**Effect of Ammoniated Fiber Explosion Combined with H\textsubscript{2}O\textsubscript{2} Pretreatment on the Hydrogen Production Capacity of Herbaceous and Woody Waste**

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Cite This: ACS Omega 2022, 7, 21433−21443

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**ABSTRACT:** An appropriate pretreatment process is an important part of the preparation of biomass energy from agricultural and forestry waste. Compared to physical and chemical pretreatments alone, the combined ammoniated fiber explosion (AFEX) + hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) pretreatment process can significantly improve the lignin degradation rate and saccharification efficiency, thus improving the hydrogen production capacity during medium-temperature dark fermentation. This study showed that the combined pretreatment increased the saccharification efficiency of herbaceous, hardwood, and softwood biomass by 58.7, 39.5, and 20.6% and the corresponding gas production reached 145.49, 80.75, and 57.52 mL/g, respectively. In addition, X-ray diffraction, scanning electron microscopy, and Fourier-transform infrared spectroscopy showed that AFEX + H\textsubscript{2}O\textsubscript{2} disrupted the structure of the feedstock and was more favorable for lignin removal. Soluble metabolites indicated that AFEX + H\textsubscript{2}O\textsubscript{2} pretreatment enhanced the butyrate metabolic pathway of the substrate and biohydrogen generation and increased the levels of extracellular polymers and microbial community structure.

1. INTRODUCTION

The overexploitation of fossil fuels, which are a non-renewable asset, has caused a series of ecological issues, such as air pollution and ozone layer depletion. ¹ The production of sustainable biomass energy from lignocellulosic feedstock has been widely used as a reliable fuel option. ² Agroforestry waste is an underutilized potential source of energy. It is widely available, easy to collect and transport, and does not harm the stability of ecosystems. ³ Therefore, the feasibility of using agroforestry waste to produce renewable biomass should be explored. ³

Currently, most agricultural and forestry waste is discarded or disposed of using incineration in landfills, which causes not only ecological damage but also wastes resources. Biomass materials have been explored as substrates for anaerobic digestion to produce biohydrogen. However, the structural properties of agroforestry biomass itself make it difficult to be utilized by microorganisms during fermentation. Therefore, the utilization of agroforestry waste requires an effective pretreatment process. ⁶

Historically, a huge number of pretreatment methods such as physical, chemical, biological, and their combined forms have been explored. Among these, alkali pretreatment methods have proven to be efficient in facilitating lignin removal for improving enzymatic efficiency. ⁵,⁶ NaOH and Ca(OH)\textsubscript{2} are the most commonly used reagents in alkali pretreatment. ⁷

However, the treatment produces black liquor and washing wastewater, which is difficult to treat and discharge. ⁸ In addition, high concentrations of alkali can cause large losses of cellulose, thus reducing biomass utilization. Therefore, the combination of diluted alkali with other methods could be an effective means of improving biomass utilization.

Ammoniated fiber explosion (AFEX) is one of the pretreatment methods that is currently receiving attention. ⁹ It has a few advantages, such as no degradation of cellulose and hemicellulose and no production of substances that hinder enzymatic digestion and fermentation. ¹⁰ However, AFEX technology alone cannot treat all three components: cellulose, hemicellulose, and lignin, simultaneously; therefore, AFEX needs to be combined with other technologies to be further developed. Hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}), a strong oxidizing agent, is inexpensive, environmentally friendly, and often used in combination with alkaline reagents in pretreatment processes to improve enzyme digestibility through lignin removal. In an alkaline environment, H\textsubscript{2}O\textsubscript{2} tends to break

Received: January 28, 2022
Accepted: June 3, 2022
Published: June 13, 2022
down to produce reactive radicals, which leads to degradation and oxidation of lignin and increases enzyme accessibility. However, hydrogen peroxide binding groups are limited to NaOH or ammonia.\(^{1,12}\) Thus, we investigated the effect of AFEX + H\(_2\)O\(_2\) on the dark fermentation of hydrogen from agroforestry biomass.

2. MATERIALS AND METHODS

2.1. Raw Materials and Chemicals. The lignocellulosic material used in this article was sourced from Changqing District, Jinan City, Shandong Province, China. Straws, poplars, and pines were dried, ground to a fine powder, passed through a 20 mesh sieve, and stored at 37 °C in sealed plastic bags. Where chemicals used for this work are not specified, they are of analytical grade. Liquid ammonia, hydrogen peroxide, cellulase, and hemicellulase used for cellulose degradation were purchased from Jinan Kost Experimental Equipment Co.

2.2. AFEX Pretreatment. During the AFEX pretreatment, the weighed feedstock was blended with water at a water stack of 0.7 (weight proportion of water to dry biomass) and the blended test was placed in a tall weight reactor. Fluid alkali was infused into the reactor to obtain 1.0 ammonia loadings (weight proportion of alkali to dry biomass). The reactor temperature increased rapidly to 130 °C and remained for a defined period. The ammonia was then released through the discharge valve (approximately 30 s). The treated biomass was removed from the reactor and dried in a fume hood (approximately 12 h) at 40 °C. The dried specimens were sealed in plastic bags and stored at -20 °C for later use. Pretreatment temperature was 130 °C and time was 15 min.

