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Effects of Colloid Milling and Hot-Water Pretreatment on Physical Properties and Enzymatic Digestibility of Oak Wood

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Abstract: A two-step process using colloid milling (CM) and hot water (HW) treatment was evaluated for its ability to improve xylose recovery and the enzymatic digestibility of oak wood. In the first step, CM pretreatment was applied at a milling (feeding) speed of 100 mL/min with four different milling times (3, 6, 9, and 12 h), and the enzymatic digestibility and physical properties of each substrate were measured. In the second step, the HW pretreatment was applied to enhance the enzymatic digestibility and xylan recovery at various reaction severities (Log R0) from 2.07 to 4.43 using 12 h colloid-milled (CM-treated) oak wood. Compared with untreated oak wood, CM not only significantly disrupted the structure of oak wood but also increased its Brunauer–Emmett–Teller surface area (42-fold) and pore volume (28-fold). The crystallinity of two-step-treated oak wood was decreased to 34.8, while the enzymatic digestibility of 12 h CM-treated oak wood was increased to 58.1% at enzyme loading of 30 filter paper units (FPU)/g glucan for 96 h. After HW treatment of CM-treated oak wood at Log R0 = 3.83, 80.7% of xylan recovery yield and 91.1% of enzymatic digestibility (with 15 FPU/g glucan at 96 h) was obtained, which was 84.2% higher than the enzymatic digestibility of untreated oak wood (6.9%).

Keywords: fractionation; woody biomass; super-colloid mill; hydrothermal pretreatment; multi-step processing

1. Introduction

In native lignocellulosic biomass (LCB), lignin and carbohydrates (i.e., cellulose and hemicellulose) are connected via a covalent- and hydrogen-bonding network with benzyl ester, benzyl ether, and phenyl glycoside functional groups [1]. LCB is inherently resistant to breakage by enzymatic degradation and microbial attack owing to its physical and chemical barriers. Barriers against enzymatic saccharification, such as the highly ordered crystalline structure of cellulose, steric interference by hemicellulose, and irreversible chemical interference of lignin, make them resistant to enzymatic degradation or microbial attack and oxidation but inhibit the enzymatic hydrolysis of LCB [2]. In addition, lignin polymers have various functional groups, such as methoxy, aliphatic/aromatic hydroxy, benzyl alcohol, ether, non-cyclic benzyl ether, and carbonyls, which are disadvantageous for enzymatic hydrolysis [3]. The main purpose of pretreatment is to break down the physical and chemical barriers of LCB and improve enzymatic digestibility, which enables the efficient bioconversion of LCB to bioproducts [4,5]. The ideal pretreatment method generally should (1) increase the surface area (i.e., porosity and pore size), (2) decrease the cellulose crystallinity, (3) depolymerize cellulose, hemicellulose, and lignin, in particular, breaking down the lignin-carbohydrate complex (LCC), (4) minimize carbohydrate loss, (5) minimize the formation of inhibitors that negatively affect the activity of fermentation microorganisms, (6) reduce the energy demand, and (7) discount the capital and operating costs [6–8].
Among the technically feasible pretreatment methods, the hot water (HW) pretreatment method has been regarded as promising for LCBs because water can be easily recovered and recycled without generating waste [9]. During the hot-water hydrolysis reaction, hydronium ions were generated from water by increasing the temperature [10]. Hydronium ions cleave the heterocyclic ether bonds and acetyl groups of hemicellulose [11]. Further, the acetic acid generated from the acetyl group of hemicellulose acts as an acid catalyst to accelerate the hydrolysis of LCB [12]. This series of processes is known as autohydrolysis. During the hydrothermal reaction, hemicellulose is first dissolved in the liquid hydrolysate and most of the cellulose is preserved as a solid residue. Under the hydrothermal reaction conditions, approximately 20–40% of lignin is typically removed and most of the insoluble lignin is retained in the residual solid [13,14]. Lignin undergoes simultaneous depolymerization and repolymerization, during which it is redistributed during the hydrothermal reaction to form “lignin droplets” on the solid surface, impeding enzyme accessibility to cellulose [15].

Meanwhile, no additional chemical catalysts are required in the HW pretreatment, which relies mostly on the acetyl and uronic groups of hemicellulose in the LCB and thereby reduces the cost of wastewater treatment [16]. Moreover, the pH of the liquid hydrolysate lies between 4 and 7, making it suitable for use in subsequent processes, such as enzymatic hydrolysis and fermentation [10]. Moreover, the non-corrosive advantage of the HW pretreatment process enables setting up a reactor for a scale-up facility at a lower cost compared with other pretreatment methods, such as acid and alkali pretreatment methods [10].

