

## Article

# Allomorphic Transformation of Cellulose for Enhancing Enzymatic Accessibility

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## Abstract

In recent decades, lignocellulosic biomass has attracted increasing attention as a sustainable alternative to fossil-fuel-based resources. However, the compact and highly crystalline structure of cellulose remains a major limitation to its effective utilization. In this study, the allomorphic transformation of cellulose was induced through chemical treatments using sodium hydroxide (NaOH) and ethylenediamine (EDA), enabling the conversion of native cellulose I into cellulose II and cellulose III, respectively. The resulting changes in the crystalline structure were systematically investigated using X-ray diffraction and Raman spectroscopy. Both NaOH- and EDA-treated celluloses exhibited enhanced enzymatic digestibility compared to untreated cellulose, consistent with the observed modifications in the crystal structure. Nevertheless, some results indicate that crystalline structure is not an absolute determining factor, but rather one of several parameters, including specific surface area, particle size, and degree of polymerization.

**Keywords:** mercerization; cellulose II; ethylenediamine; cellulose III; crystallinity index; enzymatic hydrolysis

## 1. Introduction

As concerns over environmental problems and resource depletion associated with the continued use of fossil fuels intensify, the development of sustainable alternative energy sources has emerged as a critical global challenge [1]. In this context, biomass-derived biofuels have attracted considerable attention as environmentally benign energy carriers [2]. Early biofuels, referred to as first-generation biofuels, were produced from sugar-, starch-, or oil-rich plants such as sugarcane, corn, and oilseed crops (e.g., palm oil, rape-seed, and soybean) [3]. However, this approach suffers from inherent limitations, including high production costs and direct competition with food resources [4]. Consequently, lignocellulosic biomass, which minimizes competition with food supplies, has been recognized as a promising feedstock for second-generation biofuels [5]. As an advantage, lignocellulosic biomass is abundantly available from diverse sources, including agricultural residues, forestry byproducts, and plant resources cultivated on marginal or non-arable land [6].

Cellulose, a major constituent of biomass, is the most abundant biopolymer in nature and consists of linear chains of  $\beta$ -D-glucose units linked by  $\beta$ -(1 $\rightarrow$ 4)-glycosidic bonds [7]. Sugars obtained through the hydrolysis of cellulose can be converted into a wide range of biofuels and biochemicals, including bioethanol [8]. However, extensive intra-chain hydrogen bonding between the hydroxyl groups at the C6 and C2 positions, as well as



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between the ring oxygen at C5 and the hydroxyl group at the C3 position, stabilizes the glycosidic linkages, imparting high rigidity and structural stability to the cellulose chains [7]. Moreover, inter-chain hydrogen bonds involving the hydroxyl groups at the C6 and C3 positions promote the formation of sheet-like structures, which further stack through van der Waals forces and hydrophobic interactions rather than through hydrogen bonding, resulting in a highly ordered crystalline architecture. Owing to this dense hydrogen-bonding network and layered packing, the upper and lower surfaces of cellulose crystallites exhibit relatively hydrophobic characteristics, which significantly hinder chemical derivatization and enzymatic hydrolysis [9]. Consequently, cellulose is highly resistant to chemical modification, often leading to heterogeneous substitution reactions that compromise the efficiency and reproducibility of reactions [10]. Furthermore, the high crystallinity and hydrophobic crystal planes of cellulose have been widely recognized as major factors limiting enzyme adsorption and accessibility during enzymatic hydrolysis, thereby substantially reducing the rate of hydrolysis [5,11–13]. Pretreatment processes are essential for alleviating the structural recalcitrance and enhancing the enzymatic accessibility of cellulose.

Various pretreatment methods for achieving more efficient enzymatic hydrolysis of cellulose have been extensively reported. Such approaches aim to reduce the crystallinity and improve the enzymatic accessibility of cellulose. Representative mechanical pretreatment approaches, such as ball milling [14], and chemical pretreatments, including phosphoric acid treatment [15], are known to effectively decrease the crystallinity of cellulose and promote enzymatic hydrolysis. However, ball milling is inherently limited by high consumption of mechanical energy and nonselective disruption of the structure of the treated materials, adversely affecting the process efficiency and reproducibility. Similarly, acid-based pretreatment processes, including phosphoric acid treatment, have persistent challenges associated with the disposal of acid waste, environmental burdens, and safety concerns regarding the process [5,16]. These drawbacks have prompted increasing interest in pretreatment strategies that enable more selective control over the crystalline structure of cellulose.

In this regard, pretreatment strategies based on the allomorphic transformation of crystalline cellulose have emerged as promising alternatives for enhancing the accessibility to enzymes [12,17]. Alkaline and amine treatments induce the formation of cellulose II and III, respectively, where such allomorphic transformations effectively increase the adsorption of enzymes and their access to cellulose by rearranging the crystalline structure and altering the surface properties of the crystals [5,11,12,18,19]. Cellulose II and III can be derived from native cellulose I through alkaline (e.g., NaOH) and amine (e.g., ethylenediamine) treatments, respectively [20]. Although treatments employing ionic solvents also produce cellulose II [21], their practical implementation is limited by high solvent costs and unfavorable process economics [5]. Although alkaline and amine treatments cannot be strictly classified as completely environmentally benign processes, NaOH can be efficiently recovered and recycled via the lime cycle [22], and ethylenediamine (EDA) can likewise be repeatedly recovered and reused, thereby mitigating the environmental footprint of the process [23]. These recycling capabilities suggest that pretreatment strategies based on allomorph transformation can offer a balanced combination of practicality and sustainability.

