other crops, it grows much better under salt stress and low temperatures [19]. Jerusalem artichoke (JA) has become one of the emerging energy plants considered for biofuel production [20]. Jerusalem artichoke tuber (JAT) is an excellent raw material for the production of biofuels and biochemicals and has been explored for ethanol fermentation by microbial mixed culture [21] as well as a variety of microbial strains, such as *Kluyveromyces marxianus* [22], *Klebsiella pneumoniae* [23], *Bacillus licheniformis* [24], and *Saccharomyces cerevisiae* [25–27]. Meanwhile, an efficient extraction method was used for inulin extraction from Jerusalem artichoke tubers [28]. However, studies of the utilization of Jerusalem artichoke stalk (JAS) as a lignocellulosic feedstock for the production of biofuels and biochemicals are still very limited.

For the efficient bioconversion of JAS, it is essential to reduce the pretreatment cost. In a previous study, JAS was utilized for bioethanol production using a recombinant *S. cerevisiae* strain, and H_2SO_4 or NaOH pretreatment was optimized. The pretreatment of JAS using 2% NaOH was superior to other methods and achieved the maximum ethanol concentration [29]. However, wastewater treatment was not considered. We assumed that the reuse of alkali and acid for pretreatment as well as the recovery of lignin are important issues for the biorefinery of JAS to reduce the cost of pretreatment.

In this study, an alternative strategy to the pretreatment process that includes wastewater recycling was presented using JAS as feedstock. We show here that the two-stage pretreatment strategy improved the enzymatic hydrolysis yield. In the meantime, wastewater recycling achieved more products and simultaneously reduced environmental pollution.

2. Materials and Methods

2.1. Materials

The JAS was reaped from the campus of the Dalian University of Technology (Dalian, China). JAS was chopped and dried at 60 °C for 72 h and then subjected to size reduction using a mill fitted with a 60-mesh (0.3 mm) sieve. The milled material was stored at room temperature in plastic bags used within seven days. All chemicals used were reagent grade or analytical grade and purchased from Sangon Corporation (Shanghai, China). *Trichoderma reesei* Rut C30 was selected for cellulase production in this study, kindly donated by the USDA ARS Culture Collection.

2.2. Pretreatment Process Design

The methods for the alkali pretreatment and recovery of acid-insoluble lignin were previously described by Mcintosh et al. [30]. The scheme of the designed pretreatment process is depicted in Figure 1. JAS was pretreated at 121 °C (15 psi) with 2.0% (w/v) NaOH for 30 min. The JAS and alkali solution was loaded in a 150 mL shake flask with a solid loading of 5% (w/v). NaOH-pretreated JAS and liquid fractions were separated using a Buchner funnel fitted with filter paper (No. 1, Whatman). The liquid was adjusted to pH 2.0 by concentrated H_2SO_4 (98.3%, w/v) at 60 °C and then kept at 70 °C for 1 h. Acid-insoluble lignin and recovery solution were obtained by filtering. Other insoluble substances were removed by centrifuging at 13,000 r/min for 20 min, followed by the addition of 3% (w/v) activated carbon and incubation at 80 °C for 50 min before separating the solid phase. After lignin recovery, concentrated H_2SO_4 was added to the recovered liquid, and the final H_2SO_4 concentration was adjusted to 1.5% (v/v), which was used to pretreat the NaOH-pretreated JAS using the method described previously [31].

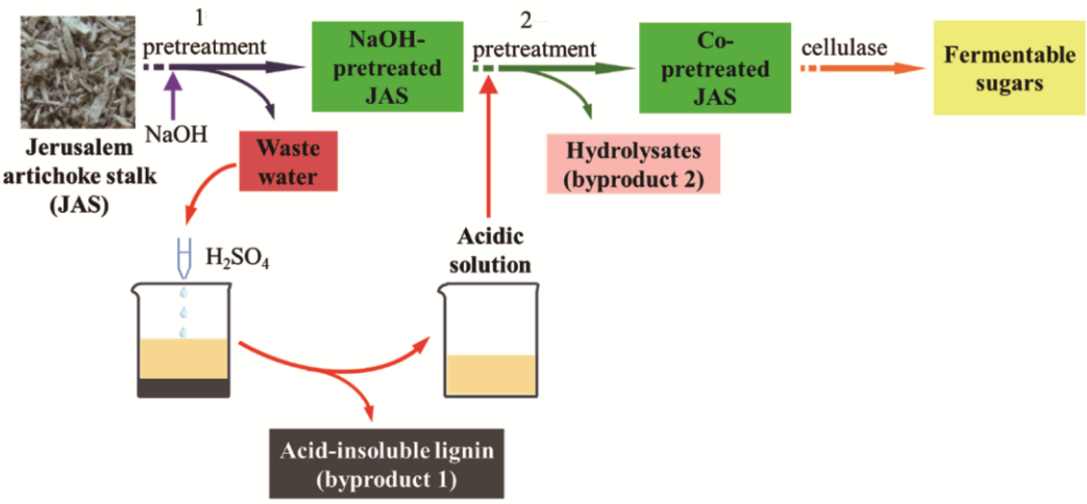

Figure 1. Schematic presentation of the designed pretreatment process.