However, HW pretreatment operates at a high operating temperature (150–230 °C) and pressure range (>5 MPa) [17]. The reaction time ranges from a few seconds to several hours, depending on the reaction temperature and liquid-to-solid ratio (typically ~10) [10]. The high reaction temperature of HW pretreatment promotes the breakdown of carbohydrates and forms byproducts (formic acid, acetic acid, levulinic acid, 5-hydroxymethylfurfural (HMF), and furfural). These byproducts act as potent inhibitors in the subsequent conversion process using enzymes and microbes. One of the most crucial issues in the design of the HW pretreatment process is increasing the economic efficiency by reducing the severity of the reaction while ensuring a high pretreatment effect.

Mechanical pretreatment methods such as grinding, chipping, milling, or comminution are also considered environmentally friendly pretreatments because of their reduced chemical usage and reaction severity during the pretreatment process, which produces fewer byproducts compared with other chemical pretreatments using acid and alkaline reagents under severe reaction conditions [18–20]. Among the mechanical pretreatment methods, the colloid mill, a grinding equipment, can reduce the particle size of a solid suspended in a liquid or reduce the droplet size in emulsions. Colloid milling (CM) can increase the space between the cellulose microfibers of the LCB, which may increase the surface area and pore volume and decrease substrate crystallinity [21]. These physical changes can be expected to improve the enzymatic digestibility of LCB, resulting in improved bioconversion yield. In addition, CM has already been developed on an industrial scale (2–10 ton/h of standard capacity); therefore, it can be used immediately in industries. Despite the many advantages of CM, few pretreatment studies have used CM for LCB. However, CM requires relatively high energy consumption and a long treatment time; nevertheless, this drawback can be overcome by combining it with additional chemical pretreatment [22–24]. This combined method of physicochemical pretreatment using CM and hot water can be practical and easily applied to the industrial process. This is because the hot-water processing is considered safe and economical because it generates less wastewater, waste material and other toxic substances, and CM can be assumed to be a practical and eco-friendly method.

In this study, oak wood was treated by a two-step process of CM treatment followed by HW treatment. Oak wood is regarded as an ideal renewable source for bioproduct production owing to its abundance and rapid growth [25]. Furthermore, as a hardwood species, it contains high levels of acetyl groups and carbohydrate (approximately 60 wt.%) contents.
These acetyl groups are favorable for HW pretreatment owing to the autohydrolytic effect. The main purpose of this study was to evaluate a two-step pretreatment process using CM followed by HW pretreatment. In the first step, deionized water (DIW)-based CM pretreatment was applied as a mechanical pretreatment to disrupt the rigid structure of the LCB. In the second step, HW pretreatment was applied under various reaction conditions to enhance enzymatic hydrolysis of the substrate and separate xylose at high concentrations. To evaluate the effect of each step, enzymatic hydrolysis, compositional analysis of residual solid and liquid hydrolysate, and physical property analyses such as scanning electron microscopy (SEM), Brunauer–Emmett–Teller (BET), and X-ray diffraction (XRD) were performed.

2. Materials and Methods

2.1. Materials

Oak wood grown and harvested in Korea was purchased from Sinwooimsan (Hongcheon-gun, Gangwon-do, Korea) in 2019. It was ground and sieved to 60–35 mesh size (0.25–0.5 mm). After sieving, oak wood powder was stored in a tightly sealed plastic container. The average moisture content of ground oak was 6.8%. The composition of oak wood was determined following the Laboratory Analytical Procedure (LAP) provided by the National Renewable Energy Laboratory (NREL, Golden, CO, USA) [26–28]. The chemical composition of untreated oak wood was 41.2% glucan, 15.7% xylan, 1.7% galactan, 0.8% arabinan, 1.0% mannan, 26.0% acid-insoluble lignin (AIL), and 4.9% acid soluble lignin (ASL). All compositional analyses were performed in triplicate. α-Cellulose (cat. no. C8002), citric acid monohydrate (cat. no. C1909), sodium citrate dihydrate (cat. no. W302600), and sodium azide (cat. no. 71290) were purchased from Sigma-Aldrich (Yongin, Gyeonggi-do, Korea). A commercial cellulase enzyme (Cellic\textsuperscript{®}CTec2; Novozymes Inc., A/S Bagsvaerd, Denmark) was used for the enzymatic digestibility test.