In this study, we aimed to identify the crystalline allomorphs of cellulose that are favorable for enhancing its enzyme accessibility and to evaluate the practical applicability of allomorph-based pretreatment strategies by applying them to lignocellulosic pulp. Ultimately, this study explores fundamental insights for the development of efficient pretreatment strategies for the production of value-added products from biomass via enzymatic hydrolysis.

## 2. Materials and Methods

### 2.1. Materials

Avicel PH-101 (FMC Corporation, Philadelphia, PA, USA) was used as a standard substrate for enzymatic hydrolysis. Avicel is a microcrystalline cellulose (MCC) with a particle size of less than 50  $\mu\text{m}$  and a cellulose I $\beta$  crystalline structure [10]. Sodium hydroxide (NaOH) and ethylenediamine (EDA) were employed for the preparation of cellulose II (Avicel II) and cellulose III (Avicel III), respectively. During the preparation process, acetic acid (99.5%), ethanol (94.5%), and acetone (99.5%) were used for pH adjustment. Unless otherwise specified, all reagents were purchased from Daejung Chemical & Metals Co., Ltd. (Siheung, Republic of Korea).

Cellic CTec2<sup>®</sup> (Novozymes, Bagsværd, Denmark), a commercial enzyme cocktail containing endoglucanase (EG), cellobiohydrolase (CBH), and  $\beta$ -glucosidase (BG), was used for enzymatic hydrolysis [8]. The enzyme–reaction mixture was prepared using sodium citrate buffer, and sodium azide was added to suppress microbial growth.

Thermomechanical pulp (TMP; Jeonju Paper Co., Ltd., Jeonju, Republic of Korea), bleached chemi-thermomechanical pulp (BCTMP; Meadow Lake Mechanical Pulp Inc., Meadow Lake, SK, Canada), softwood bleached Kraft pulp (SwBKP; Moorim P & P Co., Ltd., Jinju, Republic of Korea), and bleached sulfite pulp (Nippon Paper Industries Co., Ltd., Tokyo, Japan) were used as substrates for evaluating the effects of the conditions used in the allomorphic transformation of cellulose (determined from standard substrate-based enzymatic hydrolysis experiments) on the enzymatic hydrolysis of the lignocellulosic materials.

### 2.2. Methods

#### 2.2.1. Preparation of Cellulose Allomorphs

Cellulose II (Avicel II) was prepared via alkaline treatment according to the method reported by Agarwal et al. [24]. Briefly, 10 g of Avicel was treated with 100 mL of a 20 wt% aqueous NaOH solution under continuous stirring at ambient temperature for 24 h. After treatment, the sample was sequentially washed with acetic acid, ethanol, and distilled water until neutral pH was achieved.

Cellulose III (Avicel III) was prepared by treatment with EDA by following the procedure reported by Wu et al. [25]. In this process, 10 g of Avicel was immersed in 500 mL of EDA and stirred at ambient temperature for 24 h. After completion of the reaction, the sample was sequentially washed with ethanol and acetone until no further change in pH was observed. The Avicel II and Avicel III samples were stored under vacuum drying conditions at 50 °C until further analysis.

#### 2.2.2. Enzymatic Hydrolysis

Enzymatic hydrolysis was performed according to the method reported by Kim et al. [26], with slight modifications. Pretreated cellulose samples (0.5 g on an oven-dried basis) were used to prepare a total reaction volume of 5 mL, corresponding to a substrate concentration of 10% (*w/v*). To stabilize the enzyme activity, 4.43 mL of 0.1 M sodium citrate buffer (pH 5.0) was mixed with 0.05 mL of sodium azide solution. The enzyme cocktail was added at a loading of 10 FPU per gram of glucan, corresponding to an enzyme volume of 0.02 mL.

Enzymatic hydrolysis was performed in a shaking incubator (Hanbaek Scientific Co., Ltd., Bucheon, Republic of Korea) at 50 °C and 250 rpm. Upon completion of the reaction, the resulting mixture was heated at 100 °C for 20 min to deactivate residual active enzyme. The solid cellulose residue was then recovered by vacuum filtration, dried at 105 °C for 12 h, and weighed. The degree of enzymatic hydrolysis was evaluated based on the mass loss of the solid residue relative to the initial substrate mass. It was calculated according to Equation (1):

$$\text{Solid mass loss (\%)} = \frac{(m_0 - m_t)}{m_0} \times 100 \quad (1)$$

where  $m_0$  is the initial mass of the cellulose substrate (0.5 g), and  $m_t$  is the mass of the solid residue remaining after enzymatic hydrolysis (g).

### 2.2.3. X-Ray Diffraction (XRD) Analysis

XRD patterns of the cellulose samples were collected using an X-ray diffractometer (Rigaku Ultima IV, Rigaku Corporation, Tokyo, Japan) equipped with a fixed slit (0.19 mm). Measurements were performed using Cu-K $\alpha$  radiation ( $\lambda = 0.15418$  nm) at an accelerating voltage of 40 kV. Diffraction patterns were recorded over the  $2\theta$  range of  $5\text{--}40^\circ$ , with a step size of  $0.02^\circ$  and a scan rate of  $2^\circ/\text{min}$ .