2.3. AFEX + H\(_2\)O\(_2\) Pretreatment. In a 100 mL glass bottle, 30% H\(_2\)O\(_2\) solution and AFEX pretreated substrate were mixed in ratios of 0.5, 0.75, 1, 1.5, and 2 (w/w) and pretreated at 60 °C for 1 h, hereafter named as S1, S2, S3, S4, and S5, respectively. The treated material was washed repeatedly with distilled water until the filtrate was neutral (the pH value of the filtrate measured with an acidity meter was approximately 7), dried at 60 °C for 24 h, and then used.

2.4. Enzymatic Hydrolysis. After sample pretreatment, 8–10 g was added to the enzyme digestion vial and distilled water; sodium citrate buffer solution (pH 4.8) and antibiotics (tetracycline and cycloheximide) were added in sequence. The enzymatic flask was placed in a shaker and stirred for 1 h before cellulase, glucosidase, and xylanase were added. Fibrozyme (novozymeNS50012) and β-glucosidase (novozymeNS50010) were used at doses of 15 FPU/(g glucan) and 64 pNPUG/(g glucan). The enzymatic assimilation tests were conducted on a rotational shaker at 55 °C and 150 rpm for 72 h. After the enzymatic assimilation, the item was centrifuged at 12,000 rpm for 5 min.\(^{13}\)

The enzymatic efficiency of the substrate was calculated using the following formula

\[
\text{enzymolysis efficiency} = \frac{\text{glucose + xylose (g/L)}}{(\text{glucose} / \% \times 1.11 + \text{xylose} / \% \times 1.14) \times \text{solid loading (g/L)}} \times 100\% 
\]  

(1)

where 1.11 is the calculated change from glucan to glucose and 1.14 is the xylose conversion factor.

2.5. Preparation of Inoculum and Medium-Temperature Anaerobic Hydrogen Production Program. The biogas fermentation system consists of an arrangement of glass reaction vessels with a working volume of 500 mL, clearing out 125 mL of headspace to dispense with the hurtful impacts of hydrogen fractional weight. The saccharification product of the S4 group was used to study the effect of three pretreated biomasses on the mesophiles dark fermentation of hydrogen production, and the substrate without pretreatment was used as a control. In addition to the saccharification product, the substrate in each reactor also contained 0.5 g/L peptone.

We flushed the reactor with nitrogen for approximately 3 min to ensure an anaerobic environment and adjusted the pH to 7.0 ± 0.1; then, we incubated the reactor at 37 °C for 48 h. The batch experiment was performed three times. Amid the dim aging stage, fluid tests were collected every 6 h and gas generation was measured every 3 h,\(^{1,6}\)

The volume of gas was calculated from standard conditions (273.15 K, 101.325 kPa) as shown in eq 2.

\[
V_{\text{hydrogen}} = \frac{V_{\text{hydrogen}} (mL, \text{STP})}{273} \times \frac{273 + T}{101.325} = \frac{V_{\text{hydrogen}} (mL, T) \times (273 + T)}{101.325} \times \frac{101.325 - w}{101.325}
\]  

(2)

The experiment was conducted three times, with data recorded for each day of gas production. The fermentation broth was centrifuged at 5000 rpm for 10 min each time at approximately 0.5 mL, and the supernatant was removed for pH and volatile fatty acids (VFAs) measurements.

Three replicates were conducted, and daily production data were recorded. Each time, approximately 0.5 mL of fermentation broth was centrifuged at 5000 rpm for 10 min, and the supernatant was used to determine pH and VFAs.

2.6. Sample Composition Analysis. The chemical composition of the crude and pretreated feedstock was

| Parameter                  | pH | TS (wt %) | VS (wt % of TS) | ammonium (NH\(_4^+\)–N, mg/L) | V\(_a\) (mg/L) | TC (mg/L) | TOC (mg/L) | inorganic carbon (mg/L) |
|----------------------------|----|-----------|----------------|------------------------------|---------------|-----------|------------|------------------------|
| Typical Parameter          | 7.0 ± 0.1 | 8.6 ± 0.6 | 70.6 ± 1.8 | 584.8 ± 37.5 | 607.2 ± 45.3 | 3240 ± 154 | 2360 ± 106 | 495 ± 35 |
| 37 °C                      |     |           |               |                             |               |           |            |                        |
Table 2. Components of Untreated and AFX + H2O2-Treated Materials

