

Rapid and Complete Enzyme Hydrolysis of Lignocellulosic Nanofibrils

Raquel Martin-Sampedro,^{†,‡} Ilari Filpponen,[†] Ingrid C. Hoeger,[§] J. Y. Zhu,^{||} Janne Laine,[†] and Orlando J. Rojas^{*,†,§}

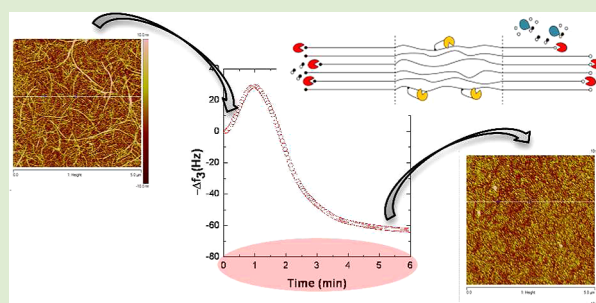
[†]School of Chemical Technology, Department of Forest Products Technology, Aalto University, 00076 Aalto, Finland

[§]Department of Forest Biomaterials, North Carolina State University, Raleigh, North Carolina 27695, United States

^{||}Forest Products Laboratory, USDA Forest Service, Madison, Wisconsin 53726, United States

S Supporting Information

ABSTRACT: Rapid enzymatic saccharification of lignocellulosic nanofibrils (LCNF) was investigated by monitoring nanoscale changes in mass via quartz crystal microgravimetry and also by measuring reducing sugar yields. In only a few minutes LCNF thin films were completely hydrolyzed upon incubation in multi-component enzyme systems. Conversion to sugars and oligosaccharides of LCNF dispersed in water occurred in about 4 h (50 °C, pH 5). In contrast, a conversion of only 57% was observed for partially crystalline cellulose (Avicel) after 9 h, under same experimental conditions. Under conditions of high enzyme loading the presence of residual lignin and other macromolecules in the cell wall of LCNF did not appear to affect negatively the recorded high hydrolysis rates. Overall, our findings suggest that deconstruction of the cell wall to nanofibrils is an effective pretreatment to facilitate rapid and complete cellulose bioconversion.



Woody biomass is the most abundant renewable resource available for the production of ethanol and other chemicals. Under current technologies lignocellulose is first subject to pretreatment to break down the various physical and chemical barriers and to make it more accessible to cellulase enzymes for subsequent saccharification and fermentation. Physical pretreatment through mechanical fibrillation of woody biomass is one avenue to remove the barrier of cellulose accessibility to cellulase enzymes.^{1,2} When conventional mechanical size reduction is used of wood alone, to the level of fibers or fibers bundles as those in wood pulp production, Class I wood size reduction, is not capable to achieve complete saccharification.³ Mechanical size reduction beyond the fiber level, to complete deconstruct the cell wall to nanofibrils, Class II size reduction, can render the cell wall to be completely cellulase-accessible.³ Although this Class II size reduction consumes a significant amount of energy, it provides a nonchemical, green route for pretreatment of lignocelluloses, without the production of undesirable compounds, and to facilitate downstream conversion and processing. Therefore, evaluation of mechanical fibrillation of lignocelluloses to nanofibrils for enzymatic saccharification has practical significance.

Due to the complexity of lignocellulose systems most studies measure the overall production of sugar after enzymatic hydrolysis based on batch sampling and offline measurements. However, temporal resolution of these measurements is limited and may not be able to resolve the dynamic phenomena in the initial stages.⁴ To solve these limitations, several in situ

