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The Evolution of Cellulose Crystallinity During the Entire Wheat Straw Organosolv Biorefinery Process and Its Relationship with Enzymatic Hydrolysis

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Abstract

Cellulose crystallinity is frequently associated with lignocellulosic biomass digestibility, yet its development during multistep biorefinery processing and its relationship with enzymatic hydrolysis remain difficult to isolate. This study investigated the evolution of cellulose crystallinity in wheat straw (*Triticum aestivum*) during Hot-Water Pretreatment (HWP), Water Pretreatment (WP), Organosolv extraction, sequential washing, and drying, and related these changes to enzymatic glucose yield. Crystallinity was determined using X-ray diffraction and an ATR-FTIR-based PLS model, while enzymatic hydrolysis was evaluated by HPLC-based glucose quantification. HWP caused a temperature-dependent decrease in crystallinity from 47.5 ± 1.3% in untreated straw to 27.0 ± 2.2% at 120 °C, whereas WP at room temperature caused no significant change. However, without subsequent Organosolv extraction, both pretreatments alone resulted in low glucose yields of approximately 10%, indicating that cellulose crystallinity is only one of several factors governing enzymatic hydrolysis efficiency. During washing after Organosolv extraction, crystallinity increased from 40.6 ± 2.1% to 54.2 ± 1.3%, whereas, under the tested conditions, glucose yield was more closely associated with the estimated residual ethanol concentration than with the change in crystallinity. Drying had the strongest effect, increasing crystallinity by up to 53.6% relative to the wet state. Overall, cellulose crystallinity should be considered as one of several interacting factors governing enzymatic digestibility, and sample moisture history must be carefully controlled when comparing crystallinity data.

Keywords: wheat straw; *Triticum aestivum*; organosolv; enzymatic hydrolysis; cellulose; crystallinity

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1. Introduction

The increasing demand for sustainable alternatives to fossil-based resources has intensified interest in lignocellulosic biomass as a renewable feedstock for biorefineries due to its potential for high sugar yields following efficient fractionation and enzymatic hydrolysis [1]. Among agricultural residues, wheat straw is one of the most abundant and economically attractive resources worldwide [2,3]. Due to its high cellulose content (34–40%), wheat straw represents a promising raw material for the production of biofuels and bio-based chemicals [4–6]. However, the efficient conversion of cellulose is hindered by the recalcitrant lignocellulosic structure, in which lignin and hemicellulose restrict enzyme accessibility and limit sugar yields during enzymatic hydrolysis [7–9].

To overcome these limitations, effective pretreatment and fractionation strategies are required. One established approach for lignocellulosic biomass is Organosolv fractionation [10]. In previous work, our research group developed a sequential treatment strategy combining relatively low-temperature Hot-Water Pretreatment (HWP) or Water Pretreatment (WP) with subsequent Organosolv extraction. This combined approach significantly improved the hydrolysability of wheat straw, achieving cellulose conversion efficiencies of up to approximately 95% under optimized conditions [11]. While these studies demonstrated the effectiveness of the pretreatment–Organosolv approach, the structural mechanisms responsible for the improved hydrolysis performance have not yet been fully clarified.

Cellulose crystallinity is one of the structural characteristics commonly associated with biomass recalcitrance and enzymatic hydrolysability. Highly ordered crystalline regions are generally considered less accessible to cellulolytic enzymes than amorphous cellulose. As a result, enzyme adsorption may be reduced and hydrolysis efficiency may be limited [12]. However, enzymatic digestibility is governed by multiple interacting factors, including lignin content, hemicellulose distribution, pore structure, surface accessibility, and particle size [13–16]. Consequently, the influence of crystallinity on enzymatic hydrolysis is often difficult to isolate and remains a subject of ongoing discussion. In lignocellulosic biorefinery processes, crystallinity may change throughout multiple processing stages. During pretreatment and Organosolv extraction, structural modifications of the cell wall occur together with lignin removal and compositional changes. These alterations can significantly affect the measured crystallinity [15]. Furthermore, subsequent processing steps may also affect cellulose structure. Washing removes residual lignin, catalyst, and organic solvent from the biomass and may therefore influence both crystallinity and enzymatic hydrolysis performance [17–19]. Drying can induce hornification, reduce pore volume, promote the reformation of hydrogen-bond networks, and alter cellulose accessibility, thereby affecting both the measured crystallinity and the hydrolysis behavior of the biomass [16].

Although numerous studies have investigated the effects of pretreatment and Organosolv extraction on biomass structure, systematic investigations of crystallinity development throughout the complete HWP/WP-assisted Organosolv processing chain remain limited. In particular, little information is available regarding the combined effects of pretreatment, Organosolv extraction, sequential washing, and drying on cellulose crystallinity and their relationship with enzymatic hydrolysis performance. Therefore, the present study investigates the evolution of cellulose crystallinity during HWP, WP, Organosolv extraction, sequential washing, and drying. By combining crystallinity measurements with enzymatic hydrolysis data, the study aims to identify the processing steps that most strongly influence crystallinity and to evaluate the extent to which crystallinity changes are reflected in the enzymatic conversion efficiency of wheat straw.

2. Materials and Methods

2.1. Experimental Workflow

The overall experimental workflow of this study (see Figure 1) comprised seven main steps: straw comminution, pretreatment, Organosolv extraction, sample washing, hydrolysis, HPLC analysis and crystallinity analysis. In addition to the hydrolysis-focused investigations, the study examined changes in the crystallinity of straw samples throughout the processing chain. Crystallinity measurements were performed on samples after comminution, after pretreatment, after Organosolv extraction, and after the individual washing steps. Furthermore, the crystallinity of selected samples was correlated with their corresponding enzymatic hydrolysis performance. This allowed the relationship between cellulose crystallinity and hydrolysis efficiency to be evaluated. This approach

enabled a comprehensive assessment of how the different processing stages affected both the structural properties of the biomass and its subsequent enzymatic digestibility.