After the second-stage pretreatment using wastewater, alkali–acid CO-pretreated JAS was obtained by filtration with filter paper (No. 1, Whatman). All separated solids were washed with tap water to a neutral pH and dried at 60 °C to constant weight.

2.3. Enzymatic Assays

Accellerase® 1500 was supplied by Genencor (Rochester, NY, USA) and stored at 4 °C. In-house cellulase was produced using cellulose and wheat bran as inducers by *Trichoderma reesei* Rut-C30 preserved in our laboratory.

The total cellulase activity was determined using filter paper (No. 1, Whatman) as recommended by NREL procedure LAP006 [32]. Xylanase and β-glucosidase activities were individually determined in 1.0 mL reaction mixtures containing 1% (w/v) oat spelt xylan or 0.5% (w/v) cellobiose dissolved in 0.2 M acetic acid/sodium acetate buffer (pH 4.8), respectively. Appropriately diluted enzyme solutions were added and incubated at 50 °C for 30 min. The released reducing sugar was measured by the dinitrosalicylic acid (DNS) method[26]. One unit (U) of each enzyme activity is defined as the amount of enzyme that produces 1 μmol of reducing sugar as glucose (xylose for xylanase) in the reaction mixture per minute. The protein content of liquid enzyme preparations was determined using a commercial Bradford protein assay reagent kit purchased from Sangon Corporation (Shanghai, China). The enzyme activities used in this study, including cellulase, xylanase, and glucosidase, are presented in Table 1.

Table 1. Enzyme activities used for Jerusalem artichoke stalk saccharification.

Enzymes	Specific Activity (U/mg Protein)	
	Accellerase® 1500	In-House Cellulase
Protein (mg mL^{-1})	27.16 ± 2.63	3.71 ± 0.58
Cellulase	4.40 ± 0.09	2.85 ± 0.21
β-Glucosidase	15.31 ± 1.33	1.00 ± 0.05
Xylanase	21.10 ± 1.25	204.80 ± 6.39

2.4. Enzymatic Saccharification

One gram of substrate was mixed with enzyme solution and then resuspended in 0.2 M acetic acid/sodium acetate buffer (pH 4.8) at a solid loading of 5% substrate concentration (*w*/*v*) containing 50 mg/L kanamycin to prevent microbial contamination. Reaction solutions were incubated at 50 °C in a water bath with shaking at 100 r/min. The samples were then immediately centrifuged at 8000 r/min for 5 min and stored at −20 °C.

2.5. Analysis Methods

Compositional analysis of raw and pretreated JAS was performed as described by the NREL analytical procedures.

Total reducing sugar was analyzed by the dinitrosalicylic acid (DNS) method recommended by NREL [33]. The concentrations of glucose, xylose, acetic acid, furfural, and HMF were determined using HPLC (Waters 410, Waters, Milford, MA, USA) equipped with an Aminex HPX-87H column (300 mm × 7.8 mm, Bio-Rad, Richmond, CA; Hercules, Ford City, PA, USA). The column oven temperature was set at 50 °C. The samples were eluted with 0.01 M H_2SO_4 at a flow rate of 0.5 mL/min.

The hydrolysis yield was calculated as follows [31]:

$$\text{Hydrolysis yield } (\%) = \frac{\text{Reducing sugar } (g) \times 0.9 \times 100}{\text{Polysaccharide in substrate } (g)} \quad (1)$$

All the presented results are the average of three parallel tests. An estimated experimental error was used to calculate the "Least significant difference" (p = 0.05). All data were analyzed using SPSS Statistics 20.0 (AsiaAnalytics, Shanghai, China).

2.6. Raw and Pretreated Material Characterization

2.6.1. Scanning Electron Microscopy (SEM) Analysis

The morphology of raw, NaOH-pretreated, and CO-pretreated JAS was analyzed with SEM (Quanta 450, FEI, Hillsboro, OR, USA). The solid samples were installed on aluminum sample stubs with double-sided carbon tape. SEM images of raw and pretreated JAS were acquired with a 20 kV acceleration voltage.

2.6.2. Fourier Transform Infrared Spectroscopy (FTIR) Analysis

FTIR was conducted using a Bruker EQUINOX55 (Bruker company, Karlsruhe, Germany). Samples were mixed with potassium bromide (KBr), and the mixture was pressed into a disc after drying. The sample spectra were scanned over the range of 400~4000 cm^{-1} with a spectral resolution of 2 cm^{-1}.