The crystallinity index (CI<sub>XRD</sub>) of cellulose was determined using the peak-height method proposed by Segal et al. [27] and calculated according to Equation (2):

$$CI_{XRD}(\%) = \frac{I_{200} - I_{am}}{I_{200}} \times 100 \quad (2)$$

$I_{200}$ : height of the 200 peak ( $2\theta = 22.5^\circ$ )

$I_{am}$ : minimum between the 200 and 100 peaks ( $2\theta = 18^\circ$ )

where  $I_{200}$  is the diffraction intensity of the (200) plane at  $2\theta = 22.5^\circ$ , corresponding to the crystalline region and  $I_{am}$  is the diffraction intensity at  $2\theta = 18^\circ$ , representing the amorphous region between the (200) and (100) planes. Baseline correction was performed by connecting the two lowest-intensity points within the  $2\theta$  range of  $7\text{--}37^\circ$  by a straight line, following previously reported procedures [13,27,28].

The crystallite size ( $L_{hkl}$ ) and interplanar spacing ( $d_{hkl}$ ) of cellulose were determined from the XRD patterns using the Scherrer equation [29] and Bragg's law [30], as shown in Equations (3) and (4), respectively:

$$L_{hkl} = \frac{K\lambda}{\beta \cos \theta} \quad (3)$$

$$n\lambda = 2d_{hkl} \sin \theta \quad (4)$$

where  $K$  is the Scherrer constant (0.9),  $\lambda$  is the X-ray wavelength,  $\beta$  is the full-width-at-half-maximum of the diffraction peak (in radians),  $\theta$  is the Bragg angle, and  $n$  is the diffraction order.

### 2.2.4. Raman Spectroscopy

Raman spectra of the cellulose samples were acquired using a confocal micro-Raman spectrometer (LabRAM Soleil, HORIBA France SAS, Palaiseau, France) equipped with a 785 nm laser operated at 20 mW. During measurement, the laser beam was focused onto the surface of the sample using a high-magnification objective lens (100 $\times$ , NA = 0.9, Nikon Corporation, Tokyo, Japan). The Raman signal was collected through a 200  $\mu\text{m}$  confocal pinhole and a 600 g/mm diffraction grating and detected using a charge-coupled device detector (Syncerity, Horiba Instruments Inc., Edison, NJ, USA).

Spectra were recorded at multiple randomly selected positions on each sample, with an acquisition time of 5 s per scan and 10 accumulations. The spectral range was set to  $200\text{--}1800\text{ cm}^{-1}$  and the corresponding spatial resolution under these conditions was approximately 1  $\mu\text{m}$ . The crystallinity index of cellulose (CI<sub>Raman</sub>) was calculated from the acquired Raman spectra using Equation (5) [31]:

$$CI_{Raman}(\%) = \frac{(I_{380}/I_{1096})}{Ac} \times 84.5 \quad (5)$$

$A_c$ :  $I_{380}/I_{1096}$  ratio of Avicel (control)

$I_{380}$ : C–C stretching vibration peak

$I_{1096}$ : C–O stretching vibrations (asymmetric glycosidic C–O–C stretching) peak

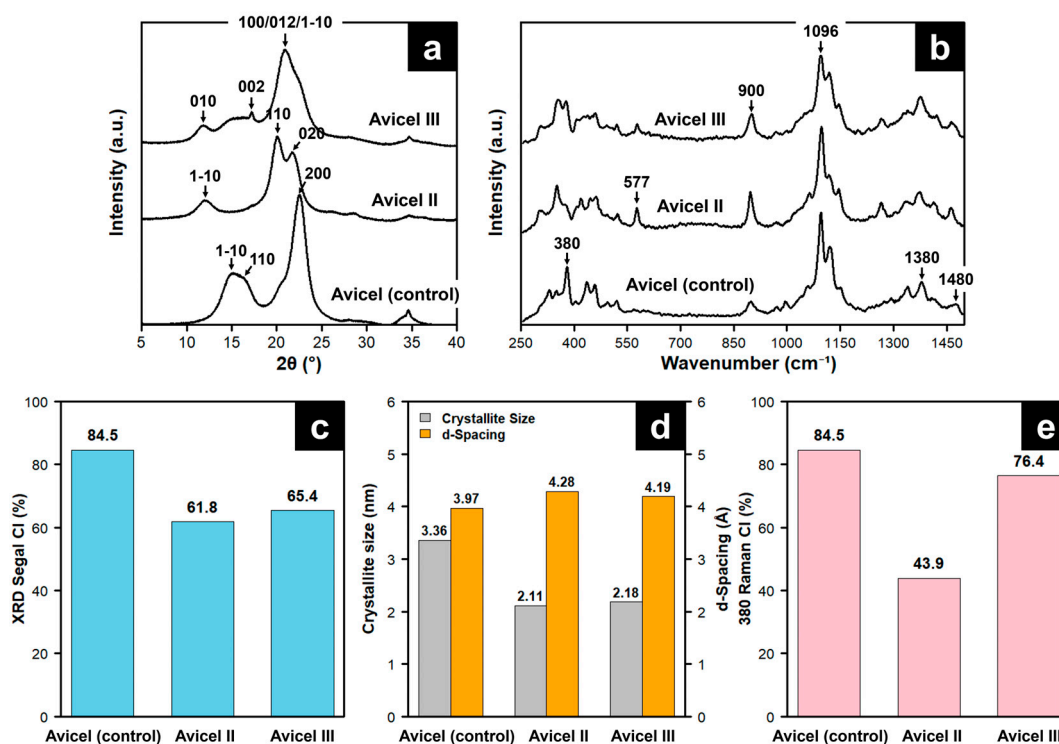
84.5:  $CI_{XRD}$  of Avicel (control)