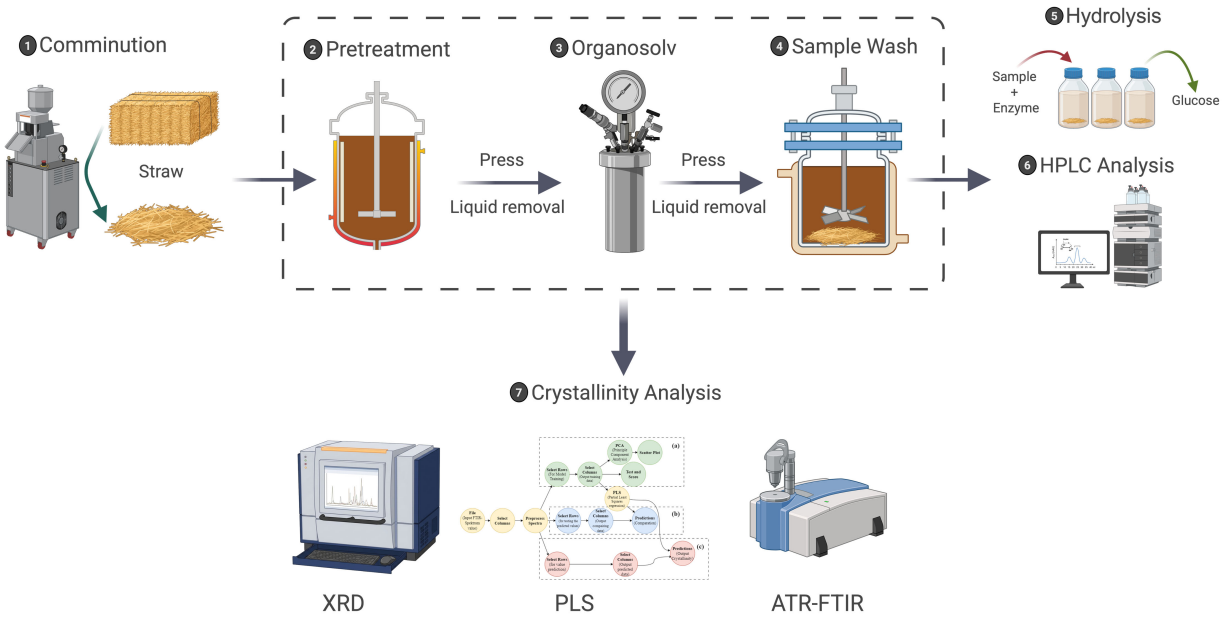

Figure 1. Schematic overview of the experimental workflow. (1) Comminution; (2) Pretreatment; (3) Organosolv; (4) Sample Wash; (5) Hydrolysis; (6) HPLC Analysis; (7) Crystallinity Analysis. Created in BioRender.com.

2.2. Raw Material and Comminution

In this study, wheat straw (*Triticum aestivum*) was used as the lignocellulosic raw material. The material was harvested from agricultural fields in the Rhineland region of Germany between June and August. After harvest, the straw was baled and transported to the laboratory facilities. Upon arrival, the material was stored in sealed plastic containers to ensure stable storage conditions and minimize environmental influences.

Straw comminution was performed using a laboratory cutting mill (SM300, Retsch, Haan, Germany). The mill was operated at 1000 rpm and equipped with a 6.0 mm sieve to control particle size during grinding. After comminution, the samples were transferred to airtight containers and stored until further use [11].

2.3. Pretreatment

Prior to Organosolv extraction, the biomass was subjected to one of two pretreatment strategies that had previously been shown to improve the performance of the overall Organosolv process. The pretreatment methods investigated in this study were Hot-Water Pretreatment (HWP) and Water Pretreatment (WP) (Figure 2).

For both pretreatment methods, 1.5 kg (dry mass basis) of comminuted wheat straw was loaded into a 50 L reactor (Model 8500, Parr Instrument Company, Moline, IL, USA). Deionized water was added at a liquor ratio of 1:19 (solid-to-liquid mass ratio). In the case of Hot-Water Pretreatment (HWP), the suspension was heated to the desired pretreatment temperature between 75 and 120 °C under continuous stirring at 50 rpm and maintained under these conditions for 90 min. For Water Pretreatment (WP), the suspension was stored at room temperature for eight days and stirred once daily for one minute at 50 rpm to ensure uniform wetting of the biomass. After completion of the respective pretreatment, the reactor contents were allowed to cool or equilibrate to room temperature, after which the biomass was removed and subjected to mechanical liquid removal prior to further processing [11].

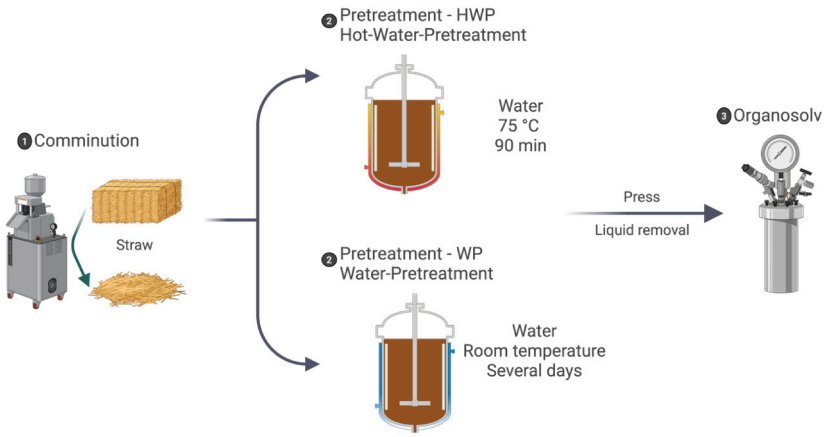

Figure 2. Schematic overview of the HWP and WP pretreatment procedures. Created with BioRender.com [11].