2.6.3. Analysis of Cellulose Crystallinity

Raw and pretreated JAS was used for X-ray diffraction (XRD) (D/MAX-2400, RIGAKU Corp., Tokyo, Japan) to obtain X-ray diffraction patterns. The samples were scanned in the range of 10~35° (2θ), with a scan step of 0.02° and a step time of 1 s. Scans were collected

at 40 kV and 100 mA under ambient temperature. The crystallinity index (CrI) of JAS was determined from the XRD data and calculated by the following equation:

$$CrI = \frac{I_{002} - I_{am}}{I_{002}} \times 100 \quad (2)$$

where I002 is the intensity of the crystalline portion of JAS at approximately 2θ = 22° and Iam is the intensity of the amorphous portion of JAS at approximately 2θ = 18° [34].

3. Results and Discussion

3.1. Chemical Composition of Raw and Pretreated Jerusalem Artichoke Stalk

The cellulose and hemicellulose contents in the JAS sample used in this study were determined to be 35.58% and 21.73%, respectively, which are similar to those of wheat straw and corn stover. However, the lignin content (18.39%) in JAS was higher than that of wheat straw and corn stover [35]. The absorption and desorption of cellobiohydrolase I (CBH I) were affected by their lignin contents; therefore, JAS is considered more difficult to digest by cellulase than corn stover [36]. The percentages of cellulose, hemicellulose, and lignin in the JAS used in this study are comparable to those reported recently [29], albeit with a higher content of hemicellulose (21.73% vs. 10.20%) and slightly lower lignin content (18.39% vs. 22.20%), possibly due to the different cultivars of Jerusalem artichoke.

To achieve a high glucose concentration in the subsequent enzymatic hydrolysis step, the cellulose content of pretreated materials needs to be improved. The combined acid and alkali pretreatment can result in a higher concentration of cellulose than single pretreatment; however, the higher energy cost is the main barrier to the combined pretreatment. If more byproducts were recovered, such as acid-insoluble lignin, and the acidic wastewater could be reused for the acid pretreatment of biomass, it would be able to reduce the cost for industrial applications using a two-stage pretreatment.

Raw JAS was subjected to two-stage pretreatment with NaOH followed by acidic wastewater treatment. The compositions of the cellulose, hemicellulose, and lignin of the non-treated JAS, NaOH-pretreated JAS, and sequentially alkali/acid-treated JAS are listed in Table 2. The recovery ratio of cellulose was very high in each step of the pretreatment, leading to an obvious increase in the cellulose concentration, but the removal ratio of hemicellulose and lignin was quite different for different pretreatment steps. In the first step of the NaOH pretreatment, 57.69% of the lignin was removed, leading to the increased content of cellulose and hemicellulose by 78.49% and 18.18%, respectively. The results indicated that NaOH could remove lignin effectively. NaOH-pretreated wastewater was used to pretreat NaOH-treated JAS after acidification and lignin separation. In this step, 31.33% hemicellulose was removed, leading to an evident augmentation in the contents of both cellulose and lignin. The cellulose content was further enhanced by 63.50% to 70.07% in the JAS after two-stage pretreatment compared to NaOH-pretreated JAS, which benefits glucose release. Dzieko'nska-Kubczak et al. pretreated Jerusalem artichoke straw with 2% HCL, 2% H_2SO_4, 2% HNO_3, 2% NaOH, and 2% KOH, and the cellulose concentration reached 56.3%, 53.09%, 60.31%, 59.3%, and 56.28%, respectively [37]. The other study reported by Li et al. achieved the highest cellulose content of Jerusalem artichoke straw at 51.18% under pretreatment with 2.8% NaOH and 3.2% H_2O_2 [38]. After two-stage pretreatment, the cellulose content was enhanced by 35.58% to 70.07% in the JAS in this study. This result is significantly better than pretreatment with acid or base alone.

Table 2. Chemical composition of raw and pretreated JAS.

Samples	Chemical Composition (%)		
	Cellulose	Hemicellulose	Lignin
Raw JAS	35.58 ± 0.11	21.73 ± 0.31	18.39 ± 0.13
NaOH-pretreated JAS	63.50 ± 0.32	25.68 ± 0.13	7.78 ± 0.05
CO-pretreated JAS	70.07 ± 0.59	17.63 ± 0.50	9.06 ± 0.01

3.2. Enzymatic Hydrolysis of Raw and Pretreated JAS

The main purpose of pretreatment technology is to increase enzyme accessibility, improving the digestibility of cellulose and therefore releasing more fermentable sugars from biomass. The concentrations of total reducing sugar, glucose, and xylose released in the enzymatic hydrolysis process of different pretreated JAS samples are presented in this study. The sugar content was determined after enzymatic hydrolysis at different reaction times under different enzyme loading levels (15, 25, and 35 FPU per gram of raw and pretreated JAS) when loading a 5% substrate concentration (w/v) in citric acid buffer (pH 4.8) at 50 °C. The hydrolysis yields of raw, NaOH-pretreated, and CO-pretreated JAS are shown in Figure 2, and the concentrations of reducing sugar, glucose, and xylose after enzymatic hydrolysis for 48 h are shown in Figure 3.