2.2. Colloid Milling (CM) Pretreatment

The untreated oak wood was treated with a SuperMass Colloider (model MKCA6-5J, Masuko Co., Kawaguchi, Japan) using an aluminum oxide grinder (model MKGA 6-120, Masuko Co., Kawaguchi, Japan). Untreated oak wood was dispersed in 6 L DIW (liquid/solid (L/S) = 20) and poured slowly into the colloid mill inlet. The interval between the two discs was adjusted to $-100\ \mu m$ and operation was conducted at a grinding stone spinning speed of 1500 rpm. One milling cycle was defined as 6.0 L of solution milled at a milling speed of 100 mL/min; therefore, one milling cycle was equivalent to 1 h of milling time. At every third cycle (i.e., 3, 6, 9, and 12 cycles or 3, 6, 9, and 12 h), a certain amount of ground sample was collected to determine the physical properties and enzymatic digestibility. To minimize structural changes, all samples were dried using a freeze dryer (model FDTA-4508, Operon Co., Ltd., Gimpo-si, Gyeonggi-do, Korea) after milling and then stored in a desiccator for the following process.

2.3. Hot Water (HW) Pretreatment

Untreated and 12 h colloid-milled (CM-treated) oak wood were treated using a stainless-steel batch reactor for HW pretreatment under various reaction conditions: temperature (120, 150, and 180 °C), time (30, 60, 90, and 120 min), and L/S = 10. The stainless steel batch reactor comprised a preheating bath (molten salt bath), reaction baths (silicone oil baths), and a cooling bath (water bath). The molten salt bath was set to 250 °C to minimize the preheating time required to reach the target temperature. The silicone oil and water bath temperatures were adjusted to the target temperature and 30 °C, respectively. Then, 4.5 g of biomass and 45.0 mL of DIW were packed into a bomb tubular-type reactor. The reactor was placed in a molten salt bath for preheating. When the reactor reached the target temperature in the molten salt bath, it was transferred to the silicone oil bath to maintain the reaction temperature. After the reaction was terminated, the reactor was transferred to a water bath for cooling. The average preheating time was less than 3 min.
under all investigated reaction temperature conditions. The bomb tubular reactor comprised SS-316 L tubing with a 21.0 mm inner diameter (ID) and 150 mm length (52.0 cm$^3$ internal volume). The temperatures of the reaction baths and reactors were continuously measured and monitored using high-temperature thermocouples (cat. no. HY-72D, Korea Hanyoung Co. Ltd., Incheon, Korea). A timer and movement controller were used to control the reaction time and movement of the reactor, respectively. Upon completion of the reaction, liquid samples discharged from the reactor were separated into two portions. One portion was subjected to hydrolysis for monosaccharide determination. The other portion was analyzed to determine the concentrations of monosaccharides, oligosaccharides, and byproducts (formic acid, acetic acid, levulinic acid, 5-HMF, and furfural). The solid samples removed from the reactor were washed with DIW, filtered, dried (at 45 °C for 72 h), and then subjected to weight-loss measurements, composition analysis, and digestibility tests.

2.4. Enzymatic Digestibility Test

Enzymatic digestibility tests of untreated and pretreated oak were performed using NREL-LAP [29]. A digestibility test was performed at 50 °C, pH 4.8 (0.05 M sodium citrate buffer), and 150 rpm using a shaking incubator (model BF-175SI, BioFree Co., Ltd., Seoul, Korea). The enzyme-loading amount was 15 and 30 filter paper unit (FPU)/g glucan basis and the average activity of cellulase was measured to be 119.4 FPU/mL. The initial glucan concentration was 1.0% (w/v) based on 100 mL of total liquid in a 250 mL Erlenmeyer flask. To prevent microbial contamination, 1.0 mL of sodium azide (20 mg/mL) was added. Samples were taken at the appropriate times (0, 3, 6, 9, 12, 24, 48, 72, and 96 h) and analyzed for glucose hydrolysis using the high-performance liquid chromatography (HPLC) system. Enzymatic digestibility was calculated using the following equations (0.9 is the conversion factor for considering monomeric glucose in the liquid sample to glucan). The α-cellulose and untreated (cutter milled only) samples were included as reference and control for digestibility under the same conditions.

$$\text{glucan digestibility (wt%)} = \frac{\text{Total released glucose (g) } \times 0.9}{\text{Initial glucan loading (g)}} \times 100$$ (1)

2.5. Composition Analysis of Untreated and Treated Oak Wood

The chemical compositions of the solid and liquid samples were determined according to the NREL-LAP procedure [27,28,30]. For untreated oak wood, the extractions were performed in two consecutive steps using water and ethanol. For the composition analysis of the extractive-free and treated solids, two-step acid hydrolysis was conducted to determine the carbohydrate (sugar) and lignin contents.