2.4. Organosolv Extraction

Organosolv extraction experiments were carried out in a 7.5 L high-pressure reactor (Model 4550, Parr Instrument Company, Moline, IL, USA). For each experiment, 100 g of biomass (dry mass basis) was loaded into the reactor and mixed with a 60 wt% ethanol–water solution at a liquor ratio of 1:19 (solid-to-liquid mass ratio). Sulfuric acid was used as the catalyst, with the catalyst loading varied according to the experimental design and expressed as a percentage of the sample dry mass.

The reactor was heated to the target extraction temperature of between 135 and 170 °C. Both the catalyst loading and the residence time at the target temperature were adjusted according to the respective experimental conditions. Following the extraction, the reactor was allowed to cool naturally to room temperature. The solid fraction was subsequently recovered and subjected to hydraulic press and liquid removal prior to further processing and analysis.

2.5. Sample Washing

Following Organosolv extraction, the samples were subjected to a washing procedure to remove residual lignin, ethanol, and catalyst from the biomass. The washing process was carried out at 60 °C to maintain a high lignin solubility and thereby facilitate the removal of lignin residues. To ensure consistent washing conditions and stable temperature control, all washing experiments were performed in a double-jacket reactor (DWK Life Sciences, Wertheim, Germany) equipped with a mechanical stirrer (HS-100D, witeg Labortechnik GmbH, Wertheim, Germany) operating at 150 rpm.

The washing procedure consisted of three sequential steps (see Table 1). Samples were collected after each washing step for subsequent crystallinity analysis, allowing structural changes in the biomass during lignin removal to be monitored throughout the process. After completion of the washing procedure, the samples were mechanically dewatered prior to further characterization.

Table 1. Overview of the sequential washing steps following organosolv extraction.

Wash Steps	Wash Solution	Liquor Ratio *
1	60% (*w*/*w*) ethanol–water solution	1:5
2	Water	1:20
3	Water	1:10

* Liquor ratio is based on the dry mass of the sample.

2.6. Sample Drying

To evaluate the influence of drying conditions on cellulose crystallinity, approximately 10 g (wet mass basis) of each sample was subjected to one of three drying methods prior to crystallinity analysis. The first method consisted of vacuum desiccation at room temperature. Samples were placed in a desiccator containing phosphorus pentoxide (P_2O_5) as a desiccant and maintained under a vacuum of approximately 100 mbar for seven days. In addition, convective drying was performed in a laboratory drying oven (UNE 500, Memmert, Schwabach, Germany) at either 50 °C or 105 °C until a constant sample mass was reached.

2.7. Crystallinity Analysis

In this study, the crystallinity of cellulose-containing samples was determined using two complementary analytical approaches: X-ray diffraction (XRD) and an attenuated total reflectance Fourier-transform infrared spectroscopy (ATR-FTIR)-based partial least squares (PLS) model.

XRD measurements were performed using an X-ray diffractometer (D2 Phaser, Bruker Corporation, Billerica, MA, USA) equipped with a Cu Kα radiation source (λ = 1.54184 Å). Wet fractionated biomass was analyzed without prior drying. For each scan, a fresh portion of wet biomass was loaded into a Bruker D83 specimen holder (Bruker Corporation, Billerica, MA, USA) made of poly(methyl methacrylate) (PMMA), which contained a 6 mm-deep specimen cavity. After loading, the sample surface was gently levelled flush with the holder rim to maintain a consistent specimen height and minimize displacement-related errors. The relatively deep cavity accommodated the wet fibrous material and helped maintain the sample in its wet state during measurement, thereby minimizing drying-induced structural alterations. Three separately loaded specimens were measured for each condition, using fresh sample material for every scan. Measurements were conducted in continuous position-sensitive detector (PSD) scan mode using a LYNXEYE detector (Bruker AXS GmbH, Karlsruhe, Germany) operated in one-dimensional mode. Diffraction patterns were recorded over a 2θ range of 5–40° with a step size of 0.1° and a counting time of 4 s per step. A total of 346 measurement points were collected. The detector slit width was set to 30 mm. A divergence slit of 0.2 mm, a scatter slit of 1.0 mm, and a 2.5° Soller module were used throughout the measurements. The crystallinity was calculated using the peak-area (A) method according to Equation (1) [20–23]. Diffraction data were processed using DIFFRAC.EVA version 5.2 (Bruker AXS GmbH, Karlsruhe, Germany).

$$Crystallinity\ [\%] = \frac{A_{crystalline}}{A_{crystalline} + A_{amorphous}} \cdot 100 \quad (1)$$

Crystallinity was also estimated using an ATR–FTIR-based PLS regression model developed within the broader research project using Orange Data Mining software (University of Ljubljana, Ljubljana, Slovenia). The calibration dataset comprised 230 cellulose-containing samples analyzed between 2021 and 2023. XRD-derived crystallinity values were used as reference values, while the corresponding infrared spectra were recorded using a Spectrum 3 ATR-FTIR spectrometer (PerkinElmer, Waltham, MA, USA) over the spectral range of 4000–550 cm^{-1}. Before model development, the spectra were smoothed using a Savitzky–Golay filter with a derivative order of 0, a fifth-order polynomial, and a window width of seven data points. The preprocessed spectra and the corresponding XRD-derived reference values were subsequently used to calibrate a PLS regression model containing 30 latent variables. The calibration model yielded a coefficient of determination of R^2 = 0.934. The resulting model was then applied to estimate the crystallinity of the

relevant process samples from their ATR-FTIR spectra. The model output represents an estimate of XRD-derived crystallinity and was not used to determine cellulose allomorphs.

