applied sciences

MDPI

Article

Drying Effect on Enzymatic Hydrolysis of Cellulose Associated with Porosity and Crystallinity

Bonwook Koo [1,*], Jaemin Jo [1] and Seong-Min Cho [2]

[1] Green and Sustainable Materials R & D Department, Korea Institute of Industrial Technology, Cheonan-si 31056, Korea; jjm1234@kitech.re.kr

[2] Department of Forest Sciences, Seoul National University, Seoul 08826, Korea; csmin93@snu.ac.kr

* Correspondence: bkoo@kitech.re.kr; Tel.: +82-41-589-8409

Received: 27 July 2020; Accepted: 7 August 2020; Published: 11 August 2020

Featured Application: Drying causes irreversible structural changes in cellulose and the changes are intimately associated with porosity, including pore volume and surface area. These changes must be considered for the application of cellulose in high value industry such as sustainable polymers that use cellulose nanofiber and sustainable sugar production.

Abstract: The effect of drying on the enzymatic hydrolysis of cellulose was determined by analysis of porosity and crystallinity. Fiber hornification induced by drying produced an irreversible reduction in pore volume due to shrinkage and pore collapse, and the decrease in porosity inhibited enzymatic hydrolysis. The drying effect index (DEI) was defined as the difference in enzymatic digestibility between oven- and never-dried pulp, and it was determined that more enzymes caused a higher DEI at the initial stage of enzymatic hydrolysis and the highest DEI was also observed at the earlier stages with higher enzyme dosage. However, there was no significant difference in the DEI with less enzymes because cellulose conversion to sugars during hydrolysis did not enhance enzymatic hydrolysis due to the decrease in enzyme activity. The water retention value (WRV) and Simons' staining were used to measure pore volume and to investigate the cause of the decrease in enzymatic hydrolysis. A decrease in enzyme accessibility induced by the collapse of enzymes' accessible larger pores was determined and this decreased the enzymatic hydrolysis. However, drying once did not cause any irreversible change in the crystalline structure, thus it seems there is no correlation between enzymatic digestibility and crystalline structure.

Keywords: drying effect; cellulose; enzymatic hydrolysis; hornification; porosity

1. Introduction

Biorefinery platforms using biomass have been studied widely in recent years because of their low carbon footprint [1]. The U.S. Department of Energy (DOE) announced the top 12 platform chemicals that can be produced from biomass and that it would consider the market and potential application of these in industry [2,3]. Most of the platform chemicals can be produced from sugars, which indicates that low cost sugar production from biomass is important to achieve a low carbon footprint [4]. Lignocellulosic biomass has been suggested as the feedstock for sustainable sugar production and various conversion processes including pretreatment and saccharification have been studied to facilitate effective sugar production from lignocellulosic biomass [5–7].

Sustainable sugar is mainly produced by enzymatic saccharification and pretreatment must be performed to improve enzyme accessibility to cellulose [8,9]. The pretreatment exposes cellulose by partial removal of hemicellulose and lignin, and it facilitates enzyme access to cellulose. After pretreatment, the exposed cellulose is likely to be partially dried and the cellulose drying affects its

enzymatic digestibility [10]. Once cellulose has been dried, the dimensions of the cellulose are changed due to the collapse of pores and shrinkage of the internal volume [11,12]. The structural change stiffens the cellulose via a process known as "hornification" [13].

The process of hornification is frequently explained as irreversible, or partially irreversible hydrogen bonding upon drying or water removal [14] due to the aggregation of the cellulose chains [15]. The cellulose chains are aggregated due to the removal of water by heating and then they are not able to fully open up to the next exposure to water [16]. The aggregation may collapse the pore structure, and thus a decrease in pore volume and surface area can be expected [12,17]. Crystallinity in cellulose fiber has been considered as one of the major properties altered by the aggregation [18]. It was reported that several cycles of drying and rewetting caused irreversible change in crystalline structure, which could not be recovered due to the growth of crystalline domains through co-crystallization [19]. However, no change in crystalline index by the recycling of paper has been reported [20].

It has been reported that pore volume is significantly decreased by the process of repetitive drying and rewetting. Repetitive recycling of delignified and alkali-extracted pulp was carried out by rewetting and drying, and it decreased the Brunauer–Emmett–Teller (BET) surface area and pore volume of cellulose, as measured by the water retention value (WRV). In a five-times recycling procedure, the BET surface area and the WRV decreased by 22.9% and 35.9%, respectively, compared with non-dried and rewet pulp [17].

In addition, in previous studies on the effect of drying, it was reported that hornification induced by drying affects enzymatic hydrolysis [21,22]. The enzymatic digestibility of dried substrate was significantly lower than that of never-dried substrate, and the reduction in enzymatic conversion was caused by a decrease in pore volume, which can be evaluated by WRV [22]. Hornification induced by drying caused the collapse of larger pores while increasing the number of smaller pores, which are not accessible to enzymes [21]. This suggests that the collapse or closure of larger pores could be a primary reason for a reduction in enzyme accessibility to cellulose, given the size of the cellulase core.

Although numerous studies have been performed to investigate the drying effect on enzymatic hydrolysis [23], little research on the effect with regard to enzyme dosage and the kinetics of hydrolysis has been performed. This study aims to investigate the drying effect on enzymatic hydrolysis at different enzyme dosages to determine the hydrolysis kinetics and the limitation of enzymatic digestibility. In addition, several properties of the cellulose structure including porosity, enzyme accessible surface area, and crystallinity were determined to investigate their effect on enzymatic hydrolysis.

