SUPPORTING INFORMATION

Rapid and Complete Enzyme Hydrolysis of Lignocellulosic Nanofibrils

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The following document is complementary to the work presented in the main contribution. It contains Experimental Methods (Table S1); results from empirical models related to binding and hydrolysis rate constants (Tables S2, S3 and S4) and, additional figures (AFM images before and after enzymatic hydrolysis as well as XPS spectra, Figures S1 and S2, respectively).

Experimental Methods

Materials. All the chemicals used were reagent-grade and were obtained from Sigma–Aldrich. Milli-Q water was used to prepare all the solutions. The multicomponent enzyme mixture (NS50013 cellulase complex) was supplied as donation by Novozymes (Bagsvaerd, Denmark). This commercial enzyme mixture from *Trichoderma reesei* fungus contains endoglucanases, exo-glucanases, cellobiohydrolases, and β-glucosidases. This cellulose complex was designed for the efficient saccharification of lignocellulosic materials with maximum activity in mild acidic conditions (pH of ca. 5) and temperatures around 50ºC.

Preparation and characterization of lignocellulosic nanofibrils (LCNF). Unbleached (from kraft cooking) and fully-bleached (after bleaching sequence A-Z/D-EOP-D-P) birch fibers were kindly

provided by UPM-Kymmene (Pietarsaari, Finland) and used to prepare LCNF. The main difference between these fibers is the lignin and hemicellulose content (Table S1). The fibers were exchanged to their sodium form as described by Solala et al.[1] and then refined in a PFI (until Schopper-Riegler degree above 90º) to enhance fiber accessibility and fibrillation efficiency. The refined fibers were processed in a high-pressure fluidizer (Microfluidizer M-110EH, Microfluidics Corp.) at VTT (Espoo, Finland). The fibers were passed five times though an intensifier pump that increased the pressure, followed by an interaction chamber, which defibrillated the fibers by shear forces and impacts against the channel walls and colliding streams. The microfluidizer was operated at a constant shear rate and the pressure was maintained at 55 MPa. The obtained slurries of LCNF1 (from unbleached fibers) and LCNF2 (from bleached fibers) were stored at 4ºC until use.

Table S1. Chemical composition (%) of the precursor birch fibers.

Fiber	Glucose	Xylose	Mannose	Acetone-extracted	Lignin
Unbleached	71.3	27.9	0.86	0.47	2.70
Fully-bleached	73.9	25.6	0.74	0.15	0.50

Preparation and characterization of LCNF films. The solid supports used for LCNF thin films were silica-coated QCM-D sensors (Q-sense AB, Västra Frölunda, Sweden) or 1x1 cm^2 silicon wafers with Si 100 native oxide top layer (Okmetic, Espoo, Finland). The solid supports were immersed in 10% NaOH solution and followed by rinsing with Milli-Q water. They were then dried with nitrogen gas and placed in a UV/ozone oven for 15 min. Polyethylene-imine (PEI) was adsorbed onto the cleaned supports in order to improve attachment and full coverage of the nanofibrils. To this end, washed silica sensors were immersed in 1 g/L aqueous solution of PEI for 15 minutes and then washed

with Milli-Q water (5 min) and dried with nitrogen gas. The solid supports were then stored in a desiccator before spin coating with LCNF, which was always done the same day.

The lignocellulosic nanofibril gels were diluted with Milli-Q water to 1.67 g/L concentration and homogenized using a Microtip-sonificator (10 minutes, 25 % amplitude). The samples were then centrifuged (45 min, 10400 rpm) with an Optima L-90k ultracentrifuge (Beckman Coulter, U.S.A) to remove remaining fibril aggregates. The clear supernatant was used for spin-coating onto the prepared PEI-silica substrates at 3000 rpm for 1 min. LCNF films were then rinsed with Milli-Q water, dried with nitrogen gas, heat-treated in an oven at 80 ºC for 10 minutes and stored in a desiccator until further use.