2.8. Hydrolysis

Enzymatic hydrolysis was performed to evaluate the effect of pretreatment and sample processing conditions on glucose release from wheat straw. For each experiment, 1 g of sample (dry mass basis) was suspended in 25 mL of 0.1 M sodium acetate buffer (pH 5.0), corresponding to a solids concentration of 40 g/L. Prior to enzyme addition, the suspension was pasteurized in an incubator (KS 4000 i control, IKA-Werke GmbH & Co. KG, Staufen im Breisgau, Germany) at 77 °C for 2 h to suppress microbial contamination. After cooling to approximately 55 °C, a commercial cellulase solution (C2610-10, US Biological, Salem, MA, USA) was added under sterile conditions at a loading of 12 wt% based on the dry mass of the sample. The enzyme solution had a specified activity of 6200–7580 U/g, where one unit (U) is defined as the amount of enzyme required to release 1 µmol of reducing sugars (expressed as glucose equivalents) per minute under standard assay conditions [11].

The sealed reaction vessels were incubated at 55 °C for 48 h. To maintain homogeneous reaction conditions and prevent sedimentation of the biomass particles, continuous shaking was applied at 130 rpm with an amplitude of 20 mm (KS 4000 i control, IKA-Werke GmbH & Co. KG, Staufen im Breisgau, Germany). All hydrolysis experiments were performed in triplicate. The glucose yield was calculated from the glucose concentration determined by HPLC and the initial total solids concentration of the hydrolysis slurry, corresponding to 40 g of dry biomass per liter, according to Equation (2) [11].

$$Glucose\ yield\ [\%] = \frac{C_{glucose}\ [g/L]}{40\ g/L} \cdot 100 \quad (2)$$

Two complementary hydrolysis procedures were used for sample characterization. Compositional analysis was carried out by total acid hydrolysis, whereas enzymatic hydrolysis was used to determine the glucose yield. Total hydrolysis was conducted according to the NREL protocol [24]. Briefly, approximately 300 mg of dry biomass was first treated with 72 wt% H_2SO_4 for 1 h. The acid concentration was subsequently reduced to 4 wt%, and hydrolysis was continued for a further 1 h. The resulting hydrolysates were used for the determination of the carbohydrate composition, while the remaining insoluble fraction was considered for lignin analysis. All measurements were performed in at least triplicate, and the reported values represent the mean of the individual determinations.

2.9. HPLC Analysis

Prior to high-performance liquid chromatography (HPLC) analysis, samples were first centrifuged at 16,100 rcf for 15 min using a centrifuge (5415 D, Eppendorf AG, Hamburg, Germany). The resulting supernatant was then diluted to fall within the respective calibration range and subsequently filtered through a 0.22 µm polyethersulfone membrane (Wicom, Heppenheim, Germany).

Sugar analysis in the hydrolysate was performed using HPLC with a system (1100 Series, Agilent Technologies, Santa Clara, CA, USA) equipped with a Repromer H+ column (300 × 8 mm, Dr. Maisch, Ammerbuch, Germany). The column temperature was maintained at 30 °C, and detection was carried out using a refractive index detector (1260 Infinity II, Agilent Technologies, Santa Clara, CA, USA) set to 35 °C. The mobile phase consisted of 5 mM sulfuric acid at a flow rate of 0.6 mL/min [11,25].

2.10. UV-Vis Analysis

The presence of dissolved lignin in the pretreatment liquor was evaluated by UV-Vis spectrophotometry using a UV-Vis spectrophotometer (Ultrospec 2100 Pro, Amersham BioScience, Buckinghamshire, UK). Prior to analysis, 1 mL of each sample was diluted with deionized water to obtain an absorbance within the linear range of the instrument. The absorbance was subsequently measured at 240 nm [24].

3. Results and Discussion

3.1. Evaluation of the Impact of Pretreatment on Sample Crystallinity and Enzymatic Hydrolysis

The effects of Hot-Water Pretreatment (HWP) and Water Pretreatment (WP) on cellulose crystallinity and enzymatic hydrolysis performance were investigated. HWP was investigated at pretreatment temperatures ranging from 75 to 120 °C. In contrast, WP was evaluated over pretreatment durations of 0–8 days. By correlating crystallinity measurements with enzymatic hydrolysis results, the influence of pretreatment-induced structural changes on biomass digestibility could be assessed.

In terms of crystallinity (Figure 3A), the untreated straw exhibited a crystallinity index of 47.5 ± 1.3%. A pronounced temperature-dependent decrease in crystallinity was observed following Hot-Water Pretreatment (HWP), reaching a minimum value of 27.0 ± 2.2% at 120 °C, corresponding to a statistically significant reduction of 43.2% relative to the untreated sample ($p < 0.05$). In contrast, Water Pretreatment (WP) performed at room temperature caused no significant changes in crystallinity over the investigated period of eight days ($p > 0.05$), with values remaining close to those of the untreated straw.