| Materials | glucan (%) | recovery | xylan (%) | recovery | lignin (%) | removal | solid recovery |
|-----------|------------|----------|-----------|----------|------------|---------|---------------|
| untreated | 41.05 ± 0.20 | 18.27 ± 0.09 | 26.00 ± 0.37 | 90.00 ± 0.45 |
| AFX       | 40.42 ± 0.20 | 17.88 ± 0.09 | 23.00 ± 0.41 | 88.64 ± 0.43 |
| S1        | 39.35 ± 0.20 | 17.26 ± 0.09 | 22.59 ± 0.25 | 85.84 ± 0.43 |
| S2        | 39.15 ± 0.19 | 17.11 ± 0.09 | 22.00 ± 0.51 | 81.88 ± 0.41 |
| S3        | 38.32 ± 0.19 | 16.73 ± 0.08 | 21.33 ± 0.24 | 79.82 ± 0.40 |
| S4        | 37.74 ± 0.19 | 14.81 ± 0.07 | 19.67 ± 0.14 | 77.98 ± 0.39 |
| S5        | 37.24 ± 0.18 | 14.76 ± 0.07 | 21.00 ± 0.59 | 74.24 ± 0.37 |
| Rice Straw | 45.88 ± 0.23 | 17.66 ± 0.18 | 38.33 ± 0.48 | 90.50 ± 0.45 |
| AFX       | 45.71 ± 0.23 | 17.51 ± 0.17 | 30.00 ± 0.44 | 85.16 ± 0.40 |
| S1        | 45.59 ± 0.23 | 17.35 ± 0.17 | 18.33 ± 0.29 | 81.08 ± 0.41 |
| S2        | 44.93 ± 0.22 | 16.57 ± 0.17 | 19.00 ± 0.60 | 76.26 ± 0.38 |
| S3        | 44.26 ± 0.22 | 16.41 ± 0.16 | 18.67 ± 0.47 | 73.12 ± 0.37 |
| S4        | 42.90 ± 0.21 | 15.08 ± 0.15 | 12.33 ± 0.48 | 73.02 ± 0.37 |
| S5        | 41.97 ± 0.21 | 15.03 ± 0.15 | 15.00 ± 0.71 | 70.82 ± 0.35 |
| Pine      | 37.10 ± 0.19 | 18.62 ± 0.19 | 33.33 ± 0.36 | 90.12 ± 0.45 |
| AFX       | 36.81 ± 0.19 | 18.57 ± 0.19 | 31.67 ± 0.69 | 87.63 ± 0.43 |
| S1        | 36.57 ± 0.18 | 18.48 ± 0.18 | 30.67 ± 1.00 | 85.28 ± 0.43 |
| S2        | 36.18 ± 0.18 | 17.75 ± 0.18 | 30.00 ± 1.21 | 78.88 ± 0.39 |
| S3        | 35.50 ± 0.18 | 17.38 ± 0.17 | 28.90 ± 0.34 | 77.16 ± 0.39 |
| S4        | 34.86 ± 0.17 | 16.57 ± 0.17 | 27.33 ± 0.53 | 72.24 ± 0.36 |
| S5        | 34.31 ± 0.17 | 12.26 ± 0.15 | 29.90 ± 0.37 | 69.02 ± 0.34 |

Examined on an oven-dry weight premise at 105 °C concurrently with the National Renewable Energy Laboratory (NREL) benchmarks program. This included cellulose (dextran), hemicellulose (xylan and arabinose), and lignin. First, 0.5 g of the sample was extracted with deionized water and anhydrous ethanol, extracted in a Soxhlet extractor for 10 h, and dried to constant weight at 105 ± 3 °C to start the corrosive hydrolysis; 0.1 g of unextracted test and 1 mL of sulfuric acid (72%, w/w) were mixed in a weight tube at 30 °C for 1 h. The solution was then diluted to 4% (w/w) with ultrapure water and then autoclaved at 121 °C for 1 h. A second acid hydrolysis step was conducted. The residues were filtered, dried, and analyzed for lignin, and the hydrolysis products were further examined to evaluate carbohydrates and acid-soluble lignin. Monomeric sugars were measured by a high-performance liquid chromatography (HPLC) instrument (Agilent 1200 arrangement, MN, USA) prepared with an Aminex HPX-87H column and a refractive index finder. HPLC conditions were based on a mobile phase of 5 mM H2SO4 at 60 °C. The column was run at 0.4 mL/min at 60 °C.

2.7. 16S rRNA Amplicon Sequencing. We evaluated the microbial community changes using high-throughput 16S rRNA gene pyrosequence sequencing. The Omega Soil Deoxyribonucleic Acid Kit (Omega Biotechnology, Norcross, USA) was used to measure the amount of deoxyribonucleic acid. Approximately 10 ng of DNA was utilized for polymerase chain reaction (PCR) enhancement and library development. PCR groundworks 338 F (ACTCCTACGGGAGGCAGCAG) and 806 R (GGACTACHVGGGTWTCTAAT) were utilized to open districts V3 and V4 on prokaryotic 16S rDNA. After filtration and evaluation, pyrosequence sequencing was performed on the MiSeq stage (Sangon Biotech, Shanghai, China) utilizing Illumina high-throughput sequencing.