where  $A_c$  is the  $\frac{I_{380}}{I_{1096}}$  ratio of Avicel used as the control sample,  $I_{380}$  corresponds to the C–C stretching vibration associated with the glucopyranose ring skeleton, and  $I_{1096}$  represents the C–O stretching vibration attributed to asymmetric glycosidic C–O–C stretching in  $\beta$ -(1 $\rightarrow$ 4)-linked cellulose [32]. The factor 84.5 corresponds the XRD-based crystallinity index ( $CI_{XRD}$ ) of the Avicel control.

### 3. Results

#### 3.1. Allomorphic Transformation of Crystalline Cellulose

To prepare crystalline samples with cellulose II and cellulose III structures, Avicel, a standard substrate for cellulose I, was treated with NaOH and EDA. Changes in the crystalline structure induced by these treatments were evaluated using XRD and Raman analyses; the results are presented in Figure 1.



**Figure 1.** (a) XRD patterns and (b) Raman spectra of Avicel samples before and after allomorphic transformation. (c) XRD-based crystallinity index ( $CI_{XRD}$  (%)) calculated using the Segal peak-height method [27]. (d) Crystallite size and d-spacing determined for the hydrophobic lattice planes: (200) for cellulose I, (110) for cellulose II, and (1 $\rightarrow$ 10) for cellulose III [19]. (e) Raman-based crystallinity index ( $CI_{Raman}$  (%)) calculated from the intensity ratio of the 380 cm<sup>-1</sup> band according to the method proposed by Agarwal et al. [31].

Figure 1a shows the XRD patterns of Avicel (control), Avicel II, and Avicel III. The Avicel control exhibited a typical cellulose I $\beta$  diffraction pattern, with characteristic peaks at  $2\theta = 14.7^\circ$ ,  $15.6^\circ$ , and  $22.5^\circ$ , corresponding to the (1 $\rightarrow$ 10), (110), and (200) crystallographic planes, respectively [13]. In contrast, Avicel II displayed diffraction features characteristic of the cellulose II structure, with prominent peaks at  $2\theta = 12.1^\circ$ ,  $20.1^\circ$ , and  $21.7^\circ$ , assigned to the (1 $\rightarrow$ 10), (110), and (020) planes, respectively [33].

For Avicel III, diffraction peaks partially attributable to the cellulose I structure were still observed; however, these peaks are interpreted as overlapping diffraction signals originating from the (110), (012), and (1–10) planes of cellulose III [34]. Notably, the primary diffraction peak at  $2\theta = 22.5^\circ$  for cellulose I shifted to approximately  $2\theta = 21.0^\circ$  after EDA treatment. This peak-shift is consistent with the report by Frech [35], who demonstrated that the main diffraction peak near  $2\theta = 22.5^\circ$  gradually shifts toward  $2\theta = 21.0^\circ$  with increasing cellulose III content.

Based on analysis of the XRD pattern, these results confirm that NaOH and EDA treatments successfully induced the allomorphic transformation of crystalline cellulose I to cellulose II and cellulose III, respectively.

Figure 1d presents the crystallite size and interplanar spacing of Avicel (control), Avicel II, and Avicel III. According to Ling et al. [19], a reduction in the crystallite size and an increase in the interplanar spacing at the hydrophobic crystal planes, namely, the (200), (110), and (1–10) planes for cellulose I, II, and III, respectively, are key for enhancing the enzymatic accessibility of cellulose during hydrolysis. As shown in Figure 1c, the crystallinity index of Avicel (control) was relatively high (84.5%), whereas that of NaOH-treated Avicel II and EDA-treated Avicel III decreased to 61.8% and 65.4%, respectively. This trend is in line with previous reports indicating that the transformation from cellulose I to cellulose II or III leads to a reduction in the crystallinity [5,11,19]. The crystallite size decreased from 3.36 nm for Avicel (control) to 2.11 nm and 2.18 nm for Avicel II and Avicel III, respectively, and the interplanar spacing increased from 3.97 Å for Avicel (control) to 4.28 Å and 4.19 Å for Avicel II and Avicel III, respectively (Figure 1d). These results suggest that the allomorphic transformation to cellulose II and cellulose III may contribute to improving the enzymatic accessibility by reducing the crystalline order and expanding the lattice spacing. Raman and infrared (IR) spectroscopy are both vibrational spectroscopic techniques; however, they differ fundamentally in the physical principles governing signal generation. IR spectroscopy measures the absorption of vibrational energy associated with changes in the dipole moment, whereas Raman spectroscopy probes inelastic light scattering (Raman scattering) arising from changes in the molecular polarizability upon laser irradiation [36]. Owing to these differences, vibrational modes that are IR-inactive may be Raman-active, and vice versa, rendering the two techniques complementary for molecular-level structural analysis [37]. However, for analyzing the crystallinity of cellulose, IR spectroscopy reflects only relative changes in the crystallinity and does not directly enable absolute quantitation of the crystallinity [13]. Consequently, Raman spectroscopy is generally regarded as a more reliable approach for analysis of the crystalline structure and quantitation of the crystallinity of cellulose [38].