The raw, NaOH-pretreated, and CO-pretreated JAS were hydrolyzed using Accellerase® 1500 with an enzyme loading of 25 FPU/g biomass (Figure 2). The results showed that raw JAS was difficult to hydrolyze by cellulolytic enzymes compared with pretreated JAS, indicating that the pretreatment process is a vital step for the hydrolysis of cellulose and hemicellulose in JAS to obtain fermentable sugars. Because the two-stage pretreatment was applied in this pretreatment design, NaOH pretreatment time has a smaller impact on the enzymatic hydrolysis than the concentration of NaOH and temperature, which was shortened to 30 min compared with that reported by McIntosh and Vancov [24]. In addition, the highest hydrolysis yield of CO-pretreated JAS achieved 73.05% at 72 h, which increased by 6.26% relative to NaOH-pretreated JAS; however, the xylose released showed a small reduction as a portion of hemicellulose was removed during acidic wastewater pretreatment.

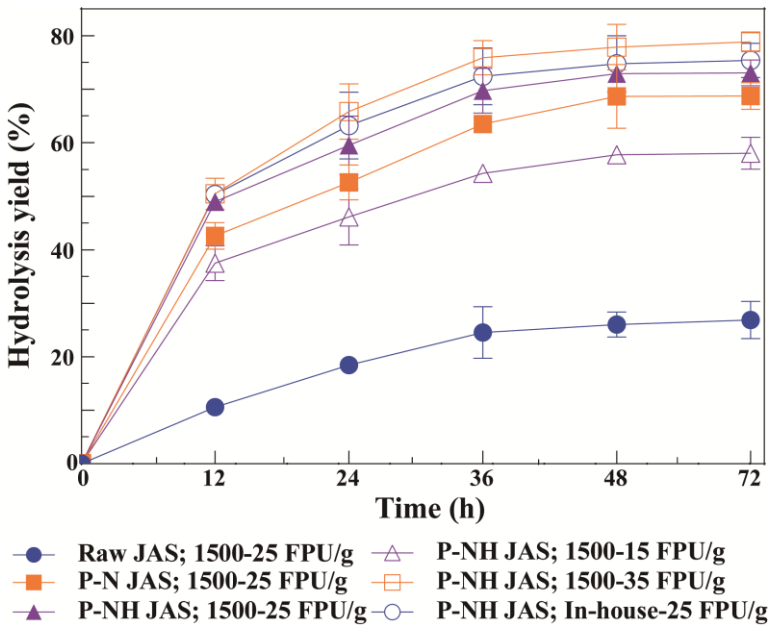

Figure 2. Enzymatic hydrolysis (5% substrate concentration, w/v; 50 °C; pH 4.8) of raw and pretreated Jerusalem artichoke stalk (JAS) using two enzyme combinations. P-N JAS: NaOH-pretreated JAS, P-NH JAS: CO-pretreated JAS; 1500: Accellerase® 1500, 1110: in-house cellulase.

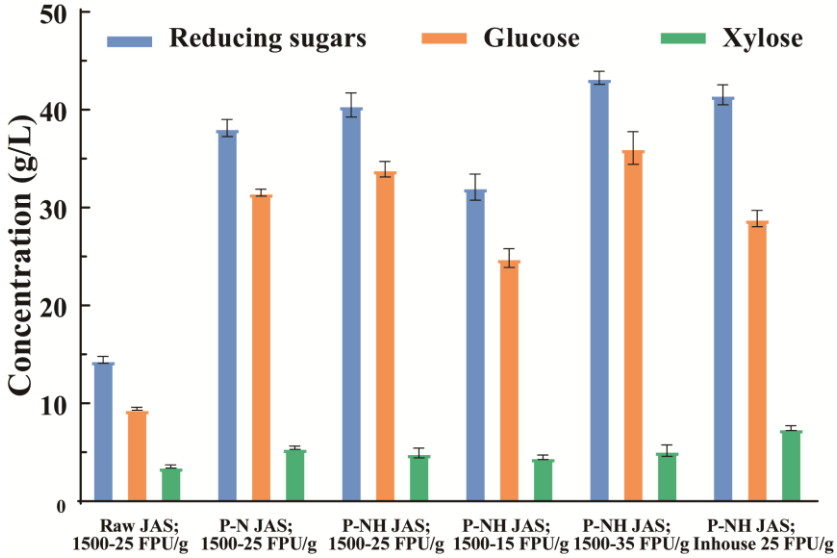

Figure 3. Sugar analysis of the enzymatic hydrolysates (5% substrate concentration, *w*/*v*; 50 °C; pH 4.8; 48 h) from raw and pretreated JAS using two enzyme combinations. P-N JAS: NaOH-pretreated JAS, P-NH JAS: CO-pretreated JAS; 1500: Accellerase® 1500, 1110: in-house cellulase.