An HPLC system (model LC-20A, Shimadzu Inc., Kyoto, Japan) with a refractive index detector (RID-20A, Shimadzu Inc., Kyoto, Japan) was used to determine the carbohydrate and byproduct contents. Monomeric sugars from the untreated and treated samples were determined using a carbohydrate analysis column (Aminex HPX-87P, Bio-Rad Inc., Hercules, CA, USA). HPLC-grade water was used as the mobile phase at a flow rate of 0.6 mL/min. The temperatures of the detector and column oven were 50 °C and 85 °C, respectively. The byproducts and sugars in the liquid hydrolysate were analyzed using an organic acid analysis column (Aminex HPX-87H; Bio-Rad Inc., Hercules, CA, USA). The 5 mM sulfuric acid solution was used as the mobile phase at a flow rate of 0.5 mL/min. The temperatures of the detector and column oven were 50 °C and 65 °C, respectively.

2.6. Reaction Severity

In this study, we applied Log $R_0$ to present the extraction behaviors of glucan and xylan after pretreatment. The concept of reaction severity, which combines the reaction
time and temperature, is often used in biomass research. The severity factor was defined by Overend and Chormet [31] as follows.

\[ \log R_0 = \log \left[ t \times e^{\left(\frac{T - 100}{14.75}\right)} \right] \]  

(2)

where \( t \) is the time (min), \( T \) is the temperature (°C), and 14.75 is an empirical parameter related to activation energy and temperature. Table 1 summarizes the reaction conditions and reaction severities (Log \( R_0 \)) tested in this study.

Table 1. Reaction severities (Log \( R_0 \)) vs. reaction temperatures and times.

| Reaction Conditions | Log \( R_0 \) |
|---------------------|--------------|
|                     | Temperature (°C) | Time (min) |
|                     | 120           | 30          | 2.07 |
|                     |               | 60          | 2.37 |
|                     |               | 90          | 2.54 |
|                     |               | 120         | 2.67 |
|                     | 150           | 30          | 2.95 |
|                     |               | 60          | 3.25 |
|                     |               | 90          | 3.43 |
|                     |               | 120         | 3.55 |
|                     | 180           | 30          | 3.83 |
|                     |               | 60          | 4.13 |
|                     |               | 90          | 4.31 |
|                     |               | 120         | 4.43 |

2.7. Gel Filtration Chromatography (GFC) Analysis

The molecular weight (MW) distribution of the liquid hydrolysate separated via HW pretreatment was measured using a gel filtration chromatography (GFC) system (model LC-10A, Shimadzu Inc., Kyoto, Japan). An aqueous SEC (GFC) column (Shodex SB-802.5 HQ, Showa Denko, Tokyo, Japan), guard column (Shodex SB-G 6 B, Showa Denko, Tokyo, Japan), and a refractive index (RI) detector (model RID-10A, Shimadzu Inc., Kyoto, Japan) were used for the analysis. The operating temperatures of the column and detector were 40 °C and 50 °C, respectively. DIW was used as the mobile phase with a volumetric flow rate of 1.0 mL/min. Before the GFC analysis, the samples were filtered with a 0.2 µm pore size syringe filter.

2.8. Physical Property Analysis

The surface morphologies of untreated and CM-treated oak wood were analyzed using scanning electron microscopy (SEM, model CX-200, COXEM Co., Ltd., Daejeon, Korea). SEM observations were conducted at an accelerating voltage of 20.0 kV and 300 × magnification.

To determine the surface area, pore size, and pore volume of the substrates, the Brunauer–Emmett–Teller (BET) and Berrett–Joyner–Halenda (BJH) models were used with a BET surface analyzer (Tristar II 3020, Micromeritics Instrument Corp.) and pretreatment equipment (VacPrep 061, Micromeritics instrument Corp.). The freeze-dried samples were degasified under vacuum for 12 h at 100 °C before analysis via nitrogen adsorption.