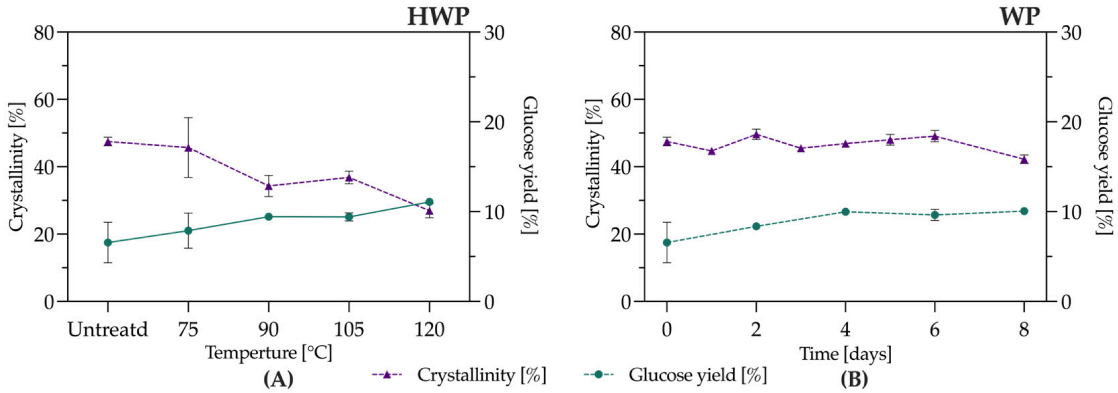

Figure 3. Effect of HWP temperature (**A**) and WP duration (**B**) on cellulose crystallinity and enzymatic glucose yield of wheat straw. Error bars indicate standard deviations of the mean ($n = 3$).

The decrease in crystallinity after HWP may reflect temperature-induced structural modifications within the lignocellulosic matrix [26–28]. Elevated temperatures promote partial hemicellulose solubilization and weaken the structural constraints surrounding cellulose microfibrils [26,27]. In addition, hot water may facilitate cellulose swelling and partial disruption of intermolecular hydrogen bonds, resulting in reduced molecular ordering. In contrast, the absence of elevated temperature during WP limits hemicellulose hydrolysis and structural rearrangements, thereby largely preserving the crystalline structure of cellulose [29–31].

The observed changes in crystallinity were accompanied by changes in biomass composition. Compositional analysis based on total hydrolysis measurements showed that the cellulose content increased from 33.09 ± 1.23% in untreated wheat straw to 37.47 ± 1.82% after HWP at 120 °C, while the hemicellulose content decreased from 26.67 ± 2.31% to 23.22 ± 1.10%. Both hemicellulose and lignin showed overall decreasing trends with

increasing HWP temperature, consistent with the partial solubilization of non-cellulosic components [26–28]. The effect of HWP on apparent crystallinity is therefore not unidirectional. Partial removal of amorphous hemicellulose and lignin can increase the relative contribution of crystalline cellulose, whereas thermal swelling and disruption of intermolecular hydrogen bonding may reduce cellulose ordering [32–35]. In the present study, however, crystallinity decreased with increasing HWP temperature. This contrasting behavior may be related to the fact that the HWP and WP samples analyzed in this section were not subjected to an additional washing step prior to crystallinity analysis. Residual hemicellulose, lignin, and other solubilized components remaining within the fiber structure may have restricted the rearrangement of cellulose chains and the formation of more ordered regions, thereby affecting the measured crystallinity. The influence of the subsequent removal of these residual components on cellulose crystallinity is examined in detail in Section 3.2.

Despite these structural changes, both pretreatment methods alone resulted in relatively low glucose yields of approximately 10% (Figure 3B), which is substantially lower than the values of around 95% obtained after subsequent Organosolv extraction [11,36]. Although partial lignin dissolution was evidenced by the brown coloration of the pretreatment liquor and the corresponding UV absorbance at 240 nm, the mild pretreatment conditions were insufficient to remove substantial amounts of lignin and hemicellulose [37–39]. Compositional analysis based on the NREL total hydrolysis method showed that, even after HWP at 120 °C, hemicellulose and lignin together still accounted for at least 60 wt% of the remaining biomass. Their persistence, together with the largely unchanged particle size and compact biomass structure, likely restricted enzyme accessibility and limited hydrolysis efficiency [40,41]. Nevertheless, HWP samples showed a statistically significant increase ($p < 0.05$) in glucose yield with increasing pretreatment temperature, reaching an overall improvement of approximately 68%. This trend suggests that increasing the HWP temperature progressively improved biomass accessibility prior to Organosolv extraction. Although pretreatment alone remained insufficient to achieve high glucose yields, the higher hydrolysis performance at elevated temperatures indicates that increasing disruption of the lignocellulosic matrix facilitated subsequent enzymatic attack.

3.2. Evaluation of the Impact of Washing on Sample Crystallinity

The washing procedure represents a critical step following Organosolv extraction, as it facilitates the removal of residual lignin, catalyst, and organic solvent retained within the biomass. Efficient removal of these components is considered essential for achieving high enzymatic hydrolysis performance and overall conversion efficiency in subsequent processing steps. Accordingly, the present study also investigated changes in cellulose crystallinity throughout the washing procedure. Multiple experiments were conducted to determine the average crystallinity following each washing step. Furthermore, the obtained crystallinity data were compared with the enzymatic hydrolysis results of selected Organosolv-treated samples to evaluate the relationship between cellulose crystallinity and hydrolysis performance.

Crystallinity analysis (Figure 4A) revealed a significant increase in cellulose crystallinity during the washing procedure ($p < 0.05$). The Organosolv-treated samples exhibited an initial crystallinity of 40.6 ± 2.1%, which increased to 46.1 ± 2.2% after the first washing step with a 60% (w/w) ethanol–water solution. A further increase to 51.5 ± 6.6% was observed after the second washing step with water, while the third washing step resulted in only a minor increase to 54.2 ± 1.3%. Overall, crystallinity increased by 13.6 percentage points, corresponding to a relative increase of approximately 33.5% compared with

the unwashed material. Most of the increase in crystallinity therefore occurred during the first two washing steps.

