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Correlating Physical Changes and Enhanced Enzymatic Saccharification of Pine Flour Pretreated by *N*-Methylmorpholine-*N*-oxide

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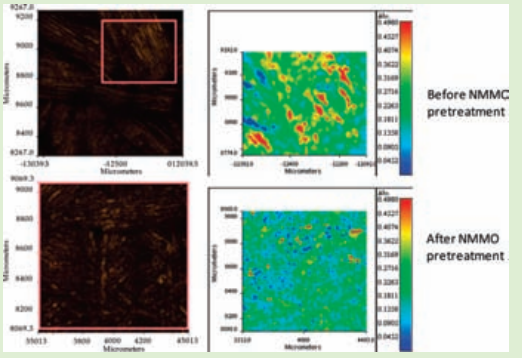
ABSTRACT: Pretreatment of lignocellulosic biomass by *N*-methylmorpholine-*N*-oxide (NMMO), a solvent used in the textile industry to dissolve cellulose for production of regenerated cellulose fibers, was observed to enhance significantly enzymatic saccharification and fermentation. The enhancement was speculated to have been caused by reduced cellulose crystallinity after dissolution and precipitation processes. This work focused on assessing several physical changes and their correlations to enzymatic saccharification of pine flour after NMMO pretreatment. Results from microstructure, surface chemical composition, and cellulose accessibility complementarily illustrated the enrichment of cellulose on pine flour surface after NMMO pretreatment. Cellulose accessibility was highly correlated to the overall glucan conversion rate. Changes in crystallinity were correlated to the initial hydrolysis rate but not overall glucan conversion rate. Findings from this work may contribute to lignocellulosic bioenergy from development of novel pretreatment technologies utilizing NMMO.



INTRODUCTION

Lignocellulosic biomass has long been envisioned as a renewable source for fuels, fine chemicals, and new materials. One of the key bottlenecks is the lack of practical, environment-friendly, and cost-effective processes. In recent years, pretreatment, that is, processes alternating native structures of biomass for enhanced simultaneous or sequential saccharification and fermentation has been recognized as a critical step of converting lignocellulosic biomass to biofuels and biochemicals.[1,2] According to a report of National Renewable Energy Laboratory, pretreatment accounts for 16−19% of capital investment in a lignocellulosic biorefinery.[3] Pretreatment also impacts production costs because of its significance to efficient conversion of biomass to fermentable sugars and subsequent fermentation and downstream processing.[4]

A variety of pretreatment technologies have been developed to change the native structure of lignocellulosic biomass physically, chemically, biologically, or in combination. Mechanical forces are simple physical pretreatment technologies to reduce particle size and thus enlarge surface area of biomass, with possible alterations of molecular structures and crystallinity of cellulose due to the heat converted from the mechanical energy.[5,6] Solvents capable of dissolving cellulose, including ionic liquids and concentrated phosphoric acid, are applied as pretreatment technologies that enable the fractionation of biomass, reduction of cellulose crystallinity, and improvement of lignin recovery.[7,8] Chemical pretreatments are very efficient technologies because native matrices in lignocellulosic biomass are readily disrupted when acids, alkaline, or oxidants are used as catalysts or reactants during pretreatment.[9−11] However, the formation of waste chemicals and undesired degradation products are concerns of chemical pretreatment technologies, in addition to high energy consumption and severity of equipment corrosion. Biological pretreatment technologies also have been studied because of potential advantages of environmental friendliness, low energy consumption, and less stringent equipment requirements because of milder conditions.[12−14] The feasibility of biological pretreatment methods is still questionable because of poor effectiveness.

Crystallinity and accessibility of cellulose are two dominant factors affecting enzymatic saccharification of lignocellulosic biomass,[15] which may be manipulated by an appropriate pretreatment technology.[16] Recently, *N*-methylmorpholine-*N*-oxide (NMMO) was observed to be a potential solvent to pretreat biomass for improved enzymatic saccharification and fermentation.[17−20] NMMO is a solvent commonly used in the textile industry to dissolve cellulose for production of regenerated cellulose products, and its nontoxic, biodegradable, nonflammable, recyclable (>99%), and reusable features make

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it a promising candidate to develop novel pretreatment technologies for lignocellulosic biorefinery.[21] Up to 30% w/w cellulose can be dissolved in NMMO/H_2O (NMMO monohydrate, NMMO dehydrate or the mixture) at a temperature between 80 and 150 °C.[22] Cellulose dissolved in NMMO loses solubility at lowered temperatures, upon dilution with water, or both and precipitates into structures with much reduced crystallinity.[23,24] Jeihanipour et al.[19] observed "dissolution, ballooning, and swelling" of high-crystalline cellulose when pretreated at 90 and 120 °C by 85, 79, and 73% NMMO, respectively. When pretreated by 85% NMMO, conversion of the crystalline cellulose I to cellulose II was observed, corresponding to the lowest crystallinity among the studied NMMO concentrations. When NMMO was applied to pretreat sugar cane bagasse (that is a byproduct after pressing sugar cane for extraction of sucrose), crystallinity of bagasse was reduced and the surface of fiber bundles was roughened.[17] The reduced crystallinity was proposed to be responsible for enhanced enzymatic saccharification after NMMO pretreatment.[17,19]