2. Materials and Methods

2.1. Sample Preparation

A fully bleached and never-dried hardwood pulp was obtained from a mill in the southeast of the United States and used in this study. The pulp was washed thoroughly with plenty of tap water. Handsheets were made using the washed pulp to prevent the formation of fiber flocks, which might affect the enzymatic digestibility of pulp due to their effect on the enzyme-accessible surface area. It has been reported that fiber bundles in a wet state can easily form very strong fiber flocks of varying size upon drying due to inter- and intra-fiber hydrogen bonding [22]. The washed hardwood pulp, which weighed about 2.0 g was disintegrated in a standard disintegrator (Lorentzen & Wettre, Kista, Sweden) for 5 min with 5% solid consistency at room temperature and then diluted to 1% solid consistency with deionized water. The diluted pulp suspension was used to make handsheets according to the TAPPI Standard method, T205 sp-95 [24] using a standard laboratory handsheet mold. The weight was 2.0 g per sheet (based on the oven-dried weight) and the handsheets were stored in a cold room at 4 °C using a plastic bag to prevent biological decomposition and drying.

2.2. Compositional Analysis

The compositional analysis of the pulp was performed according to NREL (National Renewable Energy Laboratory) Standard Procedures [25]. Sulfuric acid hydrolysis w performed in two stages with 72% and 4% of sulfuric acid. First, the pulp was hydrolyzed with 72% of sulfuric acid at 30 °C for 1 hr and then the acid was diluted to 4% for the second hydrolysis at 121 °C for 1 hr. After two stages of sulfuric acid hydrolysis, the hydrolysate from the pulp was filtrated by a 0.2 μm Milipore filter and analyzed by high-performance liquid chromatography (Agilent 1200; Agilent Technologies, Palo Alto, CA, USA) to measure the amount of structural sugars. The sugars were separated with a Shodex SP0810 column at a temperature of 80 °C with a flow rate of 0.5 mL/min using deionized water as an eluent. A refractive index detector was used to quantify the arabinose, galactose, glucose, xylose, and mannose in the hydrolysates. The chemical composition of the pulp is shown in Table 1.

Table 1. Chemical composition of the bleached hardwood pulp.

Carbohydrates						Lignin			Ash	Total Mass Balance (%)
Glu	Xyl	Gal	Ara	Man	Total	KL	ASL	Total		
79.5	16.4	ND	ND	ND	95.9	0.4	0.5	0.9	0.3	97.1

All values were calculated based on the oven-dried weight of pulp; Glu: glucan; Xyl: xylan; Man: mannan; Gal: galactan; Ara: arabinan; KL: Klason lignin; ASL: acid soluble lignin; ND: not detected.

2.3. Drying and Rewetting of the Pulp

The handsheets were subject to drying and rewet to induce fiber hornification. Two different drying methods, oven and freeze drying, were applied to determine the drying effect on enzymatic hydrolysis. Oven drying of the handsheets was conducted in a drying oven at 105 °C for 24 h and freeze drying was conducted at −40 °C in a freeze dryer for 48 h. After each drying, the dried handsheets were stored in plastic bags for enzymatic hydrolysis and analysis of those properties. Rewetting the dried pulp was performed by disintegration for 5 min at 5% of consistency and then the water was drained out by centrifuge. Rewetted pulp fibers were also stored in a plastic bag and used for enzymatic hydrolysis and analysis of those properties.

2.4. Enzymatic Hydrolysis

Commercial cellulase including β-glucosidase (C-tec2) and a xylanase (H-tec2) provided by Novozymes (Franklinton, NC, USA), were used for enzymatic hydrolysis. Cellulase dosages of 4, 10 and 20 FPU per g of pulp (5.2, 13.3 and 26.7 enzyme protein mg per g of pulp) and a ninth of the xylanase were used to determine the drying effect depending on enzyme dosage. Enzymatic hydrolysis was performed in 20 mL of 100 mM acetate buffer (pH 5.0) at a 5% (*w/v*) solids loading. The substrates and enzymes were incubated in a shaker at 50 °C and 180 rpm for 120 h. The enzymatic digestibility was determined in duplicate by the amount of sugars released during enzymatic hydrolysis based on the amount of structural sugars in the original pulp. The amount of released sugars in the enzymatic hydrolysate was quantified by the HPLC (high pressure liquid chromatography) [26].

2.5. Analysis of Pulp Properties

2.5.1. Porosity by Water Retention Value and Simons' Staining Method

When pulp fibers are dried, internal fiber volume shrinks [11] and the shrinkage prevents the accurate measurement of porosity due to the pore collapse caused by drying. Thus, the measurement needs to be done in a wet state [27] and two independent methods, water retention value (WRV) and Simon's staining method were selected to evaluate the porosity of pulp in wet state.

The WRV has been used to estimate the fiber saturation point, which correlates to the amount of water in the cell wall pores or the volume of pores, and it has been found that the drying effect induced by fiber hornification is reflected in a reduction in the WRV [22]. The WRV was determined using the

TAPPI Useful method with a centrifugal force (Eppendorf North America, Hauppauge, NY, USA) of 900 rcf (2400 rpm) for 30 min [28]. The centrifuged wet pulp was first weighed and then oven dried at 105 °C overnight. The WRV was calculated by the percentage of water retained in the dried pulp, i.e.,

$$WRV\ (\%) = [(W_{wet}\text{-}W_{dried})/W_{dried}] * 100 \quad (1)$$

in which:

- W_{wet}: weight of wet pulp
- W_{dried}: weight of dried pulp

In addition to the WRV, the Simon's staining method was performed to analyze the porosity, including the enzyme accessible surface area of the pulp, depending on the drying method. For the Simons' staining method, the 1:1 staining method were used in this study [29]. Blue dye (DB) and orange dye (DO) were dissolved in nanopure water for the preparation of each dye solution and the final concentrations of DB and DO were 1% (*w/v*), respectively. For the orange dye, only higher molecular fractions were used after the fractionation. The dye solution was then prepared with 1 mL of the DB solution, 1 mL of the DO solution, 10 mL of 10% (*w/v*) NaCl and 70 mL of nanopure water. For staining, a 25 mg of sample (dry weight) in the dye solution was incubated in a 75 °C shaking incubator at 200 rpm for 48 h. The stained samples were then filtered, rinsed with a minimum amount of distilled water and stripped with 25% (*w/v*) aqueous pyridine at 45 °C for 18 h. The dye stripping solution was then analyzed using a UV-Vis spectrophotometer to determine the concentration of DO and DB (the maximum absorbance of DB and DO was at 624 and 455 nm, respectively). The concentration of DO and DB dyes in the dye stripping solution (C_O and C_B) was determined using the following two equations, Equations (2) and (3) (using the Lambert–Beer law for a binary mixture), which were solved simultaneously [30].