methods have been proposed. Liu et al.⁴ used in situ UV–vis spectrophotometry (dual-wavelength or spectral derivative methods) to measure cellulase adsorption and enzymatic hydrolysis of lignocellulosic substrates. Igarashi et al.⁵ used high-speed atomic force microscopy (AFM) to obtain a direct real-time visualization of crystalline cellulose degradation by individual cellulase enzymes. Likewise, cellulose thin films have been used with sensing techniques such as quartz crystal microbalance (QCM), to monitor the binding and catalytic activity of cellulases in situ and in real-time.^{6–10} We reported for the first time cellulose nanofibrils from lignin-free, bleached fibers (denoted here as CNF) as suitable substrates to study enzymatic hydrolysis via QCM.⁸ However, bleached fibers may not be a practical feedstock for sugar production due to the requirements inherent to bleaching (operation and cost). Thus, feedstock containing lignin, hemicelluloses, and other macromolecules from the cell wall are more relevant. For example, lignocellulose nanofibrils (LCNF) produced from wood fibers^{11–13} may be considered. The interest in this study is related to the distinctive high hydrolysis rate observed in CNF substrates as compared to others such as regenerated cellulose or cellulose nanocrystals, CNC.⁸ Relevant to this work is the fact that only 2–3 min were required to fully degrade CNF films, while 10 and 300 min were necessary to hydrolyze films

Received: September 24, 2012

Accepted: October 25, 2012

Published: October 30, 2012

of regenerated cellulose or CNCs, respectively. To address the limitations of these earlier studies that employed substrates consisting of pure cellulose, in its various forms, we propose here LCNF as a substrate for saccharification and to confirm ultrafast degradation kinetics. Therefore, we investigate in detail the enzymatic hydrolysis of lignin-containing LCNF by using the QCM. In addition to microcrystalline cellulose used as a reference, we considered two different nanofiber substrates: LCNF obtained after microfluidization of Kraft birch fibers (LCNF1) and those obtained from same fibers after bleaching (LCNF2; see Table S1 in Supporting Information for chemical composition).

The morphology of thin LCNF films was analyzed by AFM (Figure 1a). Nanofibrils covering solid silica supports are

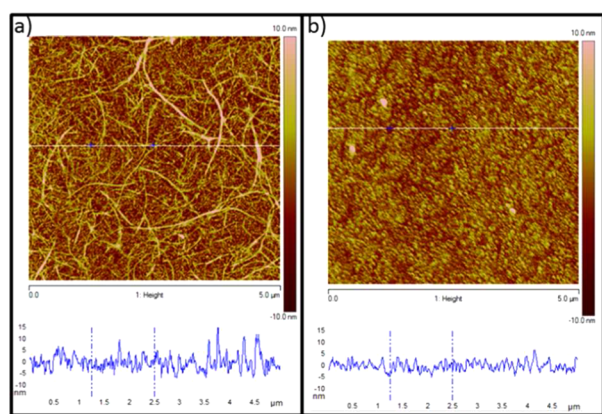


Figure 1. AFM $5 \times 5 \mu\text{m}^2$ height images of LCNF2 thin films before (left) and after (right) enzymatic hydrolysis (0.1% enzyme concentration, 15 min treatment, 40 °C, and pH 5). The height scales (bar on the right of each image) correspond to Z values between -10 and $+10$ nm. The height profiles included correspond to line scans shown in each of the AFM image.

observed on the height images of LCNF films, before enzymatic hydrolysis. LCNF1 and LCNF2 presented similar topography (see Figure S1, Supporting Information), with a large accessible surface area which are hypothesized to be major contribution in facilitating complete bioconversion. This is specially the case if the LCNF films are compared with those from regenerated cellulose, which are smooth and flat.^{6–10}

Figure 2 includes the QCM shift in frequency and energy dissipation during the enzymatic treatment of LCNF1 and LCNF2 thin films in an open-mode operation (continuous flow) of a 0.1% solution of a multicomponent enzyme system (pH 5, 40 °C; see Supporting Information for details about the enzymes used). Both QCM frequency and dissipation profiles were typical of enzymatic hydrolysis of cellulose, as previously described by us.^{7,8} In principle, binding occurs continuously while the substrate is exposed to enzymes and as long as the enzymes are active and the cellulose chains available. However, after a short, initial binding-dominant stage, concurrent hydrolysis of the film governs the signal that is registered in QCM's frequency (Δf) and dissipation (ΔD). Thus, both profiles display a turning point that is followed by an increase in frequency and dissipation energy. This effect is ascribed to the release of oligosaccharides and possibly cellulose fragments, which causes a reduction in the substrate's mass and thickness. Finally, at a given time, hydrolysis slows down and the frequency and dissipation signals reach a plateau. This indicates the depletion of the film (complete hydrolysis) or the presence