Despite these significant discoveries, molecular bases are to be studied for changes of surface composition and cellulose accessibility of lignocellulosic biomass after NMMO pretreatment. Furthermore, because enzymatic saccharification generally shows a higher sugar production rate in the initial stage of reaction, kinetics of saccharification is to be correlated to physical parameters such as cellulose accessibility and crystallinity. Pine flour was used in this work as a model biomass material to assess these parameters. Pine flour was selected because woody biomass is a group of viable feedstocks.[25] Furthermore, the swelling of pine wood chips after pretreatment by ionic liquids was recently characterized,[26] which may be integrated with fundamental characterizations in this work to facilitate future development of novel pretreatment technologies using cellulose-dissolving solvents such as NMMO.

EXPERIMENTAL SECTION

Materials. Sigmacell (pure cellulose, type 20), NMMO (50% solution in water), H_2SO_4, glucose, glucose (HK) assay kit, phenol, L-serine, glucose, and copper(II) sulfate pentahydrate were purchased from Sigma-Aldrich (St. Louis, MO). Coomassie Plus (Bradford) assay kit and bicinchoninic acid (sodium salt hydrate) were products from Pierce (Rockford, IL). Spezyme CP cellulase (product code A03117) was a gift from Genencor International (Rochester, NY). *Trichoderma reesei* (ATCC26921) cellulase (catalog no. LS002601) in the lyophilized form was purchased from Worthington Biochemical Corporation (Lakewood, NJ). Pine (*Pinus* spp.) flour samples were obtained in the milled form (that passed through a 1.0 mm sieve) from the Forest Products Center at University of Tennessee (Knoxville, TN). Other chemicals were products of Fisher Scientific (Pittsburgh, PA).

Methods. *Preparation of NMMO-Pretreated Pine Flour.* The 50% NMMO solution was concentrated to 83% using a rotary evaporator operated at 95 °C and 20 kPa under pressure. The concentrated NMMO was determined for its moisture content gravimetrically. Five grams of the 83% NMMO solution was introduced to a 50 mL screw-cap centrifuge tube incubated in a 120 °C glycerol bath. When the solution was equilibrated at 120 °C, 250 mg of pine flour was mixed with the NMMO solution, the cap tightly attached, and the suspension agitated under magnetic stirring for a preset duration. After pretreatment, pine flour was regenerated by adding 20 mL of deionized water at 25 or 95 °C under continuous stirring until the regenerated solid matters were evenly suspended. The suspension was then centrifuged at 5000*g* for 5 min to collect the precipitate, which was washed twice with 20 mL of deionized water and vacuum-dried at 40 °C and 20 kPa under pressure for 48 h.

Enzymatic Saccharification. Enzymatic saccharification was carried out in 50 mL screw-cap centrifuge tubes placed in a 50 °C shaking water bath (model C76, New Brunswick Scientific, Edison, NJ) at a setting of 180 rpm. Each tube contained 250 mg of untreated or NMMO-pretreated pine flour suspended in 20 mL of a 50 mM sodium acetate buffer at pH 5.0. The suspension was preheated to 50 °C before the addition of 48.1 filter-paper-units (FPU) of Spezyme CP cellulase/g glucan to initiate the reaction. We withdrew 100 μL aliquots of reaction suspension at preset intervals for determination of hydrolysis kinetics. The sampled suspension was incubated in a boiling water bath for 5 min to inactivate the cellulase followed by centrifugation at 10 000*g* for 2 min to collect supernatant for sugar assays. The initial rate of enzymatic hydrolysis was calculated from analysis of supernatant sampled during the first 2 h of hydrolysis. The overall conversion rate was calculated based on samples taken at 120 h of hydrolysis. (Increases in glucose concentrations during hydrolysis were observed up to ca. 24 h for most samples.)

Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy (ATR-FTIR). Prior to analysis, biomass samples (pine flour, enzyme, lignin, and Sigmacell) were vacuum-dried at 20 kPa under pressure and 40 °C for 48 h. The instrument was a Nicolet 520P spectrometer equipped with a Smart OMNI-Sampler ATR module (Thermo Fisher Scientific, Waltham, MA). FTIR reflectance spectra within a wavenumber range of 700−4000 cm^{-1} were collected at a resolution of 4 cm^{-1}. A total of 128 scans were acquired for each sample.