$$A_{455nm} = \varepsilon_{O/455}\, LC_O + \varepsilon_{B/455}\, LC_B \quad (2)$$

$$A_{624nm} = \varepsilon_{O/624}\, LC_O + \varepsilon_{B/624}\, LC_B \quad (3)$$

in which:

- A: absorbance
- ε: absorptivity
- $\varepsilon_{O/455}$ = 35.62, $\varepsilon_{B/455}$ = 2.59, $\varepsilon_{O/624}$ = 0.19, $\varepsilon_{B/624}$ = 15.62 $L{\cdot}g^{-1}{\cdot}cm^{-1}$ and L = 1 cm

2.5.2. Crystallinity by ^{13}C CPMAS Solid-State NMR Analysis

High-resolution ^{13}C CPMAS NMR spectra were collected by a Bruker Avance 200 MHz spectrometer (Bruker BioSpin Corporation, Billerica, MA, USA) with a 7 mm probe, operating at 50.13 MHz for ^{13}C, at room temperature. The spinning speed was 7000 Hz, contact pulse 2 ms, acquisition time 51.3 ms and delay between pulses was 4 s, with 20,000 scans. The adamantine peak was used as an external reference (δC 38.3 ppm). The samples were hydrated with deionized water (ca. 43%) before recording the spectra. The ^{13}C chemical shifts were given in δ values (ppm) and each peak was assigned according to the values in the literature [19]: C6-amorphous (61.3 to 62.1 ppm), C6-crystalline (65.2 to 65.8 ppm), C2, C3 and C5 (71.2 to 75.6 ppm), C4-amorphous (83.5 to 84.4 ppm), C4-crystalline (88.2 to 89.6 ppm), and C1 (104.2 to 107.8 ppm).

3. Results and Discussion

3.1. Drygin Effect on Enzymatic Hydrolysis

The effect of drying on enzymatic hydrolysis was evaluated by enzymatic digestibility, which was determined by the amount of sugars released during enzymatic hydrolysis with 10 FPU/g-pulp of cellulase. It was calculated based on the amount of structural sugars in the original pulp.

Compared with oven-dried (OD) pulp, the enzymatic digestibility of never-dried (ND) pulp increased rapidly in the initial stage of the hydrolysis, but the hydrolysis rate of the ND pulp reduced gradually as the hydrolysis proceeded (Figure 1b). A decrease in hydrolysis rate has been ascribed to several factors such as the transformation of cellulose into a structurally resistant form, inhibition of enzyme action by accumulated products, and the completeness of hydrolysis [31]. Here, it seems that the decrease in hydrolysis rate of the ND pulp can be attributed to the completeness of the hydrolysis because the digestibility leveled off at 90%. Though the enzymatic digestibility of freeze-dried (FD) pulp was slightly lower than that of the ND pulp, a similar trend was observed in the hydrolysis of the FD pulp. This suggested that freeze drying caused mild hornification, unlike oven drying [21].

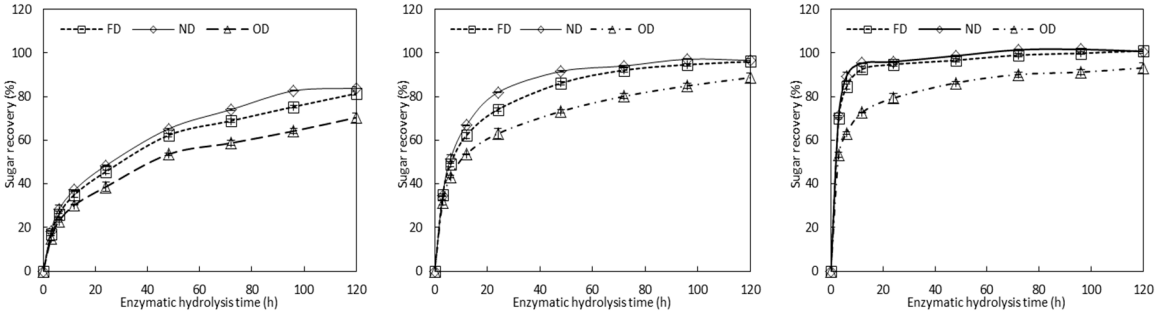

Figure 1. Enzymatic digestibility (sugar recovery) depending on the enzyme dosage of (**a**) 4 FPU/g biomass, (**b**) 10 FPU/g biomass, and (**c**) 20 FPU/g biomass.

Enzymatic hydrolysis of the ND pulp showed the highest digestibility (96.6%) when the hydrolysis was carried out with 10 FPU of cellulase for 120 h. However, the OD pulp showed significantly low digestibility though that of the FD pulp was almost similar to that of the ND pulp (96.1%) (Figure 1b). Pore volume has been considered as one of the most important properties affecting enzymatic hydrolysis [32,33] and fiber hornification induced by drying produces irreversible reduction in pore volume due to shrinkage and collapse of pores [22,32]. The reduction in pore volume decreases enzyme accessibility [22] and it reduces enzymatic digestibility due to less enzyme accessible surface area [32]. Therefore, the difference in enzymatic digestibility depending on drying, as shown in Figure 1b, should be caused by the reduction in pore volume induced by drying. However, there was a significant difference in the enzymatic digestibility of the FD and OD pulp. Again, it was considered that the better hydrolyzability of the FD pulp compared to the OD pulp, was due to the milder hornification induced by freeze drying [21]. The mild hornification can be explained by the rigidity of fibers at initial freezing [34]. The freezing gave a sort of rigidity to the fibers and this maintained the pore structure, thus minimizing pore collapse during drying. Therefore, enzymatic hydrolysis of the FD pulp showed better hydrolyzability compared to the OD pulp.