The changes in the structure and morphology of the LCNF films, before and after enzymatic hydrolysis were monitored by AFM imaging (Nanoscope IIIa Multimode scanning probe microscope from Digital Instruments, Inc., Santa Barbara, CA). The images were acquired in air in tapping mode using a J-scanner and silicon cantilevers (NSC15/AIBS from Micromasch, Tallinn, Estonia). The drive frequency of the cantilever was 325 kHz and the radius of curvature of the AFM tip according the manufacturer was less than 10 nm. At least two different LCNF films obtained for each condition were prepared and at least two different areas were analyzed on each of them. Scan sizes corresponded to 25 x 25, 10 x 10, 5 x 5 and 1 x 1 μm^2. No image processing except flattening was performed. Image analysis was performed using Nanoscope software (ver. V6.13 R1, Digital Instruments, Inc.) from which roughness and Z-sections in line profiles were determined.

The surface chemical composition of the thin films was quantified by using a X-ray photoelectron spectrometer (XPS, AXIS 165, Kratos Analytical, Manchester, UK) with mono-chromated Al Kα X-ray source. All samples were pre-evacuated overnight to obtain stable ultrahigh vacuum (UHV) conditions. Two parallel samples of the same type were subject to evaluation and for each sample at least three points were analyzed. Low scan resolutions at 80 eV pass energy and 1 eV steps were used to

determine the elemental surface composition. 1s high-resolution spectra at 20 eV pass energy and 0.1 eV steps were used for detailed carbon, C1s and oxygen, O1s peaks. The atomic concentrations were calculated from the photoelectron peak areas by using Gaussian deconvolution. The C1s spectra was divided into four different contributions of bonded carbon, corresponding to carbon without oxygen bonds (C-C and C-H) with the peak centered on a binding energy of 285eV; carbon with one oxygen bond (C-O) with binding energy of 286eV; carbon with two oxygen bonds (O-C=O) at 289.5eV. Both the oxygen/carbon atomic ratios and the C 1s high resolution curve fits were used in chemical analysis.[2]

Enzymatic hydrolysis

Enzymatic hydrolysis monitored by Quartz Crystal Microbalance with Dissipation monitoring (QCM-D). Enzyme binding and cellulosic activity on the LCNF films was monitored in situ using a QCM-D (QCM E4 model, Q-sense AB, Gothenburg, Sweden). The experiments were carried out at 40 ºC and pH 5 (0.1 M acetate buffer). The buffer solution was injected into the QCM flow module containing a LCNF substrate which was previously deposited on the QCM resonators. After the unit was filled with the background solution, the flow rate was maintained at 0.1 ml/min using a peristaltic pump. The LCNF film swelled in contact with the buffer solution, until reaching equilibrium, when a constant QCM frequency signal was registered. Then, multicomponent enzyme solution (diluted in buffer at 5, 0.1, 0.01 or 0.005% final concentrations) was continuously injected at a flow rate of 0.1 ml/min and the changes in frequency were registered until a final, stable signal was registered at the end of the process. Thereafter, the pump was stopped for 5 min and buffer solution was injected (0.1 ml/min) to rinse the system. In experiments carried out in batch mode, the enzyme flow was stopped when buffer solution initially present in the cell was fully replaced by enzyme solution and adsorption of the enzyme onto the film was observed (as a reduction in resonant frequency). No flow

condition was kept until registering a plateau signal (after adsorption and degradation of the film yielded plateau signals); then buffer solution was introduced at a flow rate of 0.1 ml/min to rinse the system. All measurements were recorded at 5 MHz fundamental resonance frequency and its overtones corresponding to 15, 25, 35, 55 and 75 MHz. The third overtone (15 MHz) was used for data processing.