The lignin sample used in FTIR-ATR analysis was prepared from pine flour by extensive enzymatic hydrolysis to a final sample with mostly lignin (EnzL), following a literature protocol[27] with slight modifications. To prepare an EnzL sample, a two-stage hydrolysis protocol was applied with a high loading level of Spezyme CP cellulase. In the first stage, a pine flour sample was incubated in a 0.05 M sodium acetate buffer (pH 5.0) at 50 °C with Spezyme CP cellulase loaded at 200 FPU/g biomass. The slurry was sampled for sugar analysis periodically, and the hydrolysis was terminated when no increase in the reducing sugar content in the reaction suspension was observed. The residual solid matter was then collected from the hydrolysis suspension by centrifugation at 10 000*g* for 2 min. The precipitate was washed twice using deionized water at 50 °C. The resultant mass was used in the second-stage hydrolysis where the identical conditions in the first stage were used. The results of sugar assay indicated that the two-stage protocol enabled the removal of >93% glucose in the corresponding pine flour samples. The obtained precipitate sample was washed by deionized water and resuspended in a 0.05 M Tris-HCl buffer at pH 8.1 for hydrolysis by trypsin at 37 °C for 20 h to remove residual proteins. Following centrifugation, the precipitate was washed twice with a 1 M NaCl solution and three times with deionized water before vacuum-drying to obtain an EnzL sample.

FTIR Imaging. A spectrum spotlight 300 FTIR imaging system (PerkinElmer Life and Analytical Sciences, Norwich, CT) with both microscopy and spectroscopy features was used to collect IR spectra and images. We pressed 200 mg pretreated or native pine flour in an accessory device of the Nicolet 520P spectrometer to 5 mm diameter pellets with a thickness of ~1 mm that were placed on the sample stage for collecting visible and FTIR images. Infrared images were generated at 6.25 μm pixel size over a selected area. All spectra were collected in the reflectance mode in the wavenumber range from 800 to 1800 cm^{-1} at a resolution of 8 cm^{-1} for a total of four scans. The spectral data were imported into the Unscrambler software (V.7.6, CAMO Software, Woodbridge, NJ) to normalize the scale of samples and convert to absorbance spectra. FTIR bands at 1270 and 1596 cm^{-1} were selected to generate spectral images.

Scanning Electron Microscopy (SEM). Pretreated or native pine flour was mounted on specimen stubs using double-sided tape for SEM observation. Samples were sputter-coated with AuPd under vacuum

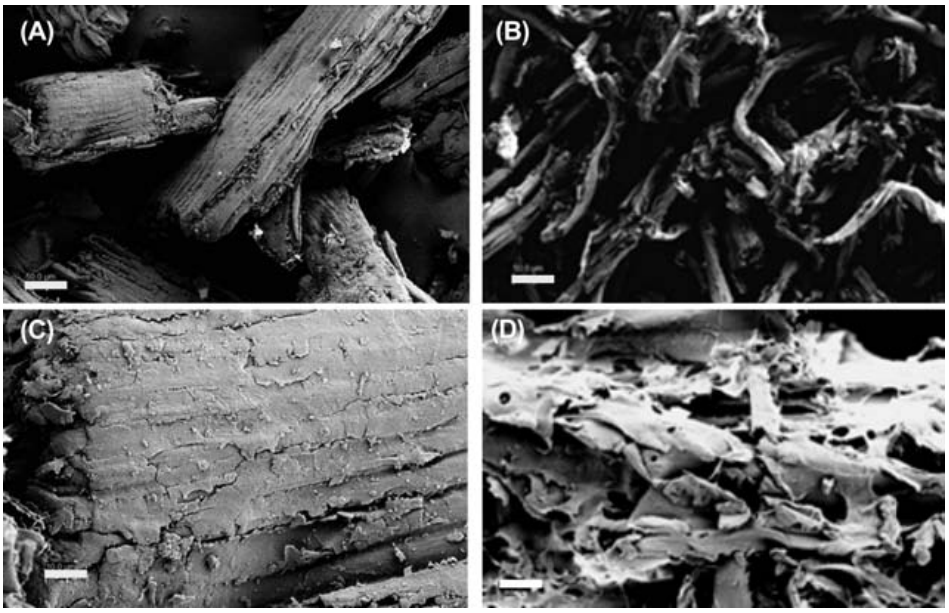

Figure 1. SEM images of pine flour before (left images) and after (right images) pretreatment using 83% NMMO at a solid/liquid mass ratio of 1:20 at 120 °C for 2 h. Magnifications are 100× for images A and B and 500× for images C and D. Scale bars = 100 μm in A and B and 20 μm in C and D.

before imaging using a LEO 1525 SEM microscope (LEO Electron Microscopy, Oberkochen, Germany).