Sequences were resolved at the genus level and analyzed for microbial community diversity.

2.8. Statistical Tests and Kinetic Model Analysis. To determine whether the effect of AFX + H2O2 pretreatment of agroforestry waste on the fermentation potential of biohydrogen was statistically significant, at each experimental level, three parallel sets of tests were performed and the data obtained were averaged. Information from the medium-temperature maturation handle were also subjected to a one-way investigation of fluctuation (ANOVA) utilizing IBM SPSS Insights 25.

Various physical, chemical, and biological factors can have a significant effect on the kinetics of the anaerobic digestion process. The cumulative hydrogen production (CHP) curve was used to estimate the hydrogen production potential. Some of the kinetic parameters are shown by a modified Gompertz model in eq 3.

\[
P(t) = P_m \exp \left\{- \exp \left[ \frac{R_m \cdot e}{P_m} (\lambda - t + 1) \right] \right\}\]

where \( P(t) = \text{CHP (mL)} \) over time \( t \) (h), \( R_m = \text{maximum rate of H}_2 \text{ generation (mL/h)} \), \( \epsilon = 2.72, \) and \( \lambda = \text{lag phase (h)} \). The \( P_m, R_m, \) and \( \lambda \) values were determined using Origin 8.5 software, and the correlation coefficient \( (R^2) \) was further evaluated.

2.9. FTIR and X-ray Diffraction Analyses. The treated tests were compacted, and the infrared spectra of the tests were obtained by employing a Fourier change infrared spectrometer (PerkinElmer Range Two, USA). The scan rate was 0.2 cm\(^{-1}\) with a resolution of 4 cm\(^{-1}\) and 32 scans in the 4000–400 cm\(^{-1}\) range.

X-ray diffractograms of the samples were obtained using an X-ray diffractometer (SmartLab SE, Rigaku, Japan). The scans...
range was set from 5 to 50° with a scan speed of 36° min⁻¹ and a scan step of 0.02°. Analysis was performed using Jade software 6.5, and eq 4 was used to calculate crystallinity (CrI)

\[
\text{CrI} = \left( \frac{I_{002} - I_{am}}{I_{002}} \right) \times 100\%
\]

(4)

where \(I_{002}\) is the 002 diffraction peak intensity at approximately 2θ = 22–22.8° and \(I_{am}\) is the diffraction peak intensity at 2θ = 18°.

2.10. Scanning Electron Microscopy and Solid-State NMR Spectroscopy Analyses. Scanning electron microscopy (SEM) images were taken with a Regulus 8220 (Hitachi, Japan), scanned at an acceleration voltage of 5 kV at a magnification of 1000× and 2000×. A small number of specimens were evenly placed into the loading platform, and the surface of the samples was plated for 2 min using an E-1010 ion sputterer (Hitachi, Japan).²¹

NMR spectra of 13C CP/MAS solids from 0 to 270 ppm were tested using a BRUKER AVANCE III HD 400 MHz instrument (Bruker, Switzerland) at a spectral resolution of 0.1 ppm. The lignin sample was dissolved with dimethyl sulfoxide and placed on an NMR spectrometer to measure the 13C NMR spectrum. Its signal properties were resolved using MestReNova12 software and compared with the literature.

3. RESULTS AND DISCUSSION

3.1. Changes in the Chemical Properties of Lignocellulose after AFEX + H₂O₂ Pretreatment. As shown in Table 2, the effect of H₂O₂ concentration loading on the recovery of solids and fractions changed after pretreatment of the feedstock. The untreated herbageous biomass had a lower lignin content than the woody type, but a relatively higher xylan content.²² Under the same conditions, the best removal rate for straw was 67.8%, while the best lignin removal rates for poplar and pine were only 24.36 and 18%. The lignin removal capacity after AFEX + H₂O₂ pretreatment was straw > poplar (hardwood) > pine (softwood). Studies have shown that lignin in herbageous biomass and hardwoods typically includes three major phenyl propane units, namely, guaiacyl, butyl, and p-hydroxyphenyl (H), whereas softwood lignin contains only G and H units, which gives softwoods greater delignification recalcitrance. As we can see from Table 2, the lignin content did not change significantly after AFEX treatment alone and its removal rate was much lower than that of the S4 group. The AFEX pretreatment effect was mainly in the structural modification of cellulose, hemicellulose, and lignin, while there was almost no removal capacity for lignin and hemicellulose. The alkaline H₂O₂ pretreatment opens the ester and ether bonds of the lignocarbon complex to reduce bio-resistance, weakening the hydrogen bonds between cellulose and hemicellulose and saponifying the ether bonds between hemicellulose and lignin.