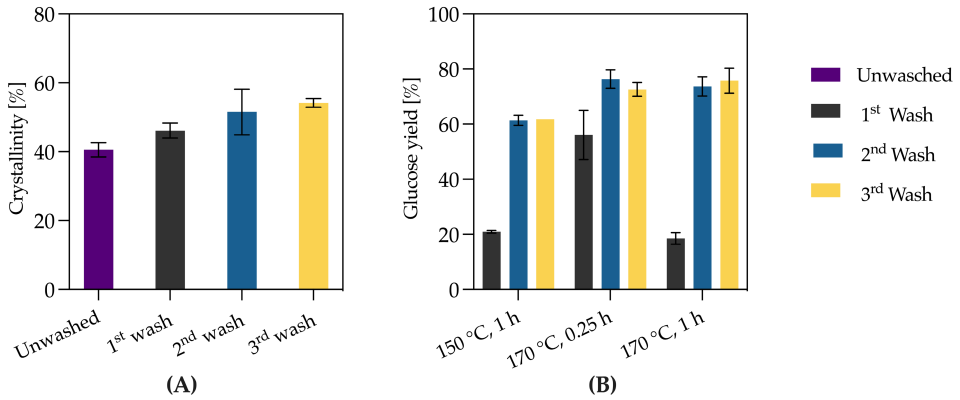

Figure 4. Effect of washing on cellulose crystallinity and glucose yield after Organosolv extraction of wheat straw. (**A**) Average cellulose crystallinity of all investigated samples after different washing steps (n = 15). (**B**) Glucose yields obtained after enzymatic hydrolysis of samples subjected to different washing steps following Organosolv pretreatment under various extraction conditions. Error bars indicate standard deviations of the mean (n = 3).

Compositional analysis further confirmed changes in the solid composition during washing. The Organosolv-treated samples contained on average 82.5 ± 5.4% cellulose, with the complete washing sequence resulting in a mean absolute increase of 2.86% in cellulose content and a corresponding mean absolute decrease of 1.73% in lignin content. These compositional changes provide additional evidence for the progressive removal of non-cellulosic material from the Organosolv-treated solid during washing.

The observed increase in crystallinity may partly result from the progressive removal of residual lignin, hemicellulose, and other low-molecular-weight compounds remaining in the biomass after Organosolv extraction (Figure 5). These components may occupy spaces between or around cellulose chains and fibrils, thereby restricting close molecular packing and the formation of intra- and intermolecular hydrogen bonds. Their removal during washing may therefore allow cellulose chains to approach each other more closely, facilitating local rearrangement and the further development or extension of ordered cellulose regions. In parallel, the removal of amorphous non-cellulosic material increases the relative contribution of crystalline cellulose to the measured diffraction signal. The smaller increase observed during the third washing step may indicate that most readily removable components had already been eliminated during the first two washing steps, leaving less scope for further compositional changes.

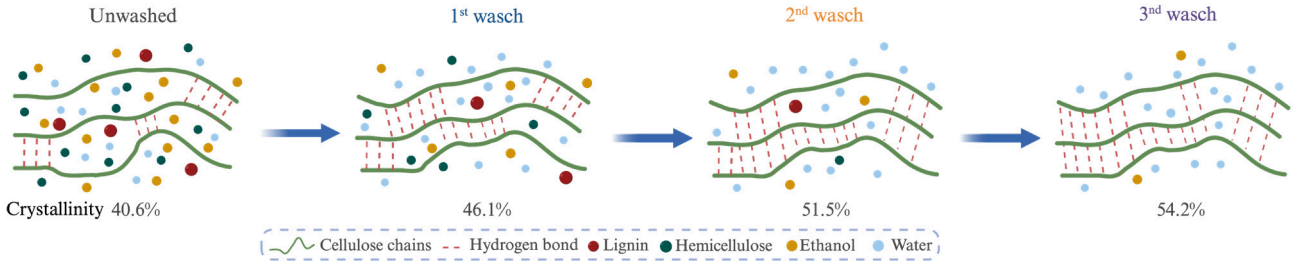

Figure 5. Proposed mechanism of crystallinity increase during sequential washing of Organosolv-treated wheat straw. Created with BioRender.com.

The XRD measurements further support this interpretation. The samples collected after the individual washing steps retained the same principal diffraction features (Figure 6),

with no distinct new reflections or systematic shifts in peak position within the resolution of the measurements. This indicates that the increase in crystallinity occurred without a detectable transition to another cellulose allomorph. Together with the progressive increase in crystallinity during washing, these patterns support an interpretation in which the removal of non-cellulosic components increases the relative contribution of crystalline cellulose while also allowing local rearrangement and ordering within the existing cellulose structure. Thus, the washing-induced increase in crystallinity appears to be associated with changes within the existing cellulose organization rather than with the formation of a different crystalline allomorph.

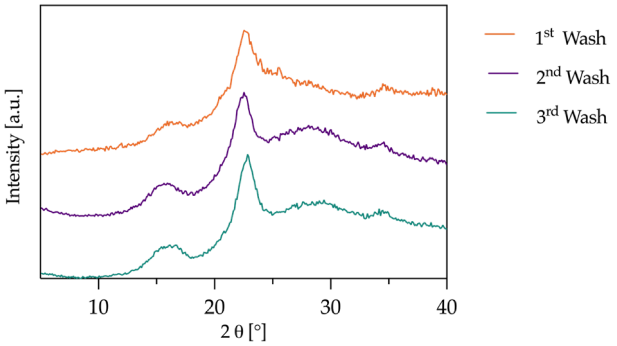

Figure 6. Representative XRD patterns of organosolv-treated wheat straw (150 °C, 4 h) after each step of the sequential washing procedure. The spectra were vertically offset for clarity.