X-ray Diffraction (XRD). Crystallinity of biomass samples was determined by X-ray diffraction using an X'Pert Pro diffraction system (Panalytical, Westborough, MA). Vacuum-dried flour samples were applied on glass plates, which were fixed on the MDR cradle. The diffraction spectrum was taken at 2.4°/min for a 2θ range of 10−30° with a step size of 0.02°. Cu Kα radiation (λ = 1.54 Å) was generated at 45 kV and 40 mA. The crystallinity was expressed as the crystallinity index (CrI) calculated by the following equation[28]

$$\mathrm{CrI} = \frac{I_{002} - I_{\mathrm{am}}}{I_{002}} \times 100\% \qquad (1)$$

where I_{002} is the diffraction intensity at 002 peak position ($2\theta \approx 22.50°$) and I_{am} is the intensity representing the amorphous region ($2\theta \approx 18.70°$).

Assessment of Cellulase Adsorption on Biomass. Langmuir adsorption isotherms were used to assess the accessibility of cellulose in pretreated or native pine flour by cellulase. The *Trichoderma reesei* (ATCC26921) cellulase was used because its mass can easily be quantified. An EnzL control sample was prepared from pine flour by the two-stage enzymatic hydrolysis protocol detailed above.

Cellulase adsorption on biomass (EnzL, pretreated and untreated pine flour) was carried out in 10 mL sealed glass vials using an end-to-end shaker at 4 °C. We suspended 50 mg of biomass solids in 5 mL of a 50 mM sodium acetate buffer at pH 5.0 containing cellulase at a concentration of 0.1−5 mg/mL, and the adsorption was allowed for 6 h. The amount of enzyme adsorbed on biomass was estimated according to the difference between initial and final cellulase contents in the suspension. All experiments were conducted in duplicate.

Langmuir adsorption parameters[27] in the following expression were determined by linear regression of the adsorption data using the Origin software (OriginLab, Northampton, MA)

$$\frac{[C_E]}{\sigma_{\mathrm{sample}}} = \frac{[C_E]}{\sigma_{\max}} + \frac{K_d}{\sigma_{\max}} \qquad (2)$$

where σ_{sample} is the amount of adsorbed cellulase (milligrams protein/milligrams biomass), $\sigma_{\max}$ is the maximum adsorption capacity (milligrams protein/milligrams biomass), $[C_E]$ is the equilibrium cellulase concentration in a suspension (milligrams protein/milliliter), and K_d is the equilibrium constant.

The cellulose accessibility was used to indicate the adsorption capacity of cellulase onto the cellulose fraction in biomass samples, estimated by comparing the adsorption capacities of cellulase onto whole biomass and lignin[27]

$$\sigma_{\mathrm{cellulose}} = \frac{\sigma_{\mathrm{PF}} - \sigma_{\mathrm{EnzL}} L_w}{C_w} \qquad (3)$$

where σ_{PF} is the maximum adsorption capacity on pine flour samples, σ_{EnzL} is the maximum adsorption capacity on EnzL, and L_w and C_w are lignin and cellulose fraction contents in pine flour samples.

Analytical Methods. The concentration of glucose hydrolyzed from biomass was determined following the protocol of the glucose (HK) assay kit.[8] The concentrations of soluble reducing sugars in hydrolyzed suspensions were determined using the BCA method.[29] The cellulase activity was determined following the procedures of the National Renewable Energy Laboratory.[30] The protein concentration was determined using the Bradford method.

RESULTS AND DISCUSSION

Physical Structures of Pine Flour before and after NMMO Pretreatment. SEM images of pine flour before and after NMMO pretreatment are shown in Figure 1 for two magnifications. Native pine flour showed ordered fiber bundles (Figure 1A,C), contrasting no ordered structures with corrugated surface for the NMMO-pretreated sample (Figure 1B,D). The SEM images (Figure 1A−D) indicate that fiber bundles in the native pine flour experienced compositional and structural reformation after NMMO pretreatment. Kuo and Lee[17] also reported increased surface roughness of sugar cane bagasse after NMMO pretreatment. Changes in surface structures of biomass are typically observed in pretreatment technologies employing solvent fractionation.[7,31] Because 83% NMMO dissolves cellulose at the pretreatment temperature of 120 °C, it is possible that cellulose was extracted from the fiber bundles during pretreatment, followed by precipitation into irregular structures during