The subsequent enzymatic saccharification was facilitated. In addition, using rice straw as an example under the same experimental conditions, high H₂O₂ could cause a continuous decreasing trend in the rate of dextran and xylan content, and the solid recovery rate reached 90% after AFEX. With the increase of H₂O₂ concentration, the content of dextran and xylan continuously decreased. Particularly, when reaching S4, the loss of glycan was more significant. After AFEX, the removal rate of lignin was 21.7%. When the proportion of H₂O₂ reached S4, the maximum lignin removal was 67.8%. Similarly, the best lignin removal results were 24.4 and 18.0% for poplar and pine wood in group S4. Thus, the optimum H₂O₂ addition for delignification was S4. In addition, the high H₂O₂ loading resulted in the loss of glucan and xylan, which was not effective for lignin removal. Therefore, the loss of solids due to high H₂O₂ loading was due to the loss of glycans rather than lignin.²³

3.2. Effect of AFEX + H₂O₂ Pretreatment on Structural Properties of Different Agroforestry Biomasses. 3.2.1. SEM Analysis. Figure 1 shows that the surface of untreated straw was smooth, dense, and rigid, with neatly arranged biomass structures. At the same time, the wax was removed from the treated straw surface, the surface roughness increased significantly, the surface structure became porous and lax, an increase in filamentous brooming was visible, a single bundle structure appeared (marked with an arrow), and the specific surface area increased, due to the removal of lignin (Table 2).²⁴ The surface structure of the treated straw was greatly disrupted, which also suggests that the pretreatment caused the straw to hydrate and swell; the liquid penetrated the straw cells, causing the complex linkages between the straw to be disrupted, which also provided more reaction sites for subsequent enzymatic hydrolysis and improved the accessibility of cellulose.²⁵ Compared to the rice straw in the figure, poplar and pine had poorer results, which is probably due to the low accessibility of the cellulase enzyme to be utilized and related to the small changes in its composition (Table 2).

3.2.2. FTIR and X-ray Diffraction Analyses. As seen in Figure S1, after pretreatment with AFEX + H₂O₂, the positions of the main absorption peaks were similar for the different samples, but the intensities were clearly different. The changes in chemical structure and content of herbaceous biomass were more significant. This was related to changes in the structure and composition of lignin, cellulose, and hemicellulose after pretreatment. The crest of rice straw at 1736 cm⁻¹ presents a place for the hemicellulose carbonyl C=O extending vibration. In contrast, the ester and carbonyl groups of poplar and pine belong to C=O stretching at 1736 cm⁻¹, which is
also attributed to hemicellulose degradation. The crest of the test at 1511 cm$^{-1}$ has a place for the lignin C=O extending with the fragrant skeleton vibration, and the diminished crest escalation was credited to the successful expulsion of lignin by the combined pretreatment. The apparent shift in the peak at 895 cm$^{-1}$ indicated that the $\beta$-glycosidic bond between cellulose and hemicellulose was broken after co-pretreatment. However, the sample showed a shift in the peak at 2910, which is due to the C−H extending vibration of the methyl and methylene groups in the lignin side chain, indicating that the cellulose was not degraded.

Due to the influence of the biomass fraction, hemicellulose and lignin are considered undefined, whereas cellulose is within the crystalline state. The CrI values improved to varying degrees after pretreatment (Figure S2). Diffraction peaks were evident for each sample around $2\theta = 18.5$ and 22.4$^{\circ}$. This indicates that AFEX + H$_2$O$_2$ pretreatment does not have any effect on the structure of the cellulose itself. The results showed that the CrI value of rice straw in group S4 increased from 27.79 to 40.54%. Poplar and pine also showed a 5−10% increase, indicating that the combination of AFEX + H$_2$O$_2$ pretreatment enhanced the removal of lignocellulose from the feedstock. In contrast, there was only a slight increase in CrI for the other concentration pretreatments, which may be due to the removal of only some non-crystalline extracts. CrI values were positively correlated with enzymatic efficiency, with most of the lignin and a few of the hemicellulose expelled amid pretreatment, driving to a reduction within the composition of the shapeless zone and an increment within the extent of the cellulose crystalline zone. The significant increase in CrI indicated an increased cellulose exposure and a loosened lignocellulosic structure, facilitating subsequent enzymatic digestion. The Fourier-transform infrared (FTIR) spectroscopy and CrI results indicated that AFEX + H$_2$O$_2$ pretreatment was more effective in herbaceous plants (Figure 2).

### 3.2.3. NMR Analysis

All untreated and pretreated samples had three distinct signals at approximately 63.4, 73.7, and 104.6 ppm and one insignificant signal at 83.5 ppm. The signals at 63.4 and 104.6 ppm consisted of chemical groups from hemicellulose and cellulose, while the signals at 73.7 and 83.5 ppm were probably from hemicellulose, cellulose, and chemical moieties in lignin. The 20.8/21.7 ppm flag of CH$_3$ within the acetyl gather of sort hemicellulose and the 173.2 ppm flag having a place for the ester gather of carbohydrates were not present in all pretreatment tests. This suggests that, in all samples, the acetyl and ester bunches of carbohydrates were vulnerable to assault by AFEX + H$_2$O$_2$.