The crystallinity of the untreated and CM-treated oak wood was determined via XRD (Rigaku Co., Japan) under the operating conditions of 40 kV and 40 mA. The samples were scanned at 4 °C/min (2θ = 10–35°, 0.02 increments). The crystallinity index (CrI) of the samples was calculated using the following equation [32]:

\[ \text{CrI} = \left( \frac{I_{002} - I_{18}}{I_{002}} \right) \times 100 \]  

(3)
where $I_{002}$ is the peak intensity corresponding to the 002 lattice plane of cellulose molecules observed at $2\theta = 22.5^\circ$ and $I_{18}$ (at $2\theta = 18^\circ$) is the peak intensity corresponding to amorphous cellulose.

3. Results and Discussion

3.1. Colloid Milling (CM) Treatment: First-Step

CM pretreatment was performed for four different milling times (3, 6, 9, and 12). To evaluate the effect of CM pretreatment, treated and untreated oak woods were subjected to an enzymatic digestibility test at cellulase loadings of 15 and 30 FPU/g glucan (Figure 1). The enzymatic digestibility (at 96 h) of untreated oak wood with 15 and 30 FPU/g glucan enzyme loadings was 6.9% and 8.4%, respectively. Conversely, 96 h enzymatic digestibility of CM-treated oak wood at all enzyme loadings was higher than 30%, regardless of milling time. Under 30 FPU/g glucan for 12 h, CM-treated oak wood demonstrated the highest enzyme digestibility of 58.1% at 96 h of enzymatic hydrolysis. Furthermore, the hydrolysis rate of the CM-treated biomass during the first 9 h was much higher than that of the untreated biomass, which was similar to that of $\alpha$-cellulose (Figure 1a). The change in digestibility occurred as the CM treatment time increased gradually, regardless of enzyme loading (15 and 30 FPU/g glucan).

![Figure 1](image)

(a) Enzymatic digestibility of untreated and CM-treated oak wood with (a) 15 FPU/g glucan and (b) 30 FPU/g glucan.

The improved digestibility of CM-treated oak wood may be attributed to a structural change in the substrate caused by CM pretreatment. Several analyses were carried out on untreated and CM-treated oak wood to determine the physical changes in the treated samples. The structural changes in the untreated and CM-treated oak wood samples were imaged using SEM, as shown in Figure 2. Figure 2a indicates that the surface of untreated oak wood has a flat and rigid structure, but the CM-treated oak wood has a significantly disordered and disrupted structure. With increasing milling time, the observed structure was more disordered (Figure 2b–e); the structure of the 12 h CM-treated oak wood (Figure 2e) showed a reticulated morphology with a considerably exposed surface. A severely exposed substrate surface enhances the enzymatic accessibility of cellulose, which has been speculated to also enhance the enzymatic hydrolysis rate and yield [9].
Figure 2. SEM images of untreated and CM-treated oak wood under 5000× magnification, (a) untreated oak wood, (b) CM-treated oak wood (3 h), (c) CM-treated oak wood (6 h), (d) CM-treated oak wood (9 h), and (e) CM-treated oak wood (12 h).

BET surface area, pore size, and pore volume analyses were performed to quantitatively evaluate the improved surface area; the results are shown in Table 2 and Figure 3. The BET surface area of untreated oak wood was measured to be 0.390 m²/g, while that of CM-treated oak wood was between 6.528 m²/g and 16.464 m²/g depending on the milling time (i.e., 3, 6, 9, and 12 h). These results indicate that CM pretreatment improved the surface area of oak wood by up to 42-fold. In addition, the pore volume significantly
increased from $2.055 \times 10^{-3}$ cm$^3$/g (untreated oak wood) to $57.800 \times 10^{-3}$ cm$^3$/g (12 h CM-treated oak wood). Even after only 3 h of CM treatment, the surface area and pore volume increased by 16.7-fold and 15-fold, respectively. However, the average pore size decreased from 65.282 nm (untreated oak wood) to 18.689 nm (12 h CM-treated oak wood) because more pretreatments may result in reduced particle size and thus pore size.

Table 2. Surface area, pore size and pore volume of untreated and CM-treated oak wood.

| Samples                  | Milling Time | Surface Area m$^2$/g | Pore Size nm | Pore Volume $\times 10^{-3}$ cm$^3$/g |
|--------------------------|--------------|-----------------------|--------------|--------------------------------------|
| Untreated                | -            | 0.3904                | 65.2820      | 2.0550                               |
| CM-treated oak wood      | 3 h          | 6.5276                | 28.8735      | 30.9220                              |
|                          | 6 h          | 11.9558               | 22.8739      | 52.5630                              |
|                          | 9 h          | 14.1727               | 20.3799      | 55.8180                              |
|                          | 12 h         | 16.4635               | 18.6886      | 57.8000                              |

Figure 3. N$_2$ physisorption isotherms of untreated and CM-treated oak wood.