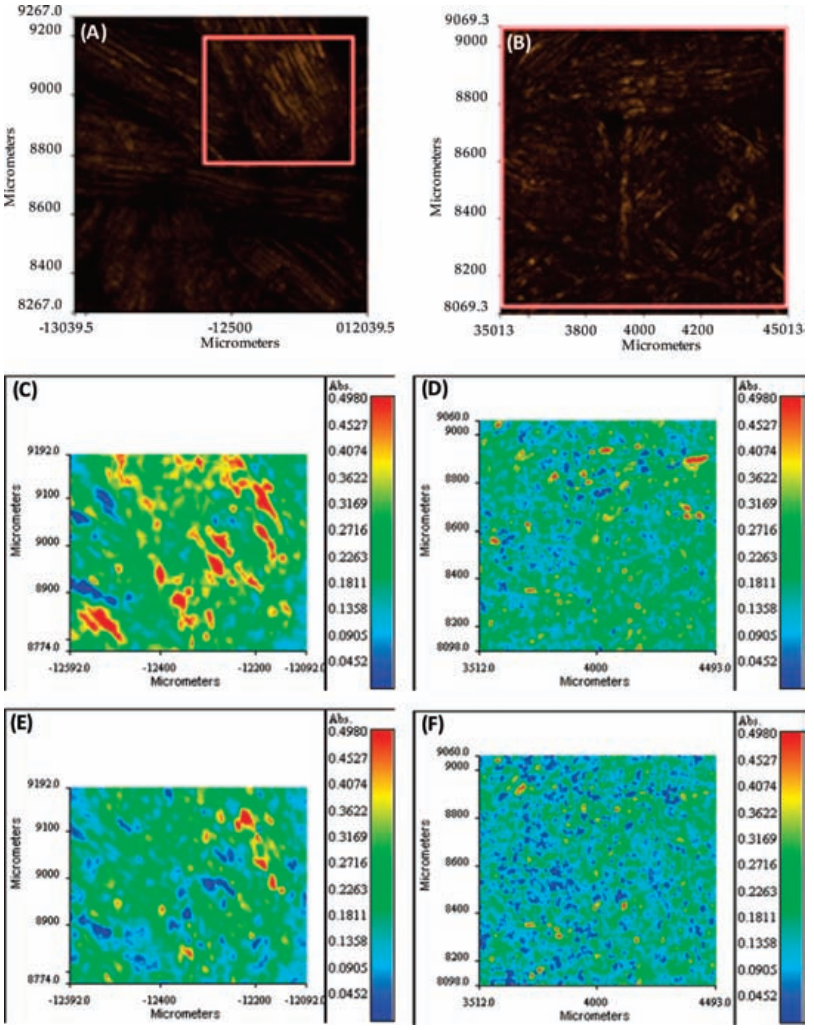

Figure 2. Visible (A and B) and FTIR spectra images (C−F) of pine flour before (left images) and after (right images) NMMO pretreatment. Spectral images generated at 1270 (C and D) and 1596 cm^{-1} (E and F) show the distribution and abundance of lignin on pine flour surfaces. Pretreatment was conducted using 83% NMMO at a solid:liquid mass ratio of 1:20 at 120 °C for 2 h.

regeneration when water was added and the water/NMMO mixture was no longer able to dissolve cellulose.

Surface Compositional Changes of Pine Flour before and after NMMO Pretreatment. Because adsorption of cellulase onto lignocellulosic biomass takes place before enzymatic hydrolysis, FTIR imaging is a powerful tool to illustrate surface compositional changes of biomass to interpret hydrolysis data, as impacted by pretreatment. Specifically in this work, the abundance and distribution of two major biomass components, that is, cellulose and lignin, on the surface of pine flour before and after NMMO pretreatment were qualitatively studied using the FTIR imaging technique by employing characteristic signals in the ATR-FTIR spectra. Although a large number of differences in FTIR spectra of cellulose and lignin have been reported,[28] the selection of signals in the FTIR spectra should minimize interferences from other biomass compounds.[32]

ATR-FTIR spectra of pure cellulose (SigmaCell), untreated pine flour, and EnzL from untreated pine flour are compared (figure not shown). The EnzL from untreated pine was used to represent the pine lignin because it is widely accepted that EnzL maintains most structural characteristics of lignin in the original biomass.[33] The FTIR absorption spectrum of the EnzL showed two bands that were not present in the spectrum of SigmaCell: vibration of guaiacyl rings at 1270 cm^{-1} and C═C stretching vibration at 1596 cm^{-1}. Therefore, absorbance information at 1270 and 1596 cm^{-1} was used to construct spectral images to illustrate the presence of lignin on pine flour surfaces.

Figure 2 shows FTIR visible and spectra images of pine flour before and after NMMO pretreatment. Similar to SEM images (Figure 1), visible images showed a change from ordered to disordered structures of fibers after pretreatment by NMMO. The spectra images constructed from absorbance at 1270 and 1596 cm^{-1} showed intense signals for untreated pine flour, signifying the abundance of lignin on the surface. After NMMO pretreatment, the characteristic absorbance referring to lignin significantly diminished, indicating the much reduced lignin content on the surface. The FTIR spectral images thus indicate increased abundance of cellulose on the surface of pine flour after NMMO pretreatment, further supporting the above speculation based on SEM images. Therefore, NMMO pretreatment can be categorized as an overall physical process that largely alters the distribution of biomass matrix components, although thermal degradation of certain compounds is also possible at the elevated pretreatment temperature.