Figure 1b shows the Raman spectra of Avicel (control), Avicel II, and Avicel III collected in the range of  $250\text{--}1500\text{ cm}^{-1}$ . Several characteristic Raman bands are commonly used for evaluating the crystallinity of cellulose, including the bands at  $380\text{ cm}^{-1}$  (cellulose skeletal C–C vibrations) [31],  $577\text{ cm}^{-1}$  (heavy-atom stretching vibrations) [24],  $1380\text{ cm}^{-1}$  (C–H bending and  $\text{CH}_2$  deformation modes) [39], and  $1480\text{ cm}^{-1}$  ( $\text{CH}_2$  scissoring and C–H bending modes) [40]. Among these, the band at  $577\text{ cm}^{-1}$  is not observed in the spectrum of cellulose I and is considered a diagnostic feature associated with allomorphic transformation induced by chemical treatments [39]. This band was clearly detected in the spectra of the Avicel II and Avicel III samples but was absent from that of the Avicel (control) sample. These Raman spectral features agree well with the XRD results (Figure 1a), confirming that NaOH and EDA treatments successfully induced the transformation of cellulose I to cellulose II and cellulose III, respectively. Agarwal et al. [39] reported that an increase in the intensity of the Raman band around  $900\text{ cm}^{-1}$  is associated with a higher relative fraction of amorphous regions in cellulose. For Avicel II and III, relatively stronger

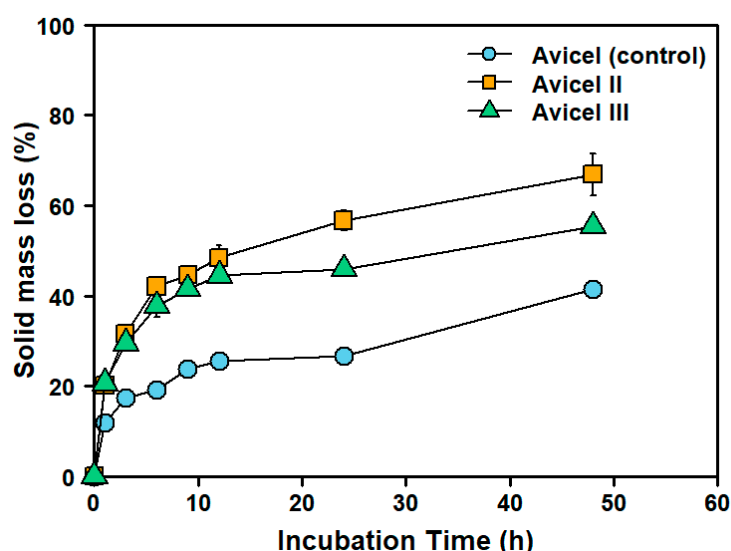
peaks (Figure 1b) were observed in this wavenumber region than for Avicel (control). This observation is in line with previous studies indicating that cellulose II and cellulose III possess lower crystallinity than native cellulose I [10,11].

The crystallinity of the cellulose samples was further evaluated using Raman spectroscopy according to the method reported by Agarwal et al. [31]. This analysis is based on the intensity ratio of the bands at  $380$  and  $1096\text{ cm}^{-1}$ , which correspond to C–C and C–O stretching vibrations, including asymmetric glycosidic C–O–C stretching [32]. From the Raman analysis, the crystallinity indices of Avicel (control), Avicel II, and Avicel III were determined as 84.5%, 43.9%, and 76.4%, respectively. As shown in Figure 1e, the crystallinity values obtained from the Raman spectra followed trends consistent with those derived from the XRD analysis; however, discrepancies were observed in the absolute values. This difference suggests that the crystallinity values for the same sample may vary depending on the analytical technique and instrumentation employed, as previously reported by Park et al. [13].

Based on the combined XRD and Raman analyses, it was confirmed that NaOH and EDA treatments successfully induced the transformation of cellulose I to cellulose II and III, respectively. Previous studies have reported that lower crystallinity, a smaller crystallite size, and a larger interplanar spacing at hydrophobic crystal planes facilitate the enzymatic hydrolysis of cellulose [41]. From this perspective, the results presented in Figure 1 suggest that the enzymatic hydrolysis cellulose II formed via NaOH treatment may be more efficient than that of cellulose III and native cellulose I.

### 3.2. Enzymatic Hydrolysis of Cellulose and Lignocellulosic Materials

The efficiency of the enzymatic hydrolysis of the cellulose samples with different crystalline structures is presented in Figure 2. The efficiency of enzymatic hydrolysis was evaluated based on the mass loss of solid residue relative to initial substrate mass after a designated reaction time. As anticipated from the structural characteristics shown in Figure 1, the extent of mass loss followed the order: Avicel II > Avicel III > Avicel (control), reflecting differences in the crystallinity, crystallite size, and interplanar spacing.



**Figure 2.** Solid mass loss after enzymatic hydrolysis of Avicel samples before and after allomorphic transformation. NaOH-treated Avicel (cellulose II) exhibited enhanced enzymatic hydrolysis compared with cellulose I and III, reflecting differences in crystalline structure as identified by XRD and Raman analyses.