Enzymatic treatments of LCNF dispersions. Enzymatic hydrolysis experiments with LCNF aqueous dispersions were carried out and the reducing sugar content at different reaction times was determined. The LCNF supernatants used to spin-coat the films were used directly as substrate in these experiments. The supernatant was mixed with buffer solution at pH 5 in several assay tubes. The assay tubes were immersed in a water bath at 40 ºC followed by enzyme solution addition. The final volume, enzyme concentration and enzyme activity – substrate ratio were 1.5 ml, 0.1% (v/v) and 100 FPU/g, respectively. The reaction was stopped at different reaction times (each assay tube corresponded to a given reaction time) and the reducing sugar content of the reaction mixture was analyzed by the Miller method.[3] Briefly, 3 ml of DNS reagent (3,5-dinitrosalicylic acid) were added into the assay tube to stop the reaction, the mixture was boiled during 5 minutes and then cooled in an ice bath. 0.5 ml of the colored solutions was centrifuged at 10,000 g for 3 min, and the supernatant was used to determine the absorbance at 540 nm in a UV- spectrophotometer. A calibration sugar curve was obtained by using glucose standard solutions to correlate the absorbance with the glucose (reducing sugar) content. The results from hydrolysis of LCNF were compared with those from microcrystalline cellulose (Avicel) used as substrate. The conditions and protocols were the same that those used for LCNF.

It should be noted that in QCM experiments the amount of fibrils in the film was much lower than in experiments with LCNF dispersions. Thus, although the enzyme concentration in the reaction mixture for experiments via QCM and cellulose dispersions was the same, 0.1% (v/v), the ratio enzyme

activity / substrate's mass was different: 100 FPU/g of substrate in dispersed LCNF experiments compared to more than 1000 FPU/g in experiments with thin films (QCM).

Empirical models to quantify binding and hydrolysis. Independent empirical models for the extent and dynamics of binding and hydrolysis were used. In order to fit key kinetic parameters to the experimental results both binding and hydrolysis data were fitted to simple Boltzmann-sigmoidal equation.

The extent of binding and hydrolysis are described by the time-dependent (*t*, min) shift of the third overtone QCM frequency Δf (Hz), which was adjusted to equation s1 and s2, respectively. The binding parameters shown in equation s1 includes 1/ τ, the adsorption rate (min^{-1}) and, Mmax, the maximum binding value (Hz) corresponding to the minimum frequency measured. W_{50} is the time (min) at which the inflection in frequency profile occurs. The hydrolytic parameters in Eq. s2 comprise the frequency at which hydrolysis ceases (B, corresponding to the plateau region at maximum frequency); the time for conversion to product to be maximized (V_{50}) and the hydrolysis rate (1/C).

$$\Delta f = A + \frac{M_{max} - A}{1 + e^{(W_{50} - t)/\tau}} \qquad \text{(s1)}$$

$$\Delta f = A + \frac{B - A}{1 + e^{(V_{50} - t)/C}} \qquad \text{(s2)}$$

The adjusted parameters after enzyme treatment of LCNF1, LCNF2 and Avicel dispersions are included in Table S2. Table S3 and S4 present the estimated parameters for hydrolysis of LCNF1 and LCNF2 films at different enzyme concentrations in open flow mode and in batch mode.

Table S2. Model parameters describing the enzymatic hydrolysis of unbleached LCNF1, bleached LCNF2 and Avicel aqueous dispersion treated with 0.1% enzyme concentration.

Substrate	B (%)	V_{50} (min)	1/C (min^{-1})	R^2
LCNF1	96±3	36±7	0.05±0.01	0.97
LCNF2	96±3	31±8	0.03±0.01	0.97
Avicel	57±2	48±15	0.01±0.01	0.99

Table S3. Model parameters describing the enzyme binding of unbleached LCNF1 and bleached LCNF2 films at different enzyme concentrations in open flow mode and in batch mode.