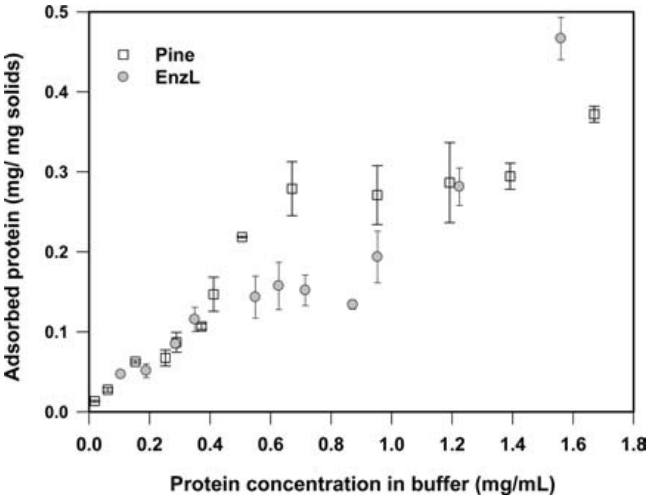

Figure 3. Langmuir adsorption isotherms for cellulase adsorbing onto pine flour pretreated by NMMO and the corresponding lignin (EnzL). Pine flour was pretreated at 120 °C for 120 min using 83% NMMO at a solid/liquid mass ratio of 1:20. The EnzL sample was prepared by extensive enzymatic hydrolysis of the pretreated pine flour.

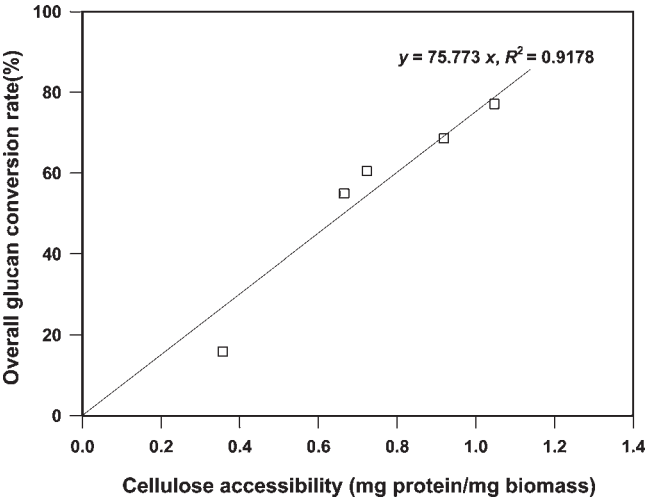

Figure 4. Linear correlation between cellulose accessibility and overall glucan conversion rate for pine flour samples pretreated at conditions described in Table 1. Samples were hydrolyzed using 48.1 FPU cellulase/g glucan at pH 5.0 and 50 °C.

Table 1. Parameters in the Langmuir Model and Estimated Cellulose Accessibility of Pine Flour Pretreated by NMMO at 120 °C for Different Durations

pretreatment time (min)[a]	σ_{PF} (mg protein/mg sample)[b]	R^2	σ_{EnzL} (mg protein/mg sample)[c]	$\sigma_{cellulose}$ (mg protein/mg sample)[d]
control	0.222	0.906	0.265	0.356
30	0.341	0.902	0.211	0.665
60	0.363	0.897	0.201	0.723
120	0.511	0.916	0.234	1.047
240	0.467	0.930	0.276	0.919

[a] Samples were regenerated at 25 °C. [b] Maximum adsorption capacity of cellulase onto pine flour samples. [c] Maximum adsorption capacity of cellulase onto corresponding lignin samples (EnzL). [d] Cellulose accessibility as defined in eq 3.

Correlation between Cellulose Accessibility and Overall Glucan Conversion Rate. Cellulose accessibility, that is, the portion of surface area contacted by cellulase, is one of the most important factors determining enzymatic digestibility of biomass. The relationship between cellulose accessibility and enzymatic hydrolysis kinetics was investigated in experiments or simulations,[8,15] in which the cellulose accessibility was determined by N_2 adsorption (BET surface), iodine adsorption, or moisture regain.[15,16] An improved method based on cellulase adsorption showed desirable reliability[27] and was adopted in this work to estimate cellulose accessibility of pine flour before and after NMMO pretreatments.