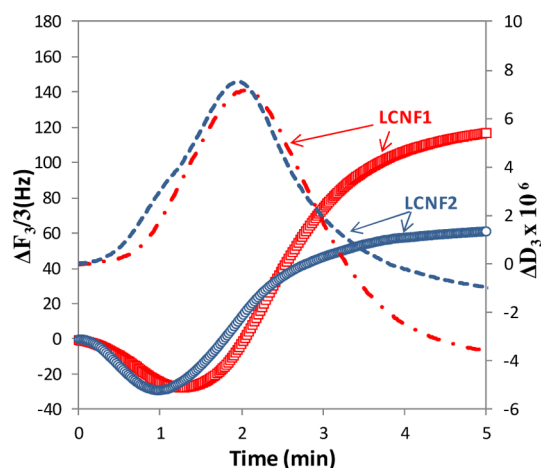


Figure 2. Shift in QCM frequency (symbols) and dissipation (dashed lines) during enzymatic hydrolysis of thin films prepared from LCNF1 (red) and LCNF2 (blue). The profiles correspond to the normalized third overtones and presented as a function of incubation time.

of recalcitrant residual material. In fact, AFM height images of the films after such stage of enzymatic hydrolysis reveal near complete degradation of the nanofibrils originally present (Figure 1b). Interestingly, XPS analyses (Figure S2, Supporting Information) indicate that no cellulose is present in the residual material after enzymatic digestion of the substrate; instead, significant nitrogen and sodium signals were recorded, indicating the presence of tightly bound proteins (enzymes) and possibly residual salts from the buffer solution, respectively. In fact, compared to AFM images of the solid silica support, there is only evidence of scattered features, which correspond to bound proteins, which also give rise to rougher surfaces (from 0.7 to 1.7 nm). Taken together, these results confirm the complete and rapid degradation of LCNF upon cellulolytic reactions. Furthermore, the small amounts of residual lignin in LCNF1 seemed not to interfere with such process (hydrolysis rate of 2.08 ± 0.53 and $2.22 \pm 0.35 \text{ min}^{-1}$ for LCNF1 and LCNF2, respectively, at 0.1% enzyme concentration, as indicated in Table S4 of Supporting Information). We note that the different QCM plateau values for LCNF1 and LCNF2 (127 and 107 Hz, respectively, according to model parameters reported in Table S4, Supporting Information) are due to differences in the initial film mass (1.63 ± 0.27 and $1.41 \pm 0.32 \mu\text{g}$, for LCNF1 and LCNF2, respectively), as was determined by measurements of QCM frequency in air at the beginning of the experiment.

Cellulose nanofibrils have both crystalline cellulose I and amorphous regions, and their degree of crystallinity is expected to be low, depending on pretreatment conditions and the intensity of the deconstruction process.¹⁴ These facts explain the observed fast hydrolysis of CNF compared to cellulose nanocrystals (with their more crystalline cellulose I structure).⁸ However, it does not explain the observation of a faster degradation of CNF compared to amorphous regenerated cellulose, as reported earlier.⁸ This is possibly due to the high surface area available in CNF for enzyme binding and subsequent hydrolysis. Alternatively, it is possible that endoglucanases attacked sections of the nanofibrils (preferably amorphous ones), close to the interface with the solid support, which could promote detachment of whole sections of the fibrils. Thus, if the latter case applies, the fast substrate mass reduction, as detected by the shift in QCM frequency, could be

related to factors others than the complete degradation of the cellulose film (e.g., to oligomers, cellobiose, or sugars).