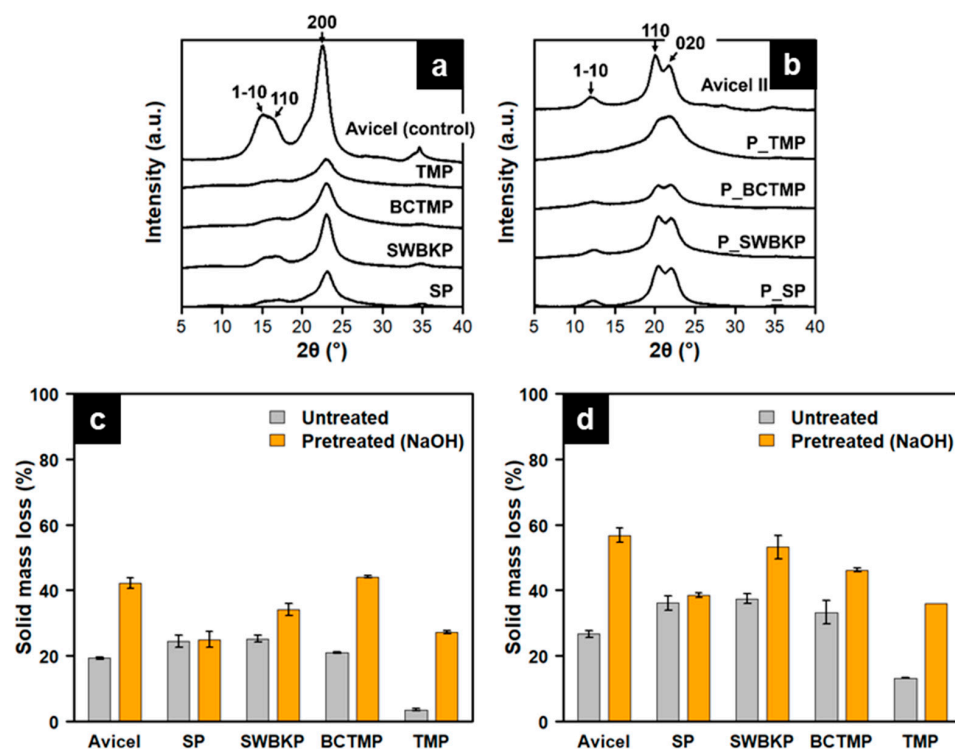
The mass loss trends for Avicel II and Avicel III were similar during the initial stage of the reaction; however, after 12 h, the mass loss of Avicel II increased more markedly. After 48 h of enzymatic hydrolysis, the mass loss reached approximately 67% for Avicel II and 55% for Avicel III, indicating that the enzymatic hydrolysis of Avicel II had the highest efficiency under identical reaction conditions. These results are in line with previous studies reporting that the transformation of cellulose I to cellulose II enhances the efficiency of enzymatic hydrolysis [12,18,19,42].

Nevertheless, considering the report by Cui et al. [11], which suggests that the efficiency of enzymatic hydrolysis may be governed more strongly by the absolute fraction of amorphous regions rather than by the cellulose allomorph itself, it may be premature to conclude that cellulose II is intrinsically more susceptible to enzymatic hydrolysis than cellulose III or cellulose I. von Schreeb et al. [10] also pointed out the limitations of predicting the accessibility and chemical reactivity of cellulose based solely on the crystalline structure and proposed the water retention value (WRV) as an alternative descriptor. In this context, a higher WRV reflects an increased contribution of amorphous regions and enhanced fiber swelling, which collectively improve the enzymatic accessibility and chemical reactivity [9,10,43]. However, WRV also has clear limitations as an indicator for assessing the enzymatic hydrolysis rate. For instance, hardwood fibers generally exhibit higher WRVs than bleached softwood pulp due to their higher xylan content, which contributes to an increased surface charge density [44]. Similarly, unbleached softwood pulp shows elevated WRV as a result of residual lignin-derived charges [45].

Despite this, both hemicelluloses and lignin are well known to hinder enzymatic hydrolysis by restricting enzyme accessibility and promoting non-productive enzyme adsorption [44]. Therefore, an increased WRV does not necessarily translate into enhanced enzymatic hydrolysis. From the authors' perspective, each of these factors represents only one of several parameters that may need to be considered when evaluating the enzymatic hydrolysis rate of cellulose in biomass samples.

Numerous previous studies have employed microcrystalline cellulose (MCC), such as Avicel and Whatman cellulose, as model substrates for evaluating the enzymatic hydrolysis of cellulose materials subjected to various pretreatments. However, MCC differs fundamentally from lignocellulosic biomass in terms of both the structural and physicochemical characteristics, where the latter contains lignin–carbohydrate complex (LCC) structures. MCC is a highly purified form of cellulose typically obtained by dissolving pulp produced via sulfite pulping or from cotton linters, followed by subsequent acid hydrolysis to remove non-cellulosic components such as hemicellulose and residual lignin [46]. In contrast, lignocellulosic biomass consists of cellulose and hemicellulose covalently linked to lignin through LCC structures, in which the hydrophobic crystal planes of cellulose are associated with the aromatic structure of lignin via hydrophobic interactions, resulting in a more complex and recalcitrant architecture [47–49].