The Cr/I was measured to evaluate the changes in physical structure. According to the milling time, the Cr/I of untreated oak wood was 48.5, while that of CM-treated oak wood decreased to 34.8 (Figure 4). The decrease in Cr/I was attributed to an increase in the amorphous portion of the LCB structure. In general, the amorphous part tends to be more easily hydrolyzed by enzymes than the crystalline portion [33,34]. It is probable that as the reaction was completed, the amorphous part of the substrate decreased, and only the crystalline part remained, thereby reducing the hydrolysis rate. Therefore, an increase in the amorphous portion of the substrate improves both the rate and yield of the enzymatic hydrolysis. Consequently, CM pretreatment can result in significant structural changes in the substrate, improving the surface area and pore volume and reducing crystallinity, thereby improving enzymatic digestibility.

3.2. Hot Water (HW) Treatment: Second-Step
3.2.1. Effect of Reaction Severity on Xylan Recovery

In the second step of the two-step pretreatment, HW treatment was conducted under various reaction conditions using CM-treated oak wood, and the results were compared with those of HW treatment only (without CM pretreatment). The effects of the chemical compositions of the solid residue and liquid hydrolysate treated by HW pretreatment on the reaction severity are presented in Figure 5. The results demonstrate that two-step
pretreatment using CM followed by HW treatment was much more effective than the HW-only treatment, even at the same $\log R_0$. It was assumed that the combined pretreatment method with mechanical treatment (CM pretreatment) resulted in additional physical and structural changes in the biomass than the chemical-only treatment. This indicates that the combined pretreatment method of CM and HW can be more effective than the single-step pretreatment method. Meanwhile, Figure 5a shows that the glucan and lignin contents of the residual solids decreased as the reaction severity increased; nevertheless, they were well preserved (over 87%) under all reaction conditions. Xylan content also decreased with increasing reaction severity; however, unlike glucan and lignin content, a substantial decrease was observed in samples at $\log R_0 > 3.25$. The decrease in xylan at $\log R_0 = 3.25$ may be attributed to auto-hydrolysis reaction by acetic acid generated from hemicellulose during pretreatment. Figure 5c summarizes the byproduct concentrations of the liquid hydrolysate, which began to be notably generated at $\log R_0 = 3.25$, and then gradually increased as the reaction severity increased. The generation of acetic acid typically indicates the beginning of auto-hydrolysis.

Meanwhile, the xylose recovery yield increased with increasing reaction severity but decreased after resulting in the highest recovery yield at $\log R_0 = 3.83$ (Figure 5b). At $\log R_0 = 3.83$, xylose recovery yields of HW-treated and CM-HW-treated oak wood were found to be 72.1% and 80.7%, respectively. Conversely, the glucose content did not significantly increase with increasing reaction severity; instead, it exhibited a slight decrease when $\log R_0$ increased from 4.13 to 4.43. The decreased yields of glucose and xylose at a high $\log R_0$ was assumed to be a result of further decomposition owing to severe reaction conditions. As shown in Figure 5c, as the reaction severity increased, the formation of various byproducts increased. Among the byproducts, acetic acid (a byproduct of hemicellulose) and furfural (a decomposition product of C5 sugars) markedly increased, while formic acid (a decomposition product of C5 and C6 sugar) and 5-HMF (a decomposition product of C6 sugar) also increased slightly. Therefore, we conclude that the appropriate HW pretreatment $\log R_0$ for CM-HW-treated oak wood is 3.83 because of the higher glucan retention yield, xylose recovery yield, and minimal formation of the byproduct in CM-HW-treated oak wood.