To further investigate the hydrolysis characteristics of CO-pretreated JAS, the materials were hydrolyzed with different enzyme doses (15, 25, and 35 FPU/g) (Figure 2). At the different enzyme dosages investigated, the hydrolysis yield rapidly increased in the first 12 h, followed by a slow increase from 12 to 36 h, and remained stable after 36 h. A hydrolysis yield of 77.90% was obtained with an enzyme dosage of 35 FPU/g at 48 h at a biomass loading of 5% (*w*/*v*). Moreover, a reducing sugar concentration of 43.28 g/L was achieved, which equals 865.58 mg reducing sugar/g CO-pretreated JAS being released. The glucose and xylose conversion rates achieved approximately 84.34% of the theoretical maximum. A similar glucose and xylose conversion rate (88% of the theoretical maximum) was reported after the pretreatment of JAS by 0.5% H_2SO_4 and 1 M NaOH, but a much higher enzyme dosage (Cellic® CTec2 cellulase, 80 FPU/g biomass) was used [29]. Our current study using 35 FPU/g biomass saved the cost of enzymes, and the NaOH-pretreated JAS was treated again using wastewater; thus, the cost of pretreatment was reduced. Additionally, there was a similar hydrolysis rate with enzyme dosages of 25 and 35 FPU/g samples in the first 12 h. To further reduce the cost of cellulase, the enzyme dosage of 25 FPU/g samples was chosen in the following experiments. Moreover, the concentrations of reducing sugar, glucose, and xylose in hydrolysates with different enzyme doses were also determined at 48 h (Figure 3). The reducing sugar released with different enzyme dosages showed the same trend of hydrolysis yield, as well as glucose, while the xylose released had little increase using Accellerase® 1500, possibly because of the low xylanase activity.

Trichoderma reesei Rut-C30 is one of the most widely used cellulase-producing strains [39–41], so the in-house crude cellulase from it was also tested in this experiment. The hydrolysis yield achieved using in-house cellulase was 3.14% higher than that of Accellerase® 1500 with the same enzyme dosage. In addition, more xylose and less glucose were released using the in-house cellulase (Figure 3), corresponding to higher xylanase activities and lower glucosidase than Accellerase® 1500 (Table 1). Supplementation with a certain amount of β-glucosidase, as previously reported, will increase glucose yield and hydrolysis efficiency [42,43]. Simultaneously, alternative strategies have been developed, and the expression of cellulase genes in *S. cerevisiae* can also solve the problem of low glucosidase activity [44,45].

Acetic acid, furfural, 5-hydroxymenthylfurfural (HMF), and phenolic compounds are formed during the pretreatment and saccharification process, which can inhibit microbial growth and sugar fermentation. In the present study, it was shown that only low concen-

trations of inhibitors were released in the hydrolysis process using Accellerase® 1500 at 25 IU/g biomass (Table 3), and the concentration of inhibitors from NaOH pretreatment was lower than that from acid pretreatment. The concentrations of the inhibitors detected in this study are within a normal range [46], and no inhibition of ethanol fermentation was observed in the follow-up experiment (data not shown).

Table 3. Inhibitor composition of hydrolysate from the different pretreatment JASs.

Pretreated Liquid	Inhibitor Composition (g/L)		
	Acetic Acid	Furfural	5-HMF
Raw JAS	0.18 ± 0.09	ND	ND
NaOH-pretreated JAS	0.19 ± 0.01	ND	ND
CO-pretreated JAS	0.43 ± 0.10	0.06 ± 0.002	ND

3.3. Byproducts Obtained during the Two-Stage Pretreatment Process

Compared with other pretreatment methods, NaOH pretreatment is more powerful in increasing the efficiency of enzymatic hydrolysis and releasing fewer inhibitors, such as acetic acid, furfural, and HMF [31]. To reduce wastewater discharge, in this study, the wastewater was further treated with sulfuric acid. Through this process design, not only was acid-insoluble lignin recovered but the resulting acidity wastewater could also be reused to treat materials again.