Accordingly, to verify if the effect of NaOH pretreatment observed in Figure 2 can be extended to actual lignocellulosic biomass, the same pretreatment conditions were applied to different pulp feedstocks, and their enzymatic hydrolysis efficiencies were evaluated. The results are presented in Figure 3. The XRD patterns of the untreated pulp samples (Figure 3a,b) exhibited characteristic diffraction peaks at  $2\theta \approx 14.7^\circ$ ,  $15.6^\circ$ , and  $22.5^\circ$ , corresponding to the (1–10), (110), and (200) crystallographic planes, respectively, which are typical of the cellulose I $\beta$  structure. After NaOH treatment, these peaks shifted to approximately  $2\theta = 12.1^\circ$ ,  $20.1^\circ$ , and  $21.7^\circ$ , corresponding to the (1–10), (110), and (020) planes, confirming the transformation of cellulose I $\beta$  to cellulose II in the lignocellulosic pulp samples.



**Figure 3.** XRD patterns of lignocellulosic samples before and after NaOH pretreatment. “Pretreated (NaOH)” denotes samples pretreated with 20% NaOH. (a) Diffraction patterns of untreated samples, exhibiting cellulose I-like features. (b) Diffraction patterns of NaOH-pretreated samples, showing cellulose II-like characteristics. (c) Solid mass loss after 6 h of enzymatic hydrolysis. (d) Solid mass loss after 24 h of enzymatic hydrolysis.

Figure 3c,d shows the solid mass loss after 6 and 24 h of enzymatic hydrolysis of the lignocellulosic pulps, respectively. After 24 h of reaction, the mass loss of the NaOH-pretreated samples increased compared to that of their untreated counterparts. Notably, the enzymatic hydrolysis efficiency increased by 15.8%, 12.9%, and 22.7% for SwBKP, BCTMP, and TMP, respectively. In contrast, no significant increase in the mass loss was observed for the sulfite pulp sample.

The observed differences in enzymatic hydrolysis behavior among the various pulps can be attributed to differences in chemical composition and structural characteristics arising from their respective pulping processes. In the case of bleached chemical pulps such as SP and SWBKP, the cellulose purity is relatively high due to the effective removal of non-cellulosic components. However, these pulps also tend to exhibit a comparatively higher crystallinity [50], which can limit enzymatic accessibility. In contrast, mechanical pulps such as BCTMP and TMP are expected to be influenced by residual lignin. Nevertheless, both hemicelluloses and lignin are largely amorphous in nature [13], and their presence does not directly correspond to crystalline constraints. Instead, factors such as the increased specific surface area and fiber disruption induced by mechanical processing must also be considered.

This result suggests inherent limitations in interpreting the enzymatic hydrolysis efficiency of cellulose solely based on changes in the crystalline structure. Enzymatic hydrolysis is influenced not only by crystallinity but also by multiple structural parameters, including the surface area, degree of polymerization, and particle size [51]. For instance, Wang et al. [5] reported that acid-hydrolysis pretreatment prior to enzymatic hydrolysis reduced the degree of polymerization of cellulose, thereby enhancing the hydrolysis efficiency.

Moreover, individual cellulolytic enzymes involved in cellulose hydrolysis exhibit distinct modes of action, making it difficult to generalize the relationship between the crystalline structure of cellulose and enzymatic activity. Cellobiohydrolase (CBH), a representative exo-acting enzyme, hydrolyzes crystalline cellulose chains from their termini to release cellobiose, whereas endoglucanase (EG) primarily cleaves internal glycosidic bonds in amorphous regions.  $\beta$ -Glucosidase (BG) subsequently converts cellobiose into glucose [13]. According to Lynd et al. [52], CBH exhibits relatively higher activity toward crystalline cellulose than EG, which preferentially acts on amorphous regions.

Overall, the results presented in Figure 3 demonstrate that NaOH pretreatment provides a measurable improvement in the enzymatic hydrolysis efficiency of lignocellulosic pulps. However, these effects cannot be fully explained by transformation of the crystalline structure alone. Therefore, the enzymatic hydrolysis of NaOH-pretreated lignocellulosic materials should be interpreted by considering not only the allomorphic transformation but also the combined effects of the degree of polymerization, surface area, and enzyme–substrate interactions.