The difference in digestibility between the ND and OD pulp decreased gradually as the hydrolysis progressed because the digestibility of the OD pulp kept increasing while that of the ND pulp had already leveled off. The OD pulp had less pore volume due to fiber hornification, and thus there was a big difference in enzymatic digestibility compared to the ND pulp in the initial stages [17]. However new pores were formed by cellulose conversion to sugars during hydrolysis and this can give enzyme the space to access remaining cellulose [35]. Thus, the difference in digestibility between the ND and OD pulps was reduced gradually as the hydrolysis progressed. It is considered that the lower enzymatic digestibility of the OD pulp at the initial stage was induced by pore collapse; however, it can be recovered at a later stage because the pore volume increased due to cellulose conversion to sugars during enzymatic hydrolysis, even though hornification leads to irreversible or partial irreversible collapse of pore and it causes the difference in digestibility at the final stage of hydrolysis [32]. In order to determine the kinetics of enzymatic hydrolysis, the Langmuir equation style fitting was used, which

enabled the prediction of the final digestibility after 120 h of enzymatic hydrolysis. The Langmuir equation style for the determination is as follows:

$$S_t = (S_{max} \cdot t)/(k + t) \quad (4)$$

where S_t is enzymatic digestibility at a time, S_{max} is the maximum digestibility predicted (%), k is the Langmuir style equilibrium constant and t is time (h). This equation can be re-organized by the Lineweaver–Burk method to linearize the rate of expression as follows:

$$1/S_t = k \cdot (1/S_{max}) \cdot (1/t) + 1/S_{max} \quad (5)$$

The kinetic parameters were inferred with an intercept at $1/S_{max}$ and a slope of $k \cdot (1/S_{max})$ and are summarized in Table 2. The predicted final digestibility of the OD pulp with 10 FPU was 82.6%, which was less than that of the ND pulp (102.0%) (Table 2). It was proved that there was a big difference between the ND and OD pulps with regard to enzyme accessibility. The difference was caused by the irreversible transformation of cellulose into a structurally resistant form by hornification and the inhibition of enzyme action by accumulated products [31]. All of the predicted digestibilities depending on enzyme dosage and drying, were similar to the real values.

Table 2. The prediction of final enzymatic digestibility depending on enzyme dosage and drying.

	Never Dried			Freeze Dried			Oven Dried		
FPU/g-Pulp	**4**	**10**	**20**	**4**	**10**	**20**	**4**	**10**	**20**
S_{max}	78.1	102.0	103.1	74.1	95.2	101.0	62.9	82.6	90.1
K	10.1	5.9	1.2	10.5	5.4	1.3	10.0	5.1	2.2

3.2. Drying Effect Depending on Enzyme Dosage

The drying effect was determined depending on enzyme dosage. Enzymatic hydrolysis was performed with 4 FPU and 20 FPU of cellulase and the enzymatic digestibility after 120 h was predicted using the same method as above with 10 FPU (Table 2).

The predicted enzymatic digestibility after 120 h increased as the enzyme dosage increased, regardless of drying. The increase in the rate of digestibility was more remarkable in the hydrolysis of the OD pulp, and the rate increased by 20.0 (ND), 24.1 (FD) and 32.3% (OD) when the enzyme dosage increased from 4 to 20 FPU. It is believed that a higher enzyme dosage can convert more cellulose to sugars in a short period of enzymatic hydrolysis, and thus the digestibility of the OD pulp increases significantly as the enzyme dosage is increased. There was a 26.0% increase in the digestibility of the OD pulp when the enzyme dosage was increased from 4 to 10 FPU, which was much higher than the 5.1% increase in digestibility when the enzymes were increased from 10 to 20 FPU. This suggested that an enzyme dosage 10 FPU was enough to improve enzymatic digestibility by pore formation during hydrolysis.

The kinetics of enzymatic hydrolysis with 4 FPU and 20 FPU are shown in Figure 1a,c. There was no leveling off on the enzymatic hydrolysis with 4 FPU. Enzymatic hydrolysis with lower enzyme dosages requires longer hydrolysis time to obtain maximum enzymatic digestibility [36], and 120 h of hydrolysis time might not be enough to achieve maximum digestibility with 4 FPU of enzyme dosage. However, the predicted digestibility with 4 FPU was 62.9%, which was slightly lower than the actual digestibility of 70.4% after 120 h (Tables 2 and 3). It was considered that hydrolysis with 4 FPU progressed steadily over 120 h and it had already reached the maximum digestibility even though it did not appear to level off. There was no further increase in digestibility with 4 FPU as a result of enzyme inhibition caused by accumulated products such as released sugars [37]. When 20 FPU was used, the enzymatic digestibility of the ND and FD pulps leveled off in 24 h and both final digestibilities were almost 100% after 120 h. Although the digestibility of the OD pulp also increased

when the enzyme dosage increased to 20 FPU, it was still lower than that of the ND and FD pulp due to the drying effect induced by fiber hornification. The enzymatic digestibility after 120 h and the predicted final digestibility of the OD pulp with 20 FPU was 93.2% and 90.1%, respectively. It indicated that the drying effect on enzymatic hydrolysis can vary depending on enzyme dosage, but there is likely to be a difference in the final enzymatic digestibility of the ND and OD pulps due to irreversible structural change in the cellulose [38]. The drying effect described by the differences in the enzymatic digestibility of the ND and OD pulp with 20 FPU was clearer at the initial stage and it was different compared to the drying effect with 4 FPU, which did not show any big differences as the enzymatic hydrolysis progressed. Thus, the drying effect can vary depending on enzyme dosage and the term "Drying effect index" (DEI) was suggested to define the drying effect. The equation for calculating the DEI is as follows (Equation (6)), and the DEI was plotted, as shown in Figure 2.

$$\text{DEI (\%)} = 100 - (\text{Digestibility of OD pulp/Digestibility of ND pulp}) * 100 \quad (6)$$