The recovery of lignin was 36.78% by H_2SO_4 at pH 2.0, and this lignin was proven to be useful in various industrial domains after modification. For example, lignin has been recognized as a highly valuable renewable polymer that can be used to produce phenolic resins and epoxies. In addition, lignin can also act as a binding and dispersing agent, for example, cement additives [47,48]. In the recovery of lignin and the detoxification process, the concentration changes of saccharides and inhibitors are shown in Table 4. After centrifugation and active carbon treatment, much fewer inhibitors were detected in the wastewater of the pretreatment. We added H_2SO_4 into the wastewater to improve the content of cellulose and at the same time avoided using high temperatures to attain the same effect to save energy. The hydrolysates after the second pretreatment were analyzed, and the concentration of reducing sugars in the hydrolysates reached 9.85 g/L, among which xylose was the principal sugar (5.82 g/L). In addition, there was 1.07 g/L glucose in the hydrolysates. Moreover, the concentration of inhibitors was very low, even lower than NaOH-pretreated liquid (Table 4), but 0.06 g/L HMF was detected (such a small concentration has no severe inhibitory effect on the growth of yeast) [49,50]. An attractive method to use the hydrolysates is for yeast seed culture after the addition of nitrogen sources such as yeast extract and peptone. Within the pretreatment process, lignin and sugars could be recovered for the purpose of the further utilization of biomass. In addition, no pollutant was released after alkali pretreatment. It is very interesting and valuable to improve alkaline pretreatment as it will simultaneously reduce the cost of treatment of wastewater and obtain byproducts.

Table 4. Sugar and composition of pretreated liquid from the different pretreatment stages.

Sugar and Inhibitor Composition (g/L)	Pretreated Liquid		
	NaOH-Pretreated Liquid	Recycling Solution	Hydrolysates
Reducing sugar	7.93 ± 0.17	1.86 ± 0.02	9.85 ± 0.05
Glucose	3.37 ± 0.13	0.41 ± 0.04	1.07 ± 0.21
Xylose	3.29 ± 0.12	0.37 ± 0.05	5.82 ± 0.07
Acetic acid	1.75 ± 0.07	0.89 ± 0.04	1.42 ± 0.11
Furfural	0.25 ± 0.00	0.01 ± 0.00	0.16 ± 0.00
5-HMF	ND	ND	0.08 ± 0.00

3.4. Structure of the Regenerated JAS Analysis

Raw and pretreated JAS were analyzed using SEM under 1500-fold magnification. Figure 4(A1–A3) shows the SEM images. A rougher surface and smaller particle size compared with the non-treated control can be observed from the NaOH-pretreated JAS. After the second pretreatment, the CO-pretreated JAS had relatively more porous and thin strip structures than the NaOH-pretreated JAS. Such structures are beneficial for the release of sugars, and the conclusions were similar to those of previous studies [51].

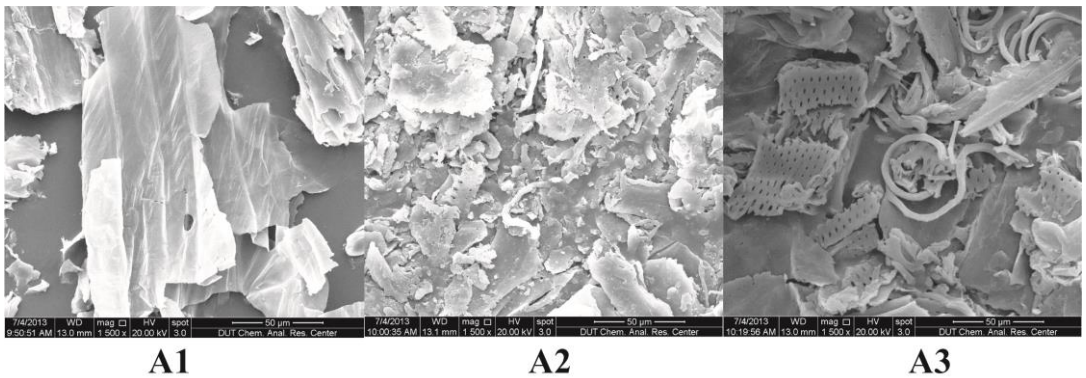

Figure 4. Characterization of the pretreated JAS. (**A**) SEM photo of JAS (1500×). **A1**, raw JAS; **A2**, NaOH-pretreated JAS; **A3**, CO-pretreated JAS.

According to previous research, the cellulose crystallinity index (CrI) increased after most chemical pretreatment methods, which is due to the degradation of lignin and hemicellulose during pretreatments, and evaluation of CrI is important for enzymatic hydrolysis [52]. In this study, CrI was calculated using XRD, and the results are shown in Figure 5. There was no significant difference in the crystallinity index (CrI) between NaOH-pretreated JAS (56.75%) and CO-pretreated JAS (57.17%), which indicated that hemicellulose was removed after wastewater pretreatment. The CrI of raw JAS (47.17%) was significantly lower than that of pretreated JAS. In summary, in the combined pretreatment, a significant portion of JAS, such as xylan and lignin, was removed, which is advantageous for cellulase to attack JAS to release more sugars.