Figure 3 shows example Langmuir adsorption isotherms of a pretreated pine flour sample and the corresponding EnzL. Both curves showed an "S" shape indicative of monolayer adsorption of cellulase at low cellulase concentrations, followed by multilayer adsorption at higher cellulase concentrations. The data corresponding to the monolayer adsorption was then fitted with the Langmuir model (eqs 2 and 3), with the obtained adsorption parameters listed in Table 1 for samples before and after NMMO pretreatment under various conditions. As shown in Table 1, the values of coefficient of determination (R^2) for these samples were between 0.864 and 0.930, indicating a good fit of the experiment data to the Langmuir model. Both σ_{PF} and cellulose accessibility increased with an increase in pretreatment time up to 120 min, followed by a slight decrease at a pretreatment time of 240 min. In contrast, there was no obvious trend for σ_{EnzI} estimated based on EnzL from pine flour samples pretreated for different durations. Because of no specific biological affinity between lignin and cellulase, the σ_{EnzL} may not have been impacted by pretreatment conditions. The increased cellulose accessibility at a longer pretreatment time indicates a better extraction of cellulose from biomass matrix that after precipitation on the pine flour surface during regeneration enables more cellulase adsorbing onto the regenerated biomass. The cellulose accessibility data thus further support results from SEM and FTIR imaging.

To correlate cellulose accessibility with hydrolysis kinetics, a relatively high loading level of cellulase (48.1 FPU per gram glucan) was applied because the relationship of structure features of pine flour and hydrolysis kinetics can be revealed with minor interferences of cellulase dosage[8] and inhibition by hydrolysis products. The correlation between cellulose accessibility and overall glucan conversion rate is shown in Figure 4 for samples in Table 1. An R^2 value of 0.92 indicates a good correlation of the two parameters. The significance of cellulose accessibility on overall digestibility of lignocellulosic biomass was not reported in the literature applying NMMO as a pretreatment method.[17−20] As discussed above, a longer NMMO pretreatment allows a larger quantity of cellulose to be extracted from the sample interior to the surface, enabling a better access by cellulase and eventually a better conversion to glucan. It should be noted that cellulase adsorbs onto biomass by specific (on cellulose) and nonspecific (e.g., onto lignin, Figure 3) mechanisms, and only the portion adsorbed on cellulose contributes to hydrolysis efficiency. The correlation in Figure 4 indicates that improved specific adsorption by cellulase after NMMO pretreatments resulted from the enrichment of cellulose on pine flour surface. Furthermore, Jeihanipour et al.[19] reported 97.9 and 100% glucose conversion from high-crystalline cellulose pretreated

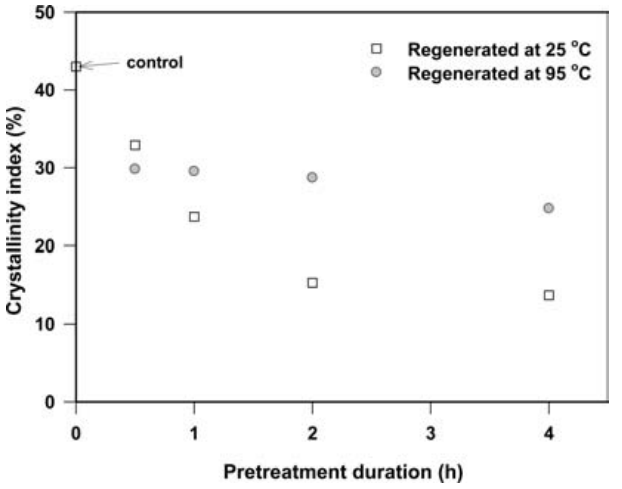

Figure 5. Cellulose crystallinity index of pine flour samples after pretreatment for different durations. Pine flour samples were pretreated using a solid:liquid mass ratio of 1:20 in 83% NMMO up to 4 h at 120 °C and regenerated at 25 or 95 °C.

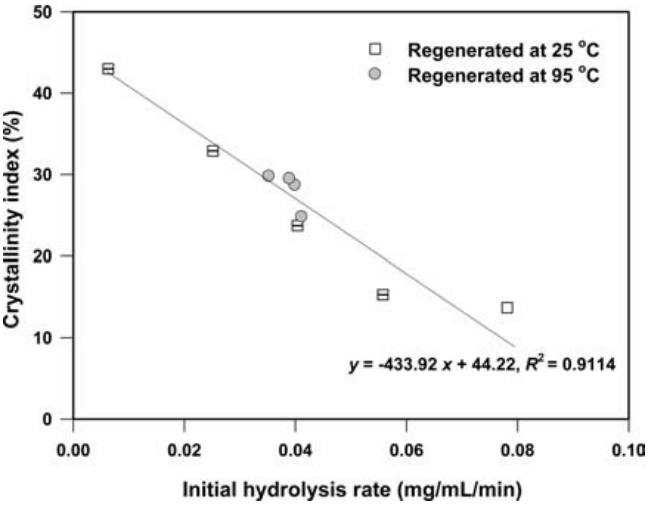

Figure 6. Linear correlation between cellulose crystallinity index and initial hydrolysis rate of pine flour samples pretreated using a solid:liquid mass ratio of 1:20 in 83% NMMO at 120 °C up to 240 min. Hydrolysis was performed with 48.1 FPU cellulase/g glucan at pH 5.0 and 50 °C.