Untreated rice straws had a 31.5 ppm signal that could be attributed to alkyl groups weakened by pretreatment. The 110−160 ppm signal was reported to be mainly from lignin aromatics and phenolics. The 130.6 ppm signal disappeared after pretreatment with rice straw, and the 134.5 ppm signal was reduced in poplar and pine wood. This compares to the larger part expulsion of lignin from herbaceous biomass amid pretreatment (Table 2). The 152.1 ppm signal for untreated poplar and 148.7 ppm for untreated pine were stronger than the 149.7 ppm signal for untreated rice straw, indicating differences in the lignin composition of wood hardwoods, softwoods, and herbaceous lignocelluloses.

### 3.3. Effect of AFEX + H$_2$O$_2$ Pretreatment on the Enzymatic Saccharification of Lignocellulose

The results of the pretreated lignocellulose hydrolysis are shown in Figure 3. The saccharification efficiency of treated rice straw biomass was increased by 58.7% compared to the original sample. The enzymatic efficiency of poplar and pine increased by 39.5 and 20.6%, respectively. AFEX + H$_2$O$_2$ pretreatment was the most effective for the glycation of herbaceous biomass. In both hardwoods and softwoods, the high lignin content after pretreatment, which leaves the cellular morphological structure intact, hinders the uptake of cellulose by the enzyme and thus affects the enzymatic efficiency. Rice straw, for example, releases a large number of reactive groups with the addition of H$_2$O$_2$. When the pH value is higher than 8 (especially in an alkaline environment), H$_2$O$_2$ is easily decomposed to produce hydroxyl radicals (−OH), hydroperoxide anions (HOO$^-$), and...
superoxide anions (O$_2^-$). These reactive groups remove lignin through degradation and oxidation, while breaking the crystalline structure of cellulose increases the contact area of the enzyme; thus, the saccharification efficiency gradually increases. When the S4 concentration was reached, which was also consistent with the pretreatment effect. Both the efficiency of saccharification and the sugar yield of the substrate decreased at higher H$_2$O$_2$ concentrations compared to those in the S4 group. The high chemical concentration resulted in a high solid loss accompanied by irreversible polysaccharide degradation and continued degradation of monosaccharides, leading to a decrease in saccharification efficiency.

3.4. Effect of AFEX + H$_2$O$_2$ Pretreatment on Hydrogen Production during Medium-Temperature Dark Fermentation. Depending on the saccharification efficiency and sugar production at different concentrations, hydrogen production was carried out using medium-temperature dark fermentation in rice straw, poplar, and pine wood as substrates in group S4.

Figure 4 shows the variation in H$_2$ production and HPR during the 84 h fermentation cycle in the anaerobic fermentation experiment. Under medium-temperature conditions, using rice straw as an example, after the initial 18 h of anaerobic fermentation reaction, the HPR of hydrogen-producing bacteria was slow, indicating that the hydrogen-producing bacteria were in the adjustment period and could not suitably exert their gas production capacity. In the subsequent 24 h, the hydrogen-producing bacteria gradually adapted to the process of anaerobic fermentation and reproduced more, with a continuous increase in gas production. The hydrogen accumulated in 72 h amounted to 1322.7 mL. Under the same temperature conditions, the CHP of rice straw, poplar, and pine was 146.65, 80.75, and 57.52 mL/g, respectively, which could be nearly doubled compared to the untreated substrate. In addition, the maximum gas production rates reached were 7.76, 4.36, and 3.05 (mL/g/h), respectively, which were also greatly enhanced compared to the control group. Less gas production was found in poplar and pine compared to rice straw, which was related to sugar production after saccharification. Therefore, AFEX + H$_2$O$_2$ can significantly increase the gas production from agroforestry waste during medium-temperature dark fermentation.

A modified Gompertz model was fitted using Origin 8.5, and the cumulative H$_2$ yields (P$_m$, R$_m$, and $\lambda$) obtained for the modified groups are shown in Table 3. The results show that the model fits the CHP data for each group, with correlation coefficients >99%. In the case of rice straw, the cumulative hydrogen yield $P_m$ value was 146.07 mL/g and the highest hydrogen yield $R_m$ was 7.62 mL/(g·h), similar to 145.50 mL/g and 7.76 mL/(g·h) shown in the figure. The kinetic parameters were larger for all samples than the control, and the hysteresis times varied between substrates under the same conditions but were all within 10–20 h.