Figure 4. Diffraction patterns and crystallinity indices (CrIs) of untreated and CM-treated oak wood.
3.2.2. Monosaccharide and Oligosaccharide in Liquid Hydrolysate

During HW pretreatment, cellulose and hemicellulose are mostly hydrolyzed as oligosaccharides (mainly glucooligosaccharide and xylooligosaccharide) in the liquid hydrolysate. The monosaccharide (xylose) and oligosaccharide (xylooligosaccharide) contents of the hydrolysate recovered from HW pretreatment and CM-HW pretreatment were analyzed and are presented in Figure 6. The oligosaccharide content of the CM-HW pretreatment hydrolysate was generally higher than that of the HW-treated hydrolysate. At $\log R_0 = 3.43$, 3.55, and 3.83, the oligosaccharide contents after HW pretreatment were 31.2%, 41.6%, and 62.5%, respectively. Conversely, at the equivalent $\log R_0$ values, the oligosaccharide contents of CM-HW pretreatment were 63.0%, 64.6%, and 65.4%, respectively. In both processes, the more severe the reaction conditions, the lower the oligosaccharide production and the greater the monosaccharide production.
Figure 6. Xylose and xylooligosaccharide contents of liquid hydrolysates (HW and HW-CM pretreatment; \( \text{Log} \ R_0 = 2.07-4.43 \)). (a) HW treatment and (b) HW-CM treatment. Reaction conditions: temperature 120 °C, 150 °C and 180 °C; reaction time 30 min, 60 min, 90 min, and 120 min; \( L/S = 10 \text{ mL/g} \).

The liquid hydrolysates obtained from CM-HW pretreatment at \( \text{Log} \ R_0 = 3.43, 3.55, \) and 3.83 were analyzed to determine the MW distribution by the GFC system, as shown in Figure 7. The highlighted range indicates a MW between 150 and 1400, which corresponds to the degree of polymerization between 1 and 10 xylose units (i.e., xylose: 150.13 g/mol, xylodecaose: 1393.17 g/mol). At \( \text{Log} \ R_0 = 3.83 \), the distribution of low MW (<150) was predominant compared with that of high MW (>1400), and the pretreatment at \( \text{Log} \ R_0 = 3.43 \) and 3.55 showed a contrasting trend. In this graph, low MW (<150) substances are assumed to be byproducts or decomposition products such as acetic acid (60.05 g/mol), formic acid (46.03 g/mol), furfural (96.08 g/mol), levulinic acid (116.11 g/mol), and 5-HMF (126.11 g/mol).

Figure 7. Molecular weight distribution of CM-HW-treated hydrolysate for reaction severity (\( \text{Log} \ R_0 \)) at 3.43, 3.55, and 3.83.
3.2.3. Enzymatic Digestibility Test of Untreated and Treated Oak Wood

To evaluate the enzymatic digestibility of HW-treated and CM-HW-treated oak wood at the reaction severities of $\text{Log } R_0 = 2.07, 2.54, 2.95, 3.43, 3.83$ and $4.31$, we conducted enzymatic hydrolysis tests following NREL LAP with enzyme loading of 15 FPU/g glucan for a hydrolysis time of 96 h (Figure 8). The digestibility of HW-treated oak wood did not exceed 30% under all reaction conditions. At the highest reaction severity ($\text{Log } R_0 = 4.13$), the digestibility of HW-treated oak wood reached only 29.1% at 96 h. Conversely, the digestibility of CM-HW-treated oak wood resulted in >58% at 96 h under all reaction conditions, and even the digestibility (at 96 h) of CM-HW-treated oak wood treated at both $\text{Log } R_0 = 3.83$ and $4.31$ was higher than that of $\alpha$-cellulose. After 96 h of hydrolysis, the digestibilities of CM-HW-treated oak wood (at $\text{Log } R_0 = 3.83$ and $4.31$) were 91.1%, 96.9%, and 78.7%, respectively. These yields are higher even compared to the enzymatic digestibilities using various chemical pretreatment methods (Table 3). However, the long reaction time of CM is still a disadvantage, which can be overcome by the optimization of treatment conditions (disc space, treatment time, and rotor speed).

![Figure 8. Enzymatic digestibility of untreated and CM-treated oak wood treated using HW with cellulase loading of 15 FPU/g glucan.](image)

Table 3. Previous study on enzymatic digestibility of oak wood using various pretreatment methods.