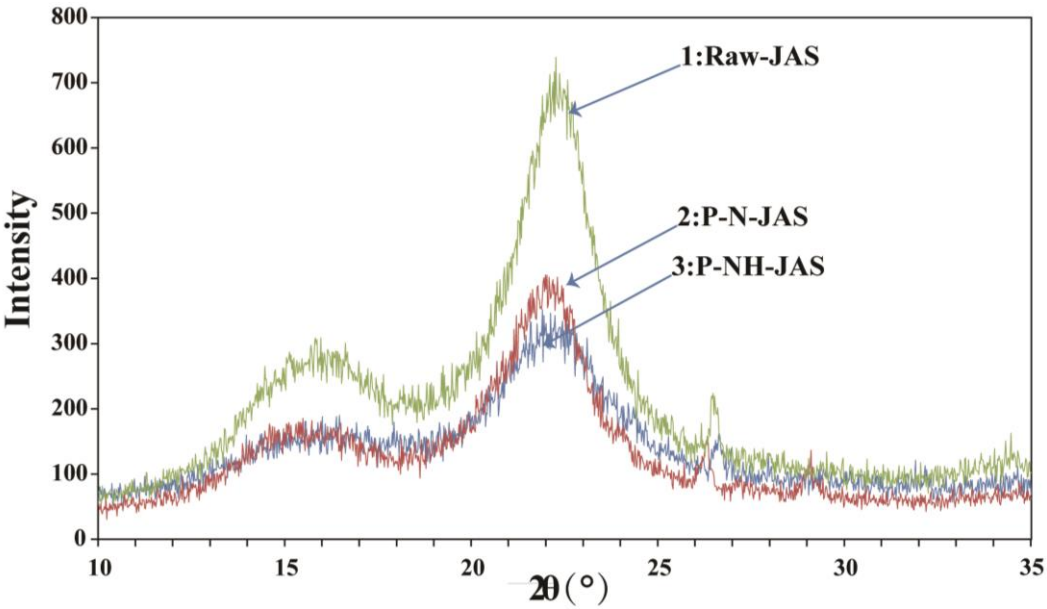

Figure 5. Comparison of X-ray diffraction patterns. P-N JAS: NaOH-pretreated JAS, P-NH JAS: CO-pretreated JAS.

The bands and spectra of the raw JAS and the pretreated JAS could be considered reflections of changes in cellulose, hemicellulose, and lignin, especially the variation in lignin, which could affect the hydrolysis yield [53]. The changes in the positions of the functional groups could be analyzed by FTIR (Figure 6). The bands at 3399 cm^{-1} and 2919 cm^{-1} correspond to the vibration of -OH and the functional group of C-H, respectively. Their existence indicated the existence of cellulose, hemicellulose, and lignin; in other words, there was no significant change in the structure before and after pretreatment (Figure 6A). The spectra at 1240 cm^{-1} represent the ester bond in the hemicellulose and lignin interaction, and the changes could be shown before and after NaOH treatment, which indicated that a certain amount of lignin was removed by the first step. Although hemicellulose was removed after pretreatment with acid, the spectra at 1240 cm^{-1} showed no significant change, which could result from the exposure of covalent bonds in the porous and multiple-rod structure caused by the pretreatment (Figure 6B). Moreover, the peak at 1104 cm^{-1}, which was exactly the peak of the covalent bond between cellulose and hemicellulose, experienced an evident increase after the first pretreatment (corresponding to the increase in surface and exposure of covalent bonds) and decreased after the second pretreatment (corresponding to the removal of hemicellulose). The variation in this peak corresponds to the contents of cellulose, hemicellulose, and lignin (Table 1).