Chemical oxygen demand was maintained at 88.7–92.4%, proving the validity of the experimental results. The pH of all fermentation systems decreased between 4.6 and 5.0 at the end of the medium-temperature dark fermentation and was relatively stable close to the final pH level. This suggests that the alkalinity provided by AFEX + H$_2$O$_2$ only slowed the decrease in alkalinity of the fermentation system and did not affect the acidification trend of the whole system. Such pH changes are seen in most steady hydrogen generation

Table 3. Effect of AFEX + H$_2$O$_2$ Pretreatment on Gompertz Coefficient and Fermentation Products

| materials           | $P_m$ (mL/g) | $R_m$ (mL/(g·h)) | $\lambda$ (h) | $R^2$ (%) | pH   | COD balance |
|---------------------|-------------|------------------|---------------|-----------|------|-------------|
| untreated poplar    | 40.20       | 1.38             | 10.06         | 99.87     | 5.0 ± 0.1 | 89.3 ± 1.2  |
| treated poplar      | 81.10       | 3.64             | 16.78         | 99.17     | 4.7 ± 0.2 | 90.2 ± 2.1  |
| untreated straw     | 62.77       | 2.28             | 11.32         | 99.40     | 4.8 ± 0.1 | 90.6 ± 1.4  |
| treated rice straw  | 146.07      | 7.62             | 20.22         | 99.58     | 4.6 ± 0.2 | 92.4 ± 2.3  |
| untreated pine      | 35.17       | 1.44             | 9.13          | 99.78     | 4.8 ± 0.2 | 88.7 ± 1.8  |
| treated pine        | 57.74       | 2.45             | 12.29         | 99.22     | 4.8 ± 0.1 | 89.5 ± 1.9  |

Figure 4. Effect of AFEX + H$_2$O$_2$ on biohydrogen production from agroforestry waste: (a) H$_2$ yield (37 °C); (b) corresponding maximum hydrogen production rate (HPR) (37 °C).
frameworks, suggesting that the ultimate pH concentration had no inhibitory impact on H₂. 39

Statistical analysis of the hydrogen production, acetate, butyrate, and soluble organic matter (SMPs) concentrations was conducted using ANOVA and showed that the P-values for all of these were <0.01. This indicates that the AFEX + H₂O₂ pretreatment had a critical effect on the experimental results (Table 4).

### Table 4. Analysis of Variance for Fermentation Parameters

| materials    | BioH₂ yields | SMPs | acetate | butyrate |
|--------------|--------------|------|---------|----------|
| poplar       | <0.01        | <0.01| <0.01   | <0.01    |
| rice straw   | <0.01        | <0.01| <0.01   | <0.01    |
| pine         | <0.01        | <0.01| <0.01   | <0.01    |

3.5. Effect of AFEX + H₂O₂ Pretreatment on the Hydrogen Production Pathway. The butyrate and ethanol metabolic pathways are the two most important metabolic pathways in medium-temperature dark fermentation for hydrogen production, and they have a high hydrogenation capacity. pH, SMP, and alkalinity may indirectly affect the stability of the fermentation system and even cause ecological damage. AFEX + H₂O₂ pretreatment of dark fermentation for hydrogen production produced mainly SMPs (ethanol, acetate, butyrate, and propionate), whose accumulation was closely related to hydrogen production. Figure 5 shows the distribution of the different components on the metabolites. Medium-temperature treatment of rice straw was most effective in group S4, with concentrations of 1.62 and 2.71 g/L for acetic acid and butyrate, respectively. The SMP concentration of 5.05 g/L was 25.07% higher than that of the control (Figure 5a). Compared to the control, the SMP concentrations were 19.51 and 15.11% higher in treated poplar and pine, respectively. It was demonstrated that AFEX + H₂O₂ improved the substrate conversion. Within the S4 bunch of rice straws, after the slack stage, the substrate to begin with delivered a huge sum of acetic acid derivation by maturation, driving to an overabundance of NADH and H⁺. At the same time, the metabolism in the acetate type does not oxidize NADH and H⁺, and when the acidic end formed by acetate is excessive, a negative feedback mechanism is generated due to low pH, driving the coupling with the butyric acid cycle mechanism (eqs 5 and 6). As a result, after 24 h, the level of

![Figure 5](https://example.com/figure5.png)

**Figure 5.** Effect of AFEX + H₂O₂ pretreatment on soluble microbial products (SMPs) and glucose: (a) SMPs (mesophilic); (b) SMPs and glucose in group S4 rice straw (mesophilic); (c) SMPs and glucose in group S4 pine (mesophilic); (d) SMPs and glucose in group S4 poplar (mesophilic).
butyrate increases dramatically. Although the butyrate metabolic pathway is unable to oxidize the excedent NADH and H⁺ (eq 7), coupling with the acetic acid derivation metabolic pathway decreases the collection of NADH and H⁺ while advancing substrate digestion system, thus helping to regulate the homeostasis of the fermentation system. The results showed that AFEX + H₂O₂ pretreatment of agroforestry waste was more favorable to butyric acid-type fermentation.

\[
\begin{align*}
C_6H_{12}C_6 + 4H_2O + 2NAD^+ & \rightarrow 2CH_3COO^- + 2HCO_3^- + 2H_2 + 6H^+ \Delta G^0 = -215.7 \text{ KJ/mol} \\
NADH + H^+ & \rightarrow NAD^+ + H_2 \Delta G^0 = -21.9 \text{ KJ/mol}
\end{align*}
\]