The highest DEI, which represents the biggest drying effect, increased as enzyme dosage increased and were revealed at the earlier stages of hydrolysis (Figure 2). The highest DEIs were 22.3 (4 FPU), 24.1 (10 FPU) and 29.3 (20 FPU) and these were observed at 96, 24 and 6 h, respectively. Enzymatic hydrolysis of the OD pulp began in the non-collapsed part first, followed by hydrolysis of the collapsed part because enzyme access to the non-collapsed part was easier than access to the collapsed part [39]. Higher enzyme dosage resulted in the biggest difference in digestibility (DEI) at the earlier stage (29.3 at 6 h) because there was a considerable difference in the amount of collapsed pores in the ND and OD pulp. The ND pulp was hydrolyzed quickly due to the small amount of collapsed pores and the fast hydrolysis of the non-collapsed pores in the ND pulp by more enzymes resulted in a big difference in the DEI in 6 h. As the hydrolysis progressed, the reversible collapsed pores were converted to partial collapsed pores by rewetting in buffer solution and the cellulose conversion to sugars induced by progress in the hydrolysis provided more pore volume in the cellulose [40]. Thus, the DEI with 20 FPU decreased as hydrolysis progressed, which meant that there was less difference in enzymatic digestibility between the ND and OD pulp. Therefore, it was concluded that more enzymes caused a higher drying effect at the initial stage of enzymatic hydrolysis and the time for the highest drying effect as revealed earlier. Once the drying effect reached maximum, the drying effect was reduced as hydrolysis progressed when 10 and 20 FPU of enzymes were used. When 4 FPU of enzymes was used, however, there was little difference in the DEI as hydrolysis progressed, unlike 10 and 20 FPU of enzymes. It was considered that the slower rate of hydrolysis with less enzymes caused more inhibition due to a decrease in enzyme activity, and thus, new pores formed during hydrolysis could not dramatically enhance enzyme accessibility in 120 h. In summary, the drying effect on enzymatic hydrolysis increased as the enzyme dosage increased, and the highest DEI occurred in the earlier stages with higher enzyme dosage. As enzymatic hydrolysis with 10 and 20 FPU of enzymes progressed, the drying effect decreased gradually due to the formation of pores in the OD pulp during hydrolysis. When 4 FPU was used, however, a decrease in the DEI was not observed in 120 h because there was not enough pore formation from enzyme inhibition.

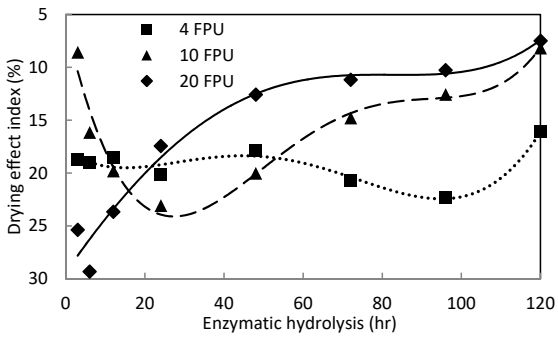

Figure 2. Drying effect index depending on enzyme dosage and hydrolysis time.

3.3. Evaluation of Drying Effect Using Water Retention Value and Simons' Staining Method

The water retention value (WRV) has been used to characterize the drying effect on enzymatic hydrolysis induced by fiber hornification because it can easily measure the pore volume of pulp in a wet state by measuring the amount of water in cell wall [22].

It was previously found that the WRV of the OD pulp decreased significantly due to collapse and shrinkage of pores and the decrease in the WRV correlated well with enzymatic digestibility [22,32]. In this study, the OD pulp with lower enzymatic digestibility, as shown above, revealed a significantly lower WRV, which decreased by 36.4% and 33.3% based on the ND and FD pulps, respectively (Table 3). The decrease in WRV related to the lower enzymatic digestibility was also caused by less enzyme accessibility to cellulose, induced by the decrease in pore volume [22]. However, as enzymatic hydrolysis progresses, new pores are formed through cellulose conversion to sugars and the pore formation increases the pore volume in cellulose. Therefore, the WRV of the OD pulp hydrolyzed for 24 h increased drastically from 105.7 to 203.0%. As described, the kinetics of enzymatic hydrolysis showed that the bigger drying effect at initial stages, which is due to less pore volume caused by the collapse of pores and pores formed by cellulose conversion during hydrolysis, can supply enzymes with space to access cellulose. Therefore, the difference in enzymatic digestibility between the ND and OD pulps will decrease.

Table 3. Water retention value (WRV) and enzymatic digestibility depending on drying.

FPU/g-Pulp	Never Dried	Freeze Dried	Oven Dried	Oven Dried after 24 h of EH with 10 FPU/g-Pulp
WRV (%)	166.1	158.4	105.7	203.0
EH with 4 FPU/g-pulp	83.9 ± 2.2	81.4 ± 0.6	70.4 ± 0.8	NA
EH with 10 FPU/g-pulp	96.6 ± 0.5	96.1 ± 0.4	88.7 ± 0.1	NA
EH with 20 FPU/g-pulp	100.7 ± 0.9	101.0 ± 0.2	93.2 ± 0.4	NA

The Simons' staining method can be used to evaluate the enzyme accessible surface area on cellulose using adsorption and desorption of dyes [26,30]. The pores are segregated depending on the diameter of two different dyes, which have a different affinity for hydroxyl groups in cellulose, and thereby, enzyme accessible surface area of cellulose can be measured in a wet state. The large orange-colored dye molecules can only penetrate larger pores and adsorb in preference to the small blue-colored dye molecules as a result of their greater affinity for hydroxyl groups in cellulose [29]. The large orange-colored dye molecule is mainly composed of two fractions that have hydrodynamic diameters of 5–7 and 12–36 nm [29]. The 5–7 nm diameter is very similar to that of the catalytic core of *Trichoderma reesei* endoglucanase [21]. Therefore, enzyme accessible surface area can be evaluated by the amount of adsorbed orange dye [26], even though the adsorption behavior onto the cellulose surface may be different to enzymes, and also the drying effect on enzymatic hydrolysis has been assessed effectively by the Simons' staining [21].