Importantly, all samples from the washing series were mechanically dewatered and analyzed in the wet state without an additional drying step. This experimental design allows the observed increase in crystallinity to be associated specifically with the washing procedure, without introducing structural changes caused by subsequent drying. The results therefore demonstrate that sequential washing itself can substantially alter the measured cellulose crystallinity of Organosolv-treated wheat straw. Although the individual contributions of non-cellulosic component removal and local cellulose rearrangement cannot be quantitatively separated, the combined crystallinity and XRD results provide consistent evidence that washing modifies the structural organization of the cellulose-rich solid.

The enzymatic hydrolysis results (Figure 4B) followed a similar trend. The lowest glucose yield was obtained after the first washing step, most likely due to residual ethanol in the biomass. At this stage, the ethanol concentration in the hydrolysis slurry was approximately 10% (*w*/*w*), a level reported to negatively affect cellulase activity and stability [42]. Consequently, the inhibitory effect of ethanol likely outweighed any potential benefit associated with the relatively low cellulose crystallinity.

After the second and third washing steps, the residual ethanol concentration decreased to approximately 1.6% and 0.26%, respectively. According to the findings of Chen and Jin (2006), the inhibitory effect of ethanol on cellulase activity is expected to be only minor at these concentrations [43]. Despite the continued increase in crystallinity, no statistically significant differences in glucose yield were observed between these washing steps. This finding suggests that the additional crystallinity increase from 51.5% to 54.2% was insufficient to produce a measurable effect on enzymatic hydrolysis. Overall, the results indicate that residual ethanol was the primary factor influencing hydrolysis performance after the first washing step, whereas the relatively small crystallinity changes observed thereafter had only a minor impact on glucose yield under the investigated conditions.

3.3. Evaluation of the Impact of Drying Method and Temperature on Sample Crystallinity

In addition to pretreatment, Organosolv extraction, and washing, sample drying represents another important factor that may influence the measured cellulose crystallinity. Drying can alter the moisture state and structural organization of cellulose and may therefore affect the crystallinity values obtained during analysis. To evaluate this effect, wet samples were compared with samples subjected to different drying methods and temperatures. This comparison was used to assess the extent to which drying conditions and sample preparation history influence the interpretation of cellulose crystallinity.

The results of the experiment showed (Figure 7A) that the Organosolv-extracted samples exhibited the lowest crystallinity in the wet state, reaching 42.0 ± 1.1%. However, all drying methods resulted in a significant increase in crystallinity. Drying in a vacuum desiccator increased crystallinity by 24.7% compared to the wet sample, whereas oven drying produced an average increase of around 53.6%, with the highest value observed at 50 °C (64.5 ± 4.5%). A similar trend was observed for samples subjected to Organosolv extraction without pretreatment. While increasing the extraction time reduced the crystallinity by an average of 18.5% compared to untreated straw (Figure 7B), subsequent drying increased the measured crystallinity by an average of 20.7%.

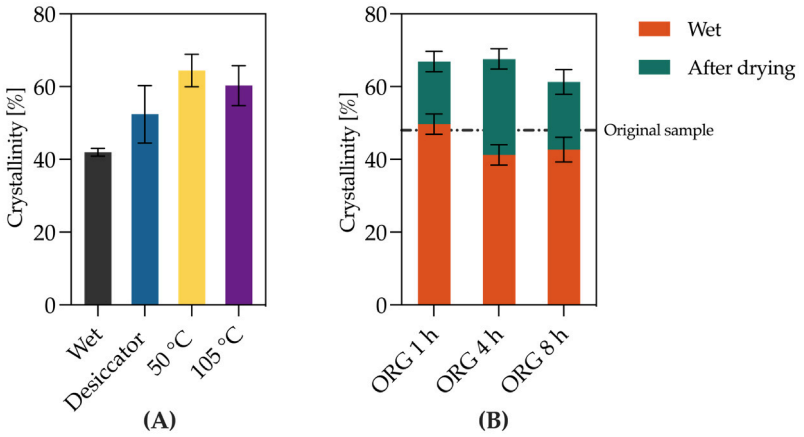

Figure 7. Effect of drying conditions on the crystallinity of Organosolv-treated wheat straw. (**A**) Crystallinity of HWP-pretreated and subsequently Organosolv-extracted (2 h) samples measured in the wet state and after drying under different conditions. (**B**) Changes in crystallinity of non-pretreated wheat straw subjected to Organosolv extraction at 135 °C for different residence times (1–8 h) and subsequently dried in a vacuum desiccator. Error bars indicate standard deviations of the mean (n = 3).

These findings show that measured cellulose crystallinity in lignocellulosic biomass depends on both processing conditions and the drying procedure applied before analysis. The drying-associated increase is consistent with dehydration-related changes in cellulose association and packing, including closer contact between adjacent microfibrils, as illustrated schematically in Figure 8 [20,44–46]. Accordingly, the observed changes are interpreted as variations in relative cellulose ordering during drying rather than as a polymorphic transformation. The magnitude of the crystallinity increase depended on the drying conditions. Vacuum-desiccator drying produced a moderate increase, whereas drying at 50 °C resulted in the highest crystallinity. The value obtained at 105 °C was slightly lower than that at 50 °C but remained substantially higher than that of the wet sample. Overall, the main difference was observed between the wet and dried states, while drying temperature modulated the magnitude of the increase without producing a monotonic temperature-dependent response [47,48].