### 3.6. Effect of AFEX + H₂O₂ Pretreatment of Agroforestry Waste on Microbial Community Structure

To explore the fermentation mechanism of AFEX + H₂O₂ pretreatment in agroforestry squander to advance hydrogen generation, the microbial communities were analyzed in a slime collected from bio-H₂ maturation reactors utilizing high-throughput 16S rRNA pyrophosphate sequencing. The number of substantial arrangements for each test extended from 58,407 to 81,546, with a coverage of 0.999. This result illustrates the sequencing reliability. The Shannon index, an important parameter of bacterial abundance, showed a
reduction in the treated substrate of 0.37–0.56. This indicates that the diversity of the microbial community was significantly reduced after pretreatment with AFEX + H2O2. Figure 6 portrays the changes within the microbial community at the phylum and genus levels.

Firmicutes were the dominant phylum in all samples that could use glucose to produce hydrogen. Under the same conditions of medium-temperature fermentation, the abundance microbial community of the S4 rice straw group increased from 56.06 to 80.33%, while the abundance of the microbial community in poplar and pine increased only by 20 and 2%, respectively (Figure 6a). The pretreated bioreactor was more favorable to the growth and enrichment of Firmicutes under medium-temperature conditions. Other dominant clades were Bacteroidetes and Patescibacteria, which, unlike Firmicutes, do not form endophytic bundles to adapt to their environment, and their main role is to degrade complex organic matter (e.g., cellulose, proteins, etc.). A symbiotic relationship has been reported between most Bacteroidetes and Patescibacteria species, whereas Firmicutes species can survive independently. In addition, species contrasts could be dissected using Fisher’s correct test with 95% certainty intervals. The results are shown in Figure 7a,d,e. Significant changes were seen at the phylum level for Firmicutes, Bacteroidetes, and Patescibacteria, explaining that AFEX + H2O2 pretreatment optimized the microorganisms of the phylum.

The relative plenitude of microbial genera inside the mesophilic reactor is shown in Figure 6b. At the genus level, the microbial communities were similar for all samples, with 16–20 genera showing abundances above 1%. Of these, the abundance of Clostridium sensu stricto 1 expanded by 13, 11, and 2% individually after pretreatment. The abundance of Anaerocolumna, on the other hand, increased only slightly. Clostridium, a typical genus of dark-fermenting hydrogen-producing bacteria, can produce hydrogen from monosaccharides (glucose and xylose). The Clostridium sensu stricto 1 was found to be the predominant hydrogen-producing bacterium in biohydrogen production systems positively correlated with substrate utilization and SMPs, similar to previous studies. Fisher’s precise test results showed that pretreatment optimizes the microorganisms at the genus level (Figure 7b,d,e). However, another phenomenon could be observed through the heat map. The abundance of Clostridium butyricum was higher within the treated than within the untreated gather. High hydrogen production activity was obtained in the reactor with C. butyricum. Aly et al. reported a high positive correlation between C. butyricum and H2 and H2u content. This suggests that pretreatment greatly improved...
the interaction between the dominant microorganisms, leading to a more butyric acid-directed fermentation pathway. This shows more thermodynamic advantages.

The results of the medium-temperature fermentation showed that the AFEX + H2O2 pretreated material had a higher hydrogen production and significantly improved the microbial community structure. In terms of the different materials, the AFEX + H2O2 pretreatment was more suitable for herbaceous plants compared to hardwood and softwood.

4. CONCLUSIONS
AFEX + H2O2 pretreatment was a viable strategy for the removal of lignin from herbaceous biomass, such as rice straw, and could substantially promote its enzymatic saccharification efficiency. However, it was less effective for woody biomass, such as hardwoods and softwoods. Microstructural changes such as SEM, X-ray diffraction (XRD), and 13C NMR indicated that lignin may be associated with the intact cellular morphology, which is maintained during pretreatment, thus blocking cellulase access to cellulose and reducing its saccharification efficiency. Most importantly, AFEX + H2O2 significantly improved the hydrogen production performance of dark fermentation of agroforestry waste. The CHP of rice straw, poplar, and pine was 146.65, 80.75, and 57.52 mL/g. Moreover, the increased abundance of C. butyricum contributed to the H2 production by the butyric acid-type fermentation pathway.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c00598.

FTIR and XRD profiles of straw, poplar, and pine after AFEX + H2O2 pretreatment and effect of different materials on the relative abundance thermograms of the first 30 microbial species (PDF)

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Notes
The authors declare no competing financial interest.

ACKNOWLEDGMENTS
This work was supported by the major projects of tackling key industrial of Shandong’s New-Traditional Kinetic Energy Conversion and the Shandong Province Key R&D Program (major science and technology innovation project): 2021CXGC010802.

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