As result of the Simons' staining, we found that the amount of orange dye adsorbed in the ND and OD pulps were 154.4 and 75.9 mg per g of cellulose, respectively, and the drying process decreased more than half of the accessible surface area in larger pores by pore collapse (Table 4). However, the amount of blue dye, which adsorbed on the surface area of smaller pores, increased from 36.7 to 62.1 mg per g of cellulose. The increase in the adsorption of blue dye was likely caused by the change of larger pores to smaller pores due to the collapse of larger pores, and thus, the surface area of smaller pore increased [21]. The increase in enzyme accessible surface area enhanced enzymatic digestibility through better enzyme adsorption, and it was more remarkable at the initial stage. The total amount of adsorbed dye decreased from 191.0 to 138.0 by drying, which suggested that the formation of the smaller pores and disappearance of larger pores by pore collapse in cellulose decreased the total surface area. It is well known that most pretreatment processes open up the cell wall structure by mechanical shearing force and/or chemical removal of components and this forms new pores in

cellulose [41]. Thus, enzymatic hydrolysis could be enhanced by increasing the accessibility through pore formation. The drying process decreased enzyme accessibility and reduced enzymatic hydrolysis. This can be explained by using the opposing mechanism of pretreatment on enzyme accessibility and enzymatic hydrolysis. The drying effect was more remarkable at the initial stage and then it decreased as hydrolysis progressed. However, the irreversible change in structure made a difference to the maximum enzymatic digestibility.

Table 4. Amount of adsorbed dye depending on drying methods.

Pulps	Amount of Adsorbed Dye by Simons' Staining (mg·g^{-1})		
	Orange Dye	Blue Dye	Total
Never dried	154.4 ± 2.3	36.7 ± 2.0	191.0 ± 4.3
Oven dried	75.9 ± 3.0	62.1 ± 5.8	138.0 ± 8.8

3.4. Evaluation of Drying Effect on Crystalline Structure Using ^{13}C CPMAS Solid-State NMR Analysis

The ultrastructure of cellulose has mainly been determined by ^{13}C CPMAS solid-state NMR spectroscopy [42,43], and the solid-state NMR characterized changes associated with drying pulp [44] and bleached pulp by hornification has also been reported [19].

The ^{13}C solid-state NMR analysis was performed in wet conditions for the ND, OD and FD pulps and the spectra with signals assigned are shown in Figure 3 [19]. The ^{13}C NMR spectrum of the pulp was mainly dominated by signals assigned to cellulose. Signals assigned to C-4 and C-6 were partially separated into two clusters, labeled i and s, which were assigned to the interior and surface of the crystalline domains, respectively. Broadening of the NMR signal and relative sharpening of C-4 and C-6 signals on the surfaces of crystalline domains by partial drying has been reported, but the signal broadening and sharpening of C-4 and C-6 signals can be reversed by rewetting the pulp [19]. The ^{13}C NMR spectra of all three pulps were quite similar because both the FD and OD pulps were analyzed in a wet state. Although it has been reported that several repetitions of drying and rewetting cause the difference to peak at 89.4 ppm due to the irreversible drying effect [19], it was not observed in this study because drying was performed only once and rewetting for NMR analysis might reverse the drying effect. Therefore, it can be concluded that drying once did not cause an irreversible change in crystalline structure, and therefore, the decrease in enzymatic digestibility does not seem to be related to the structure of cellulose. However, it might be related to cellulose structure if the pulp was dried more than once.

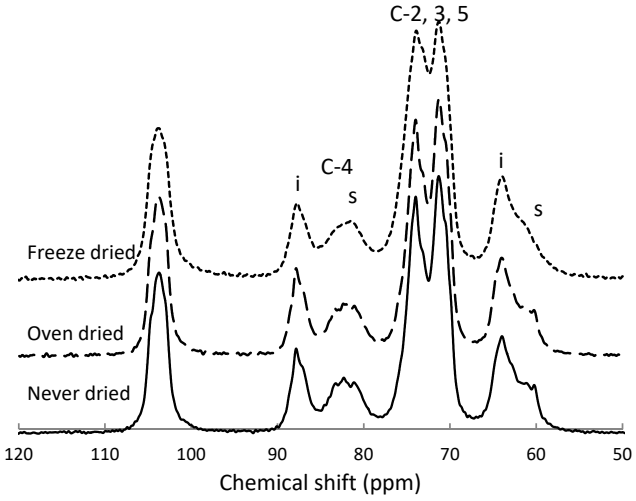

Figure 3. 13C solid-state NMR spectra of never-dried, freeze-dried and oven-dried pulps.

4. Conclusions

The drying effect was evaluated by the difference in enzymatic digestibility in the ND and OD pulp, which was found to vary depending on enzyme dosage. The drying effect index (DEI) was defined as the difference in enzymatic digestibility between the OD and ND pulps and it was concluded that more enzymes cause a higher DEI at the initial stage of enzymatic hydrolysis, and the highest DEI was also observed in the earlier stages with the higher enzyme dosage. Once the DEI reached maximum, it began to reduce as enzymatic hydrolysis progressed due to pore formation by cellulose conversion to sugars. However, there was no big difference in the DEI with lower enzyme dosage because slower hydrolysis with less enzymes caused more enzyme inhibition. Thus, the pores formed by cellulose conversion during hydrolysis with less enzymes could not improve enzyme accessibility after 120 h.

It was also found that the drying effect was well correlated with porosity in cellulose, as measured by the water retention value (WRV) and Simons' staining. The porosity in cellulose was reduced during drying by pore collapse and thus, enzyme accessibility decreased accordingly. The pore collapse was partially reversed by rewetting the pulp, however, there was an irreversible structural change that decreased the final enzymatic digestibility of the OD pulp. However, irreversible change in crystalline structure by drying once was not observed through the 13C solid-state NMR, and thus, decreases in enzymatic digestibility did not seem to be related to the crystalline structure of cellulose.