We carried out experiments in an attempt to explain the reasons why a distinctive fast shift in QCM frequency was observed after incubation of lignocellulose nanofibrils in cellulase solution (that might indicate a fast kinetics of degradation). The following key questions were addressed: (i) Is LCNF released in the buffer solution due to a weak anchoring between the fibrils and the surface of the QCM resonator? (ii) Is it possible that enzymes attack nanofibrils close to the anchoring solid support and produce detachment of whole fibrils? Otherwise, (iii) are the fibrils in LCNF completely hydrolyzed to sugars upon enzyme treatment?

The first possibility was ruled out after exposing films of LCNF1 and LCNF2 with enzyme-free buffer solutions (100 $\mu\text{L}/\text{min}$, pH 5, 40 $^{\circ}\text{C}$) for several hours. It was observed that the QCM frequency shift and dissipation did not change significantly during the treatment (data not show). Neither the AFM images after buffer injection showed any further removal of fibrils (Figure 3a). Therefore, it can be concluded that the LCNF films were robust and the films were strongly anchored onto the sensor surface.

In order to clarify if whole nanofibrils were removed from the substrate we conducted a simple experiment under similar conditions used in QCM continuous operation but outside the flow cell. A film of LCNF2 supported on a silica wafer was

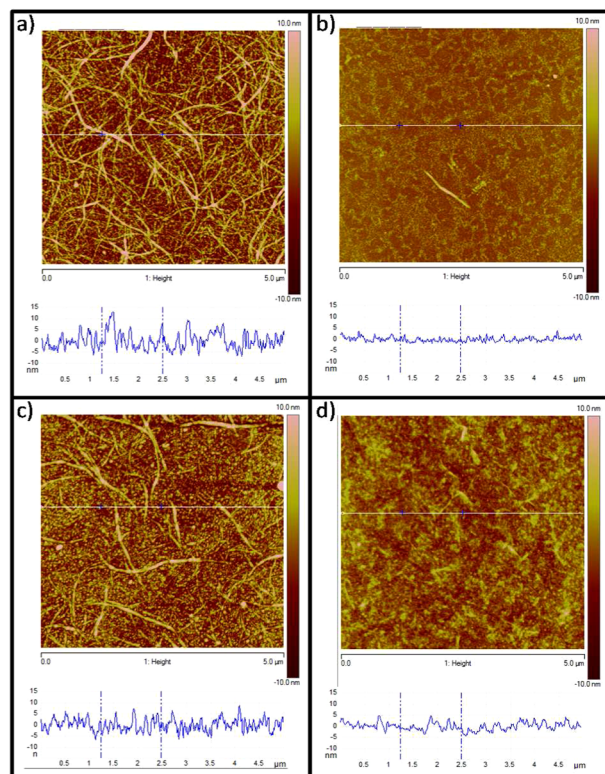


Figure 3. AFM $5 \times 5 \mu\text{m}^2$ height images of thin films of LCNF2 after (a) buffer treatment, (b) enzymatic treatment with no flow or buffer rinse, (c) injection of enzyme followed by a fast rinse with buffer upon enzyme adsorption, (d) injection of enzyme stopped upon enzyme adsorption (batch mode). The height scale (bar at the right of each image) corresponds to Z values between -10 and $+10$ nm. The height profile included in the bottom panels correspond to line scan shown in each AFM images.

immersed in buffer for 1 h at 40 $^{\circ}\text{C}$, then few drops of the same enzyme solution (0.1%) were deposited onto the film. After 20 min of incubation at 40 $^{\circ}\text{C}$, the film was dried, without any rinsing or shear (for example, if a drying stream of air was used) but in an oven at 80 $^{\circ}\text{C}$ for 10 min. AFM imaging indicated the absence of any residual fibrils after enzyme treatment (Figure 3b), which substantiates the possibility that fibrils were fully degraded or saccharified by the enzyme system.