The enzymatic hydrolysis results provide functional context for the observed changes in crystallinity. Glucose yield decreased from 79.0% for the never-dried sample to 6.4% after drying at 50 °C and 2.6% after drying at 105 °C. Notably, the sample dried at 105 °C exhibited slightly lower crystallinity than the sample dried at 50 °C, yet its glucose yield was substantially lower. This shows that cellulose crystallinity is an important structural parameter but does not, by itself, fully explain the loss of enzymatic hydrolysability caused by drying. Previous studies indicate that drying can simultaneously increase cellulose ordering, promote hornification, reduce fiber swelling, and restrict pore accessibility [16,49–51]. Chen et al. reported that increasing drying temperature or duration in unbleached wheat-straw pulp increased cellulose crystallinity while reducing fiber swelling and altering the fiber-wall pore structure [49]. Koo et al. likewise showed that drying-induced hornification reduced pore volume and enzymatic hydrolysis even without an irreversible change in crystalline structure [16]. Luo and Zhu related the loss of lignocellulose saccharification after drying to reduced water retention and restricted enzyme accessibility through pores in the fiber wall [50], while Salmén and Stevanic showed that microfibril aggregation and hornification can occur without a corresponding measurable change in cellulose crystallinity [51]. Taken together, these studies support the view that crystallinity and physical accessibility may change concurrently during drying, but their effects on enzymatic hydrolysis are not necessarily proportional.

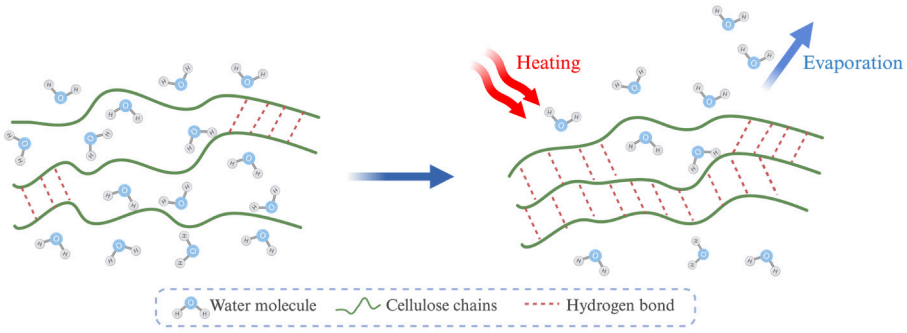

Figure 8. Proposed mechanism of drying-induced cellulose ordering through bound-water removal and hydrogen-bond reformation. Created with BioRender.com.

Therefore, biomass samples should ideally be maintained in the wet state after Organosolv extraction and subsequent washing whenever enzymatic digestibility is to be evaluated. Avoiding drying minimizes irreversible structural changes that may reduce cellulose accessibility and artificially underestimate the effectiveness of the pretreatment or extraction process. Consequently, both the moisture state and drying history of biomass samples should be carefully controlled and reported when comparing crystallinity measurements and enzymatic hydrolysis results across different studies.

4. Conclusions

The results of this study demonstrate that cellulose crystallinity in wheat straw changes substantially throughout the entire biomass processing chain and is influenced not only by pretreatment and Organosolv extraction, but also by subsequent washing and drying procedures. Hot-Water Pretreatment (HWP) significantly reduced cellulose crystallinity from 47.5 ± 1.3% in untreated straw to 27.0 ± 2.2% at 120 °C, corresponding to a reduction of 43.2%. In contrast, Water Pretreatment (WP) performed at room temperature caused no significant changes in crystallinity. Despite this pronounced structural modification, both pretreatment methods alone produced relatively low glucose yields of approximately 10%, indicating that crystallinity reduction alone was insufficient to achieve efficient enzymatic hydrolysis.

The washing experiments further revealed that cellulose crystallinity increased continuously during the removal of residual lignin, catalyst, and solvent. Crystallinity increased from 40.6 ± 2.1% after Organosolv extraction to 54.2 ± 1.3% after the final washing step, corresponding to a relative increase of approximately 33.5%. Representative XRD patterns obtained after the individual washing steps retained the same principal diffraction features, with no obvious new reflections or systematic shifts in peak position within the resolution of the measurements. These results provide no evidence of a detectable transition between cellulose allomorphs, suggesting that the observed increase in crystallinity was associated with changes in ordering within the existing cellulose structure rather than with a change in crystalline form. However, this increase was not accompanied by a corresponding decrease in glucose yield. Once residual ethanol concentrations had been reduced from approximately 10% to below 2%, no statistically significant differences in hydrolysis performance were observed between the second and third washing steps. These findings indicate that residual solvent concentrations exerted a greater influence on enzymatic hydrolysis than the relatively small crystallinity differences generated during washing.

The most pronounced crystallinity changes were observed during sample drying. While the wet Organosolv-treated samples exhibited a crystallinity of only 42.0 ± 1.1%, vacuum-desiccator drying increased this value by 24.7%. The strongest effect was observed after oven drying at 50 °C, where crystallinity increased from 42.0 ± 1.1% to 64.5 ± 4.5%, corresponding to a relative increase of approximately 53.6%. Similar trends were observed for Organosolv-treated samples extracted for different residence times. These results demonstrate that drying can substantially alter the measured crystallinity of lignocellulosic biomass and may therefore influence the interpretation of structure-property relationships. In addition to increasing crystallinity, drying may induce hornification and particle agglomeration, further reducing cellulose accessibility [49,50].

Overall, the results show that cellulose crystallinity alone cannot adequately explain enzymatic hydrolysis performance. Although crystallinity changed significantly during pretreatment, washing, and drying, the hydrolysis results were more strongly influenced by factors such as lignin removal, residual solvent content, biomass accessibility, and sample moisture state. Consequently, crystallinity should be considered as one of several interacting parameters governing biomass digestibility rather than as an isolated predictor of hydrolysis efficiency. Furthermore, because sample preparation procedures can alter crystallinity by more than 20–50%, crystallinity data reported in different studies should be compared with caution unless identical washing, drying, and storage conditions are applied.

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