Author Contributions: Conceptualization, B.K.; methodology, B.K.; formal analysis, B.K. and J.J.; investigation, B.K. and S.-M.C.; resources, B.K.; data curation, S.-M.C.; writing—original draft preparation, B.K.; writing—review and editing, B.K.; visualization, J.J.; supervision, B.K.; project administration, B.K. All authors have read and agreed to the published version of the manuscript.

Funding: This study was carried out with the support of the R & D Program for Forest Science Technology (Project No. 2020226A00-2022-AC01) provided by the Korea Forest Service (Korea Forestry Promotion Institute).

Acknowledgments: The NMR analysis in this work was performed by Rui Katahira, a research chemist at the National Renewable Energy Laboratory.

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

References

1. Li, T.; Takkellapati, S. The current and emerging sources of technical lignins and their applications. *Biofuel Bioprod. Biorefin.* **2018**, *12*, 756–787. [CrossRef] [PubMed]
2. Bozell, J.J.; Petersen, G.R. Technology development for the production of biobased products from biorefinery carbohydrates-the US Department of Energys "Top 10" revisited. *Green Chem.* **2010**, *12*, 539–554. [CrossRef]
3. Biddy, M.J.; Scarlata, C.; Kinchin, C. *Chemicals from Biomass: A Market Assessment of Bioproducts with Near-Term Potential*; National Renewable Energy Laboratory and Department of Energy: Washington, DC, USA. Available online: https://www.osti.gov/biblio/1244312 (accessed on 10 August 2020). [CrossRef]
4. Wheeldon, I.; Christopher, P.; Blanch, H. Integration of heterogeneous and biochemical catalysis for production of fuels and chemicals from biomass. *Curr. Opin. Biotechnol.* **2017**, *45*, 127–135. [CrossRef] [PubMed]
5. Koo, B.; Treasure, T.H.; Jameel, H.; Phillips, R.B.; Chang, H.M.; Park, S. Reduction of enzyme dosage by oxygen delignification and mechanical refining for enzymatic hydrolysis of green liquor-pretreated hardwood. Appl. *Biochem. Biotechnol.* **2011**, *165*, 832–844. [CrossRef] [PubMed]
6. Mata, T.M.; Martins, A.A.; Caetano, N.S. Bio-refinery approach for spent coffee grounds valorization. *Bioresour. Technol.* **2016**, *247*, 1077–1084. [CrossRef]
7. Nguyen, Q.A.; Cho, E.J.; Lee, D.S.; Bae, H.J. Development of an advanced integrative process to create valuable biosugars including manno-oligosaccharides and mannose from spent coffee grounds. *Bioresour. Technol.* **2018**, *272*, 209–216. [CrossRef]
8. Galbe, M.; Zacchi, G. Pretreatment: The key to efficient utilization of lignocellulosic materials. *Biomass Bioenerg.* **2012**, *46*, 70–78. [CrossRef]
9. Rajendran, K.; Taherzadeh, M.J. Chapter 3 Bioprocessing of Renewable Resources to Commodity Bioproducts. In *Pretreatment of Ligocellulosic Materials*; Wiley: Hoboken, NJ, USA, 2014.