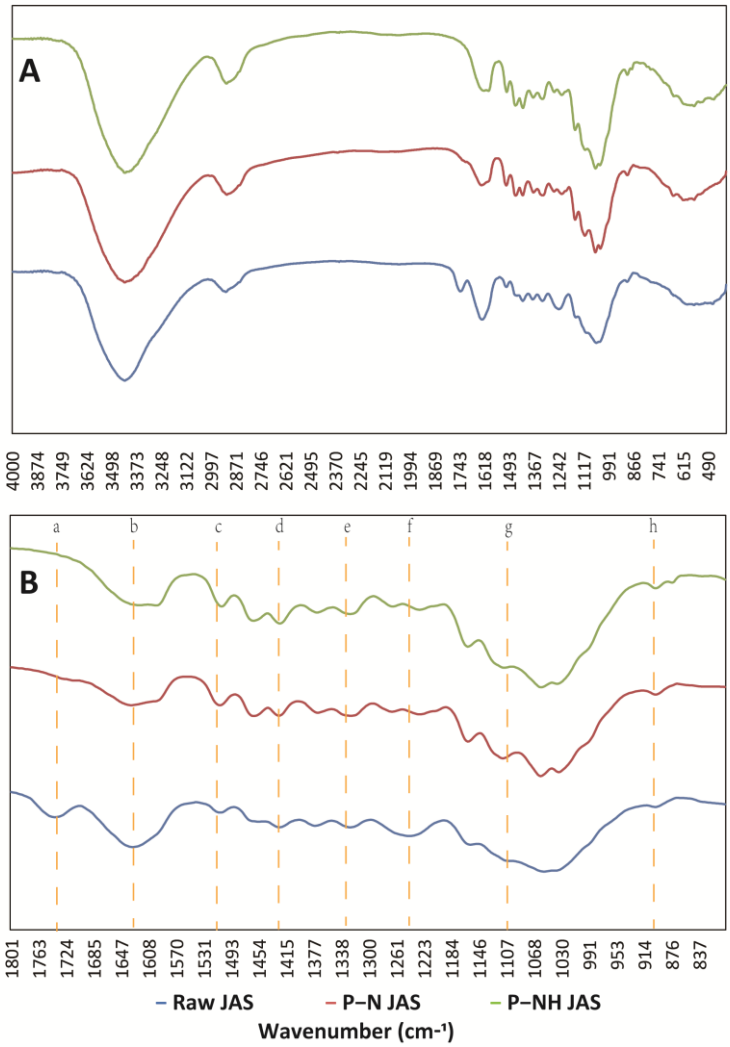

Figure 6. FTIR spectroscopy fingerprints of raw and pretreated JAS. (**A**) The wavenumber range is 500 cm^{-1}–4000 cm^{-1}. (**B**) The wavenumber range is 800 cm^{-1}–1800 cm^{-1}. P-N JAS: NaOH-pretreated JAS, P-NH JAS: CO-pretreated JAS. (a) 1735 cm^{-1}, (b) 1630 cm^{-1}, (c) 1515 cm^{-1}, (d) 1425 cm^{-1}, (e) 1329 cm^{-1}, (f) 1240 cm^{-1}, (g) 1104 cm^{-1}, (h) 897 cm^{-1}.

The peak at 1630 cm^{-1} (indicates -C-O-) reflected the content of lignin (Figure 6B). After the first pretreatment, a significant decrease in lignin was observed, and then an increase after the second pretreatment was shown (but still less than that of raw materials), which is consistent with the results stated in Table 1. However, other peaks of lignin at 1515 cm^{-1} (aromatic skeletal from lignin) and 1329 cm^{-1} (syringyl and guaiacyl condensed lignin) had no significant change, which explained that lignin still existed in the pretreated JAS in a considerable amount. The absorption peak of hemicellulose is at 1735 cm^{-1}, indicating a change in the C=O bond in hemicellulose. Both the raw JAS and the NaOH-pretreated JAS obtained after the first pretreatment showed this peak (the peak appeared both in the raw JAS and NaOH-pretreated JAS obtained after the first pretreatment), which indicated the existence of hemicellulose. However, the peak (1735 cm^{-1}) after the 2nd pretreatment was not obvious, which indicated that hemicellulose was removed by acid pretreatment. The peaks at 1425 cm^{-1}, which represent the CH_2 in crystalline cellulose, diminished after pretreatment, and the peak at 897 cm^{-1} corresponds to the bending vibration of cellulose. After the pretreatments, these two peaks both had enhanced ratios among all the peaks, indicating an increase in the cellulose content. The variation in the concentrations of cellulose, hemicellulose, and lignin is consistent with the FTIR analysis results (Table 1).

4. Conclusions

In this study, raw JAS was subjected to two-stage pretreatment with NaOH followed by acidic wastewater treatment. In the first step of NaOH pretreatment, 57.69% lignin was removed, leading to the increased content of cellulose and hemicellulose by 78.49% and 18.18%, respectively. After further pretreatment, 31.33% hemicellulose was removed, leading to the cellulose content being further enhanced to 70.07%. The pretreated JAS showed excellent sugar release (72.90% hydrolysis yield by 25 FPU/g sample in 48 h), while at the same time, acid-insoluble lignin (36.78% lignin recovery) and hydrolysates (9.85g/L reducing sugar) were obtained. In addition, the concentration of inhibitors was very low (0.06 g/L HMF). Furthermore, the structure of the JAS surface was disrupted by pretreatment and increased the cellulose content, thus improving the hydrolysis efficiency by SEM, XRD, and FTIR analyses.

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Abbreviations

The following abbreviations are used in this manuscript:

JA	Jerusalem artichoke
JAS	Jerusalem artichoke stalk
JAT	Jerusalem artichoke tuber
P-N JAS	NaOH-pretreated Jerusalem artichoke stalk
P-NH JAS	Combined alkali-acid pretreated Jerusalem artichoke stalk
NREL	National Renewable Energy Laboratory
DNS	Dinitrosalicylic acid
HMF	Hydroxymenthylfurfural
HPLC	High-Performance Liquid Chromatography
SEM	Scanning Electron Microscopy
FTIR	Fourier Transform infrared spectroscopy
XRD	X-ray diffraction
KBr	potassium bromide
CrI	Crystallinity index
CBHI	Cellobiohydrolase I
FPU	Filter Paper Unit
T. reesei	*Trichoderma reesei*

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