To further verify this hypothesis, two additional QCM experiments were carried out with LCNF2 films. The first consisted of enzyme injection (constant flow rate of 100 $\mu\text{L}/\text{min}$, enzyme concentration of 0.1%) followed by a fast rinse with buffer (flow rate of 300 $\mu\text{L}/\text{min}$) shortly after the enzymes bound onto the substrate. The objective of this rinse with high flow rates was to achieve effective removal of the enzyme after contact with the substrate, to determine if the fibrils are subject to detachment under flow, by shear forces. The shifts in frequency and dissipation monitored in these experiments are shown in Figure 4a. A fast adsorption of the enzyme (decrease

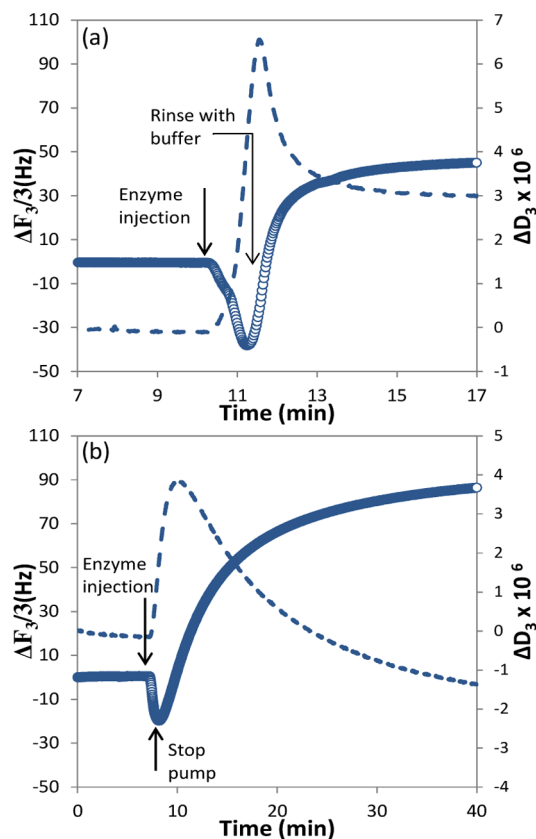


Figure 4. Change in QCM frequency and dissipation during enzymatic hydrolysis of LCNF2 thin films: (a) injection of enzyme followed by a fast rinse with buffer upon enzyme adsorption; (b) injection of enzyme stopped upon enzyme adsorption (batch mode). The frequency (circles) and dissipation (dashed line) profiles are the normalized third overtones as a function of time.

in frequency) followed by an initial degradation of the LCNF2 substrate (increase in frequency), similar to those observed in the QCM experiments described before, were observed. However, after rinsing with buffer, the enzymes were removed from the film, and the hydrolysis halted. Thus, an incomplete degradation of the film took place, as indicated by the maximum frequency shift reached in the plateau region

(approximately 55% lower than that observed after complete degradation). AFM imaging (Figure 3c) confirmed the observed incomplete degradation, because fibrils are easily identified after the treatment, although in lower amounts with respect to the original film (Figure 1a).

An additional QCM experiment consisted of an enzymatic treatment in batch mode. More specifically, enzyme flow (100 $\mu\text{L}/\text{min}$ of 0.1% multicomponent enzyme solution) was stopped after registering the onset of adsorption of the enzymes onto the substrate (decrease of frequency shift). Therefore, in this experiment, the hydrolysis was continued by enzymes already in contact with the film, with no contribution from fresh or excess enzymes flowing in the system. As indicated by the shift in QCM frequency (Figure 4b), although the adsorption rate was similar to that in which the same enzyme solution (0.1%) was injected at constant flow (open flow system, Figure 2), the hydrolysis process was slower (hydrolysis rates of 0.28 ± 0.05 and $2.22 \pm 0.35 \text{ min}^{-1}$ for batch and open flow modes, respectively, according to model parameters reported in Table S4, Supporting Information). This indicates that replenishment of enzymes is effective in increasing the hydrolysis. Nevertheless, the degradation of the fibrils was also complete in this batch or close flow experiment, as can be observed in AFM images (Figure 3d). Note however, that for complete hydrolysis 50 min incubation was required compared to 5 min under continuous enzyme flow.