### 3.3. Technical Implications and Limitations

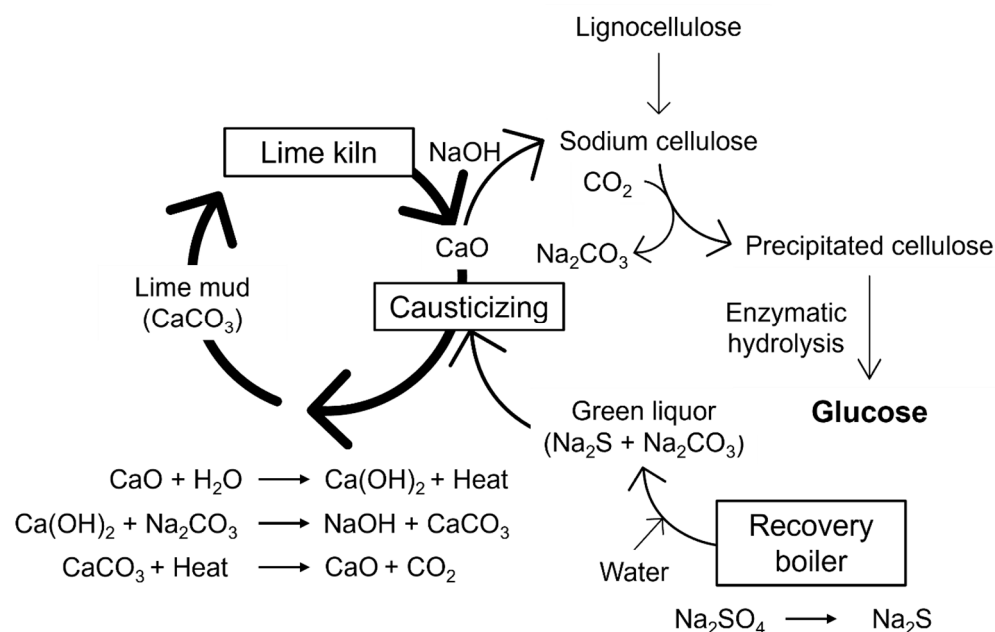
Based on the results obtained in this study, the transformation of the crystalline structure of lignocellulosic pulp into cellulose II via NaOH pretreatment appears to contribute positively to the efficiency of enzymatic hydrolysis by reducing the crystallinity, decreasing the size of the crystallites, and increasing the interplanar spacing. These structural modifications synergistically enhance the accessibility of cellulose to enzymes, thereby facilitating enzymatic hydrolysis to a certain extent.

However, a process configuration that involves transformation of the crystalline structure through alkaline treatment followed by neutralization via acid treatment prior to saccharification may introduce additional complexity and increased costs given the increased number of unit operations. From the perspective of overall process efficiency, these factors should be carefully considered and further evaluated.

Nevertheless, when viewed in the context of industrial Kraft pulping processes (Figure 4), potential strategies for mitigating these limitations can be envisioned. In the chemical recovery cycle of Kraft pulping, sodium carbonate ( $\text{Na}_2\text{CO}_3$ ), a major component of green liquor, can be regenerated into NaOH through the reaction with calcium hydroxide ( $\text{Ca}(\text{OH})_2$ ). Furthermore, pH adjustment following alkaline treatment can be achieved by injecting carbon dioxide, thereby enabling the utilization of  $\text{CO}_2$  generated during the reburning step of the causticization process (Figure 4). According to Wang et al. [5], integrating such chemical recovery and recycling strategies may partially offset the economic burden associated with the increased complexity of the process.

Overall, these considerations suggest that NaOH-based allomorphic transformation of the crystal structure has potential as a pretreatment strategy for enhancing the enzymatic hydrolysis of lignocellulosic pulps, provided that process integration and chemical recovery schemes are appropriately designed to ensure economic and operational feasibility.

Future work will focus on optimizing NaOH pretreatment conditions, including concentration and treatment time, across a broader range of pulp samples, together with more detailed time-course analyses to better elucidate enzymatic hydrolysis kinetics. In addition, further characterization parameters such as the degree of polymerization and surface morphology will be incorporated to provide a more comprehensive understanding of structure–hydrolysis relationships.



**Figure 4.** Flow chart of a hypothetical mill for enzymatic hydrolysis of lignocellulosic products in the chemical recovery cycle during Kraft pulping. Redrawn from Wang et al. [5] with slight modifications.

Although EDA pretreatment showed lower performance than NaOH pretreatment in the present study, systematic investigations of EDA-based treatments under varied concentrations and treatment durations will also be conducted. Despite its known toxicity, EDA is a regenerable solvent and has been reported to maintain pretreatment performance upon reuse [23]. Therefore, analogous to NaOH recovery via lime-based regeneration processes, recyclable EDA systems may offer potential environmental and economic advantages if effective recovery and reuse strategies are implemented. This hypothesis warrants further investigation in future studies.

#### 4. Conclusions

XRD and Raman spectroscopic analyses confirmed that NaOH and EDA treatments induce the transformation of cellulose I to cellulose II and cellulose III, respectively. These allomorphic transformations of the crystalline structure reduced the crystallinity, increased the interplanar spacing, and decreased the crystallite size, thereby enhancing the efficiency of enzymatic hydrolysis of a standard cellulose substrate (Avicel). When NaOH pretreatment, which was most effective for increasing the efficiency of enzymatic hydrolysis, was applied to lignocellulosic pulps, the hydrolysis of certain pulp samples improved, demonstrating the potential applicability of allomorph transformation-based pretreatment strategies to lignocellulosic materials. However, the improvements were not comparable across all samples, indicating that the efficiency of enzymatic hydrolysis cannot be explained solely by changes in the crystalline structure. Rather, it is governed by the combined effects of multiple factors, including the content of lignin and hemicellulose, degree of polymerization, particle size, accessible surface area, and enzyme-specific modes of action. These findings suggest that the efficiency of enzymatic hydrolysis of lignocellulosic pulps should be interpreted from a multifaceted perspective that incorporates substrate accessibility and enzyme–substrate interactions rather than relying on crystallinity alone as a single descriptor. Future studies should focus on optimizing process parameters such as the reaction time, temperature, and enzyme loading, as well as on developing integrated analytical frameworks for quantitation of the enzymatic hydrolysis efficiency based on both structural and kinetic factors.

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