10. Mandels, M.; Hontz, L.; Nystrom, J. Enzymatic hydrolysis of waste cellulose. *Biotechnol. Bioeng.* **1974**, *16*, 1471–1493. [CrossRef]
11. Fernandes Diniz, J.M.B.; Gil, M.; Castro, J. Hornification-its origin and interpretation in wood pulps. *Wood Sci. Technol.* **2004**, *37*, 489–494. [CrossRef]
12. Park, S.; Venditti, R.A.; Jameel, H.; Pawlak, J.J. A novel method to evaluate fibre hornification by high resolution thermogravimetric analysis. *Appita J.* **2006**, *59*, 481–485.
13. Ferreira, S.R.; Silva, F.; Lima, P.R.L.; Filho, R.D.T. Effect of hornification on the structure, tensile behavior and fiber matrix bond of sisal, jute and curauá fiber cement based composite system. *Constr. Build. Mater.* **2017**, *139*, 551–561. [CrossRef]
14. Kato, K.; Cameron, R. A review of the relationship between thermally-accelerated ageing of paper and hornification. *Cellulose* **1999**, *6*, 23–40. [CrossRef]
15. Weise, U. Hornification: Mechanisms and terminology. *Paperi Ja Puu.* **1998**, *80*, 110–115.
16. Welf, E.; Venditti, R.; Hubbe, M.; Pawlak, J. The effects of heating without water removal and drying on the swelling as measured by water retention value and degradation as measured by intrinsic viscosity of cellulose papermaking fibers. *Prog. Pap. Recycl.* **2005**, *14*, 1–9.
17. Chen, Y.; Wan, J.; Ma, Y. Effect of noncellulosic constituents on physical properties and pore structure of recycled fibre. *Appita J.* **2009**, *62*, 290–295.
18. Nazhad, M.M. Fundamentals of Strength Loss in Recycled Paper. Ph.D. Thesis, University of British Columbia, Vancouver, BC, Canada, 22 December 1994. V6T 1Z4. [CrossRef]
19. Newman, R.H. Carbon-13 NMR evidence for cocrystallization of cellulose as a mechanism for hornification of bleached kraft pulp. *Cellulose* **2004**, *11*, 45–52. [CrossRef]
20. Wistara, N.; Young, R.A. Properties and treatments of pulps from recycled paper. Part, I. Physical and chemical properties of pulps. *Cellulose* **1999**, *6*, 291–324. [CrossRef]
21. Esteghlalian, A.; Bilodeau, M.; Mansfield, S.; Saddler, J. Do enzymatic hydrolyzability and Simons' stain reflect the changes in the accessibility of lignocellulosic substrates to cellulase enzymes? *Biotechnol. Prog.* **2001**, *17*, 1049–1054. [CrossRef]
22. Luo, X.; Zhu, J. Effects of drying-induced fiber hornification on enzymatic saccharification of lignocelluloses. *Enzyme Microb. Technol.* **2011**, *48*, 92–99. [CrossRef]
23. Li, Y.; Li, B.; Mo, W.; Yang, W.; Wu, S. Influence of residual lignin and thermal drying on the ultrastructure of chemical hardwood pulp and its enzymatic hydrolysis properties. *Cellulose* **2019**, *26*, 2075–2085. [CrossRef]
24. TAPPI T205 sp-95. *Forming Handsheets for Physical Tests of Pulp*; TAPPI Press: Atlanta, GA, USA, 1995; Available online: https://www.tappi.org/content/sarg/t205.pdf (accessed on 10 August 2020).
25. NREL. In *Determination of Structural Carbohydrates and Lignin in Biomass*; Laboratory Analytical Procedure (LAP), US Department of Energy, National Renewable Energy Laboratory: Golden, CO, USA, 2008. Available online: https://www.nrel.gov/docs/gen/fy13/42618.pdf (accessed on 10 August 2020).
26. Koo, B.; Jameel, H.; Phillips, R.; Chang, H.; Park, S. Reduction of enzyme dosage for enzymatic hydrolysis by the mechanical refining and oxygen bleaching post-treatments. *Appl. Biochem. Biotechnol.* **2011**, *165*, 832–844. [CrossRef] [PubMed]
27. Koo, B.; Park, S. A method to evaluate biomass accessibility in wet state based on thermoporometry. *Methods Mol. Biol.* **2012**, *908*, 83–89.
28. TAPPI UM256. *Water Retention Value (WRV)*; TAPPI Press: Atlanta, GA, USA, 1981; Available online: https://imisrise.tappi.org/TAPPI/Products/01/UM/0104UM256.aspx (accessed on 10 August 2020).
29. Yu, X.; Atalla, R. A staining technique for evaluating the pore structure variations of microcrystalline cellulose powders. *Powder Technol.* **1998**, *98*, 135–138. [CrossRef]
30. Chandra, R.; Ewanick, S.; Hsieh, C.; Saddler, J. The characterization of pretreated lignocellulosic substrates prior to enzymatic hydrolysis, part 1: A modified Simons' staining technique. *Biotechnol. Prog.* **2008**, *24*, 1178–1185. [CrossRef] [PubMed]
31. Lee, Y.H.; Fan, L. Kinetic studies of enzymatic hydrolysis of insoluble cellulose: Analysis of the initial rates. *Biotechnol. Bioeng.* **1982**, *24*, 2383–2406. [CrossRef]
32. Ioelovich, M.; Morag, E. Effect of cellulose structure on enzymatic hydrolysis. *BioResources* **2011**, *6*, 2818–2835.
33. Sun, S.; Sun, S.; Cao, X.; Sun, R. The role of pretreatment in improving the enzymatic hydrolysis of lignocellulosic materials. *Bioresour. Technol.* **2016**, *199*, 49–58. [CrossRef]

34. Hribernik, S.; Kleinschek, K.S.; Rihm, R.; Ganster, J.; Fink, H.P.; Smole, M.S. Tuning of cellulose fibres' structure and surface topography: Influence of swelling and various drying procedures. *Carbohydr. Polym.* **2016**, *148*, 227–235. [CrossRef]
35. Buschle-Diller, G.; Fanter, C.; Loth, F. Structural changes in hemp fibers as a result of enzymatic hydrolysis with mixed enzyme systems. *Text. Res. J.* **1999**, *69*, 244–251. [CrossRef]
36. Kristensen, J.B.; Felby, C.; Jørgensen, H. Yield-determining factors in high-solids enzymatic hydrolysis of lignocellulose. *Biotechnol. Biofuels* **2009**, *2*, 11. [CrossRef]
37. Jing, X.; Zhang, X.; Bao, J. Inhibition performance of lignocellulose degradation products on industrial cellulase enzymes during cellulose hydrolysis. *Appl. Biochem. Biotechnol.* **2009**, *159*, 696–707. [CrossRef] [PubMed]
38. Spinu, M.; Dos Santos, N.; Le Moigne, N.; Navard, P. How does the never-dried state influence the swelling and dissolution of cellulose fibres in aqueous solvent? *Cellulose* **2011**, *18*, 247–256. [CrossRef]
39. Wang, Q.; He, Z.; Zhu, Z.; Zhang, Y.H.P.; Ni, Y.; Luo, X.; Zhu, J. Evaluations of cellulose accessibilities of lignocelluloses by solute exclusion and protein adsorption techniques. *Biotechnol. Bioeng.* **2011**, *109*, 381–389. [CrossRef] [PubMed]
40. Zeng, M.; Mosier, N.S.; Huang, C.P.; Sherman, D.M.; Ladisch, M.R. Microscopic examination of changes of plant cell structure in corn stover due to hot water pretreatment and enzymatic hydrolysis. *Biotechnol. Bioeng.* **2007**, *97*, 265–278. [CrossRef] [PubMed]
41. Lee, S.; Teramoto, Y.; Endo, T. Enzymatic saccharification of woody biomass micro/nanofibrillated by continuous extrusion process I-Effect of additives with cellulose affinity. *Bioresour. Technol.* **2009**, *100*, 275–279. [CrossRef]
42. Atalla, R.; VanderHart, D. The role of solid state 13C NMR spectroscopy in studies of the nature of native celluloses. *Solid State Nucl. Magn. Reson.* **1999**, *15*, 1–19. [CrossRef]
43. Hult, E.L.; Iversen, T.; Sugiyama, J. Characterization of the supermolecular structure of cellulose in wood pulp fibres. *Cellulose* **2003**, *10*, 103–110. [CrossRef]
44. Hult, E.L.; Larsson, P.; Iversen, T. Cellulose fibril aggregation—an inherent property of kraft pulps. *Polymer* **2001**, *42*, 3309–3314. [CrossRef]