In order to determine if LCNF was completely hydrolyzed to sugars (i.e., the third question stated previously), incubation with aqueous dispersions of cellulose substrates were carried out and the reducing sugar content in the reaction mixture was quantified at different times. The enzymatic hydrolysis of both LCNF1 and LCNF2 was compared with that of microcrystalline cellulose (Avicel) subject to same enzyme treatment. The experimental data (Figure 5) were fitted to a Boltzmann-

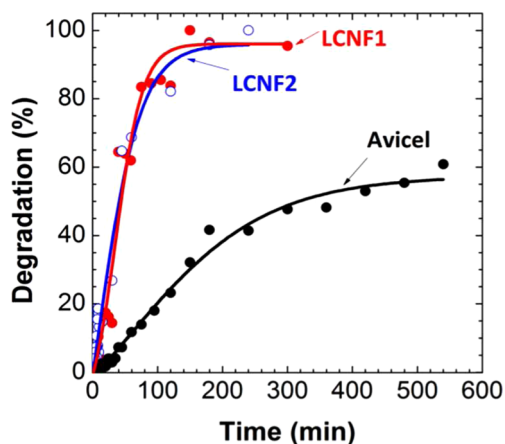


Figure 5. Percentage of mass degraded to reducing sugar during enzymatic hydrolysis of LCNF1, LCNF2, and microcrystalline cellulose (Avicel). Red, blue and black circles are the experimental data for LCNF1, LCNF2, and Avicel, respectively.

sigmoidal equation and the hydrolytic parameters determined (Supporting Information). Complete LCNF hydrolysis to reducing sugars took place in 4 h compared to a 57% hydrolysis of Avicel in 9 h. This supports the hypothesis of a distinctive, rapid enzymatic hydrolysis of LCNF substrates, likely due to the completely accessible cellulose surface. However, in the case of microcrystalline cellulose, with high

crystallinity index the largest cellulose crystals were non hydrolyzed during the treatment.⁸

Finally, the dynamics of the hydrolysis of LCNF1 and LCNF2 thin films was compared at several enzyme concentrations (QCM experiments). The experimental results were fitted to a kinetic model (see Supporting Information). Results showed a similar general behavior as a function of incubation time upon hydrolysis of both LCNF films. An increase in both binding and hydrolysis rates was observed with the increase of enzyme concentration, as expected. Similar results have been reported by other authors studying the enzymatic degradation of regenerated cellulose.^{6,7} However, compared to these reports, the hydrolysis of LCNF in the present case was remarkably faster than that of regenerated and amorphous cellulose. Overall, these results indicate that lignocellulose nanofibrils are subject to rapid enzymatic saccharification, making them a suitable substrate for bioconversion.

■ ASSOCIATED CONTENT

📄 Supporting Information

Experimental methods, empirical binding, and hydrolysis models and additional figures and tables, as indicated in the text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

■ AUTHOR INFORMATION

✉ Corresponding Author

*Tel.: +1-919-513-7494. Fax: +1-919-515-6302. E-mail: ojrojas@ncsu.edu.

📍 Present Address

‡Cellulose and Paper Laboratories, Forestry Products Department, CIFOR-INIA, Ctra de La Coruña Km 7.5, 28040 Madrid, Spain.

📝 Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was performed as a part of the Lignocell Project (Value-added materials and functional structures from lignocellulose). R.M.-S. is grateful to the Spanish Ministry of Science and Innovation for funding her stay in Aalto University and NC State via the FPI Fellowship BES2007-14528.

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