by 85% NMMO at 120 °C for 1 and 2.5 h, respectively, after 72 h of enzymatic hydrolysis. In contrast, although our hydrolysis was conducted for up to 120 h, the overall glucan conversion rates in our study were all <80% under the studied conditions, possibly resulting from incomplete extraction of cellulose at the studied pretreatment conditions.

Correlation between Crystallinity and Initial Hydrolysis Rate. The excellent solubility of cellulose in NMMO was a major motivation for the development of relevant pretreatment technologies.[22] As discussed above, the original fibrous structure of native pine flour was modified after pretreatment by NMMO, and the extraction and precipitation processes likely change the crystallinity of pine flour. The reduced crystallinity was reported for studies based on cellulose[19] and sugar cane bagasse[17] after NMMO pretreatment. However, correlation between crystallinity and saccharification data has not been attempted. Figure 5 shows CrI of pine flour samples pretreated in 83% NMMO at 120 °C for different durations, followed by regeneration at 25 or 95 °C. At the regeneration temperature of 25 °C, an increase in pretreatment time resulted in decreased crystallinity of pretreated pine flour samples, but the decrease in crystallinity was less significant when the pretreatment time was extended from 2 to 4 h. Figure 5 suggests that a longer treatment time enables a larger quantity of cellulose being extracted from the cell wall that subsequently results in a higher percentage of precipitated cellulose after regeneration and thus reduced crystallinity. For the comparable treatments using a regeneration temperature of 95 °C, a lower CrI was also observed for a longer pretreatment time, but the crystallinity was much higher than the corresponding treatment regenerated at 25 °C. It is likely that precipitation of cellulose is faster at a lower regeneration temperature, and the shortened precipitation reduces the possibility for cellulose extracted from pine flour to recrystallize to crystalline structures.

The initial hydrolysis rates of pine flour samples are plotted in Figure 6 to show the correlation with crystallinity. The untreated pine flour sample had the highest CrI and the lowest initial hydrolysis rate. A decrease in CrI after a longer pretreatment time corresponded to a higher initial hydrolysis rate. The sample pretreated for 4 h and regenerated at 25 °C had the lowest crystallinity index (13.7%, compared with 43.0% of the native pine flour) that corresponded to a 12-fold increase in the initial rate of hydrolysis. Overall, an excellent linear correlation ($R^2 = 0.91$) was observed between cellulose crystallinity and initial hydrolysis rate for pine flour samples. Conversely, correlation between overall glucan conversion and crystallinity was poor ($R^2 = 0.669$, not shown). This observation disagrees with the literature hypothesis that the improved overall sugar conversion was due to reduced crystallinity after NMMO pretreatment.[17,19]

Our observation was in a good agreement with a two-phase model proposed by Ryu et al.[15] for simultaneous hydrolysis of pure cellulose present in both crystalline and amorphous structures. Biophysically, amorphous cellulose has much higher affinity for adsorption of cellulase and thus hydrolysis rate,[34] which may have been responsible for a higher initial hydrolysis rate for pine flour samples with a lower CrI. Therefore, NMMO pretreatment, by effectively decreasing the crystallinity of cellulose in pine flour, resulted in a faster conversion of cellulose to sugars in the early stage of enzymatic saccharification that is critical to practical production.

CONCLUSIONS

Our work was in agreement with the literature observations of altered physical structure, reduced crystallinity, and enhanced enzymatic saccharification after pretreatment by NMMO. These observations resulted from the solubility of cellulose in NMMO that enabled the extraction of cellulose from lignocellulosic matrix and precipitation into a less-crystalline structure after regeneration. New observations from our work included reduced lignin concentration on pine flour surface, lower crystallinity after regeneration at a lower temperature, and increased cellulose accessibility for samples pretreated for a longer time at a same pretreatment temperature. Fundamentally, we showed a linear correlation between cellulose accessibility and overall glucan conversion rate as well as that between cellulose crystallinity and initial saccharification rate. The overall glucan conversion rate, however, was not correlated to the decrease in crystallinity

after NMMO pretreatment. Our study may provide insight to develop solvent fractionation technologies using industrial, green, and biodegradable solvents such as NMMO for profitable biorefinery.

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