| Pretreatment Method | Conditions ( ) | Enzymatic Hydrolysis ( ) | Yield (%) |
|---------------------|----------------|--------------------------|-----------|
| Hot water           | 170 °C, 60 min | 60 FPU $^4$, 72 h $^5$   | 76.5 [35] |
| $\text{H}_2\text{SO}_4$ | 0.2 wt.%, 170 °C, 15 min | 60 FPU $^4$, 72 h $^5$   | 93.8 [35] |
| Ammonia             | 20 wt.%, 170 °C, 60 min | 60 FPU $^4$, 72 h $^5$   | 66.1 [35] |
| $\text{H}_2\text{O}_2$ | 3.2 wt.%, 170 °C, 60 min | 60 FPU $^4$, 72 h $^5$   | 76.5 [35] |
| [Emim][OAc] $^1$    | 98.0 wt.%, 45 °C, 40 min | 80 h (no information for enzyme loading) | 59.3 [36] |
| [NMMO] $^2$         | 85.0 wt.%, 130 °C, 120 min | 15 FPU $^4$, 24 h $^5$   | 72.2 [37] |
| CM-HW $^3$          | CM: 12 h, HW: 170 °C, 120 min | 15 FPU $^4$, 96 h $^5$   | 96.9 This work |

$^1$1-ethyl-3-methylimidazolium acetate, $^2$N-methylmorpholine-N-oxide, $^3$Colloid milling-hot water pretreatment, $^4$FPU/g glucan, $^5$hydrolysis time.

In general, xylan removal can be regarded as one of the key factors that improves enzyme digestibility by removing the steric hindrance of biomass and improving enzyme
accessibility to cellulose [35]. Figure 5a indicates that the xylan removal of HW-treated ($\log R_0 = 4.31$) and CM-HW-treated ($\log R_0 = 3.83$) oak wood was 85.5% and 83.5%, respectively. Although xylan removal was approximately at a similar level, the enzymatic digestibility of HW-treated and CM-HW-treated oak wood was 29.1% and 91.1%, respectively. Based on our characterization study of the physical structure/properties (Figures 2 and 4 and Table 2), this variation in enzymatic digestibility may be caused by structural changes in the CM-treated oak wood.

### 3.2.4. Overall Mass Balance

The overall mass balance for the production of sugars from oak wood using the two-process scheme is shown in Figure 9. Figure 9a shows the mass balance of the single-step pretreatment followed by enzyme saccharification, while Figure 9b shows a two-step pretreatment followed by enzyme saccharification. All mass balances were established based on 100 g of oak wood input for processing and 15 FPU/g glucan enzyme loading in the saccharification step. To calculate the mass balances, pretreatment conditions (at $\log R_0 = 3.83$) were adopted for both the HW-only and CM-HW treatments. In the HW-only treatment, 11.3 g of xylan was recovered in the liquid hydrolysate, and then 8.3 g of glucose was obtained after enzymatic hydrolysis of 100 g of oak wood. In the case of two-step pretreatment using the CM-HW process, 12.7 g of xylan was recovered in the liquid hydrolysate, and a considerable amount of glucose (34.8 g) was obtained by enzymatic hydrolysis because of a high saccharification yield (91.1%). Only 3.4 g of glucan remained in the residual solid, indicating that high-purity lignin can be obtained as a byproduct.

![Figure 9](image-url)

**Figure 9.** Mass balances of sugar production processes using two different pretreatment methods (a) HW-only pretreatment and (b) CM-HW pretreatment.
4. Conclusions

In this study, we evaluated the synergistic pretreatment effect of a two-step process using CM and HW treatment. The changes in physical properties such as surface area, pore volume, and crystallinity of a treated solid caused by CM were observed to be significant, such that the enzymatic digestibility of LCB was substantially enhanced from 6.9% for untreated oak wood to 91.1% for two-step pretreated oak wood. In addition, two-step pretreatment resulted in high glucan retention (92.7%) and xylan recovery (80.7%), while minimizing the degradation of carbohydrates. Therefore, CM pretreatment successfully overcame the shortcomings of the HW-only treatment under severe reaction conditions, such that it resulted in lower degradation of carbohydrates than in the HW-only treatment, while achieving higher 96 h glucan digestibility of the treated solid (91.1% of CM-HW-treated and 18.8% of HW-treated oak wood). Hence, combining CM and HW pretreatments can be regarded as an effective chemical-free pretreatment method for LCB conversion.

Author Contributions: T.H.K. (Tae Hoon Kim), as the first author, conducted all experiments, summarized the data, and drafted the manuscript. S.H.P. and T.D.T.L. conducted the experiments and analyzed the data. T.H.K. (Tae Hyun Kim) and K.K.O. contributed equally as co-corresponding authors; specifically, they designed the reactor system as well as the overall study and experiments, interpreted the results, wrote the manuscript, and finalized the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the Technology Development Program to Solve Climate Changes of the National Research Foundation (NRF), funded by the Ministry of Science and ICT (2017M1A2A2087627).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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