Enzyme concentration	LCNF Substrate	$-M_{max}$ (Hz)	W_{50} (min)	$1/\tau$ (min^{-1})	R^2
5%	LCNF1	52±5	0.5±0.05	11.5±0.6	0.9993
	LCNF2	29±12	0.3±0.02	14.7±0.5	0.9988
0.1%	LCNF1	28±1	0.7±0.09	5.3±0.8	0.9984
	LCNF2	33±5	0.6±0.10	6.6±0.6	0.9975
0.01%	LCNF1	14±3	1.2±0.11	2.6±0.2	0.9976
	LCNF2	14±2	0.9±0.10	3.3±0.2	0.9961
0.005%	LCNF1	21±4	2.2±0.16	1.4±0.3	0.9958
	LCNF2	20±2	2.5±0.17	1.2±0.1	0.9956
0.1% - Batch	LCNF2	20±1	0.5±0.10	5.0±0.7	0.9918

Table S4. Model parameters describing the enzymatic hydrolysis of unbleached LCNF1 and bleached LCNF2 films at different enzyme concentrations in open flow mode and in batch mode.

Enzyme concentration	LCNF Substrate	B (Hz)	V_{50} (min)	1/C (min^{-1})	R^2
5%	LCNF1	158±18	0.3±0.07	8.88±0.54	0.9953
	LCNF2	80±6	0.4±0.09	8.90±0.65	0.9950
0.1%	LCNF1	127±25	1.2±0.23	2.08±0.53	0.9831
	LCNF2	107±15	1.0±0.18	2.22±0.35	0.9880
0.01%	LCNF1	112±11	5.9±0.21	0.34±0.08	0.9741
	LCNF2	97±9	6.0±0.32	0.27±0.09	0.9717
0.005%	LCNF1	132±13	10.3±0.84	0.18±0.06	0.9824
	LCNF2	113±9	12.1±0.07	0.13±0.02	0.9669
0.1% - Batch	LCNF2	103±10	5.2±0.12	0.28±0.05	0.9625

Complementary Figures S1 and S2

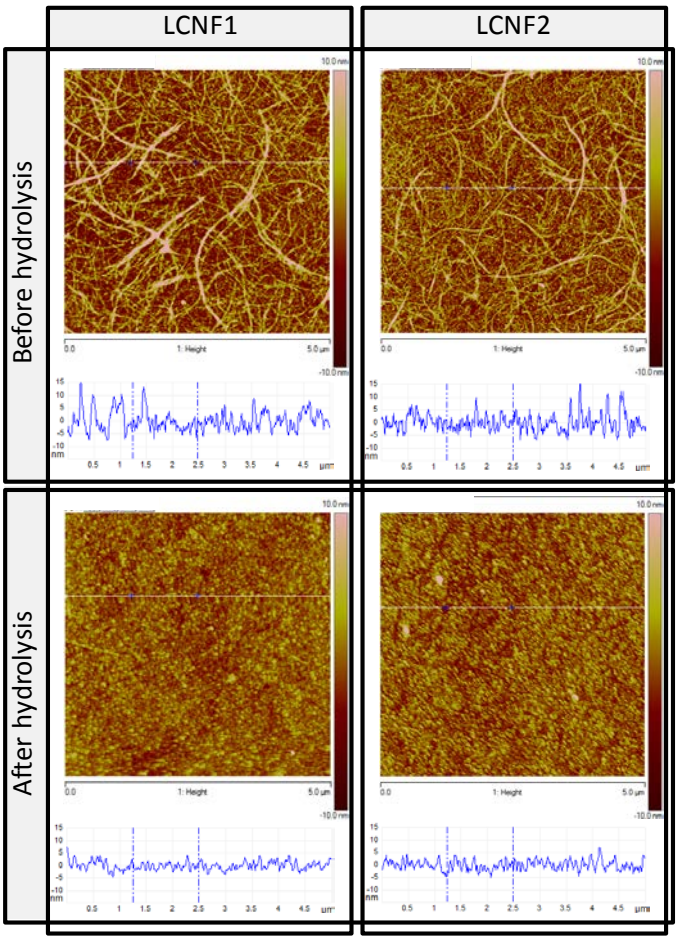

Figure S1. AFM 5 x 5 μm^2 height images of LCNF1 and LCNF2 thin films before (top) and after (bottom) enzymatic hydrolysis. The height scale (bar on the right of each image) corresponds to Z values between -10 and +10 nm. The height profiles included correspond to line scans shown in each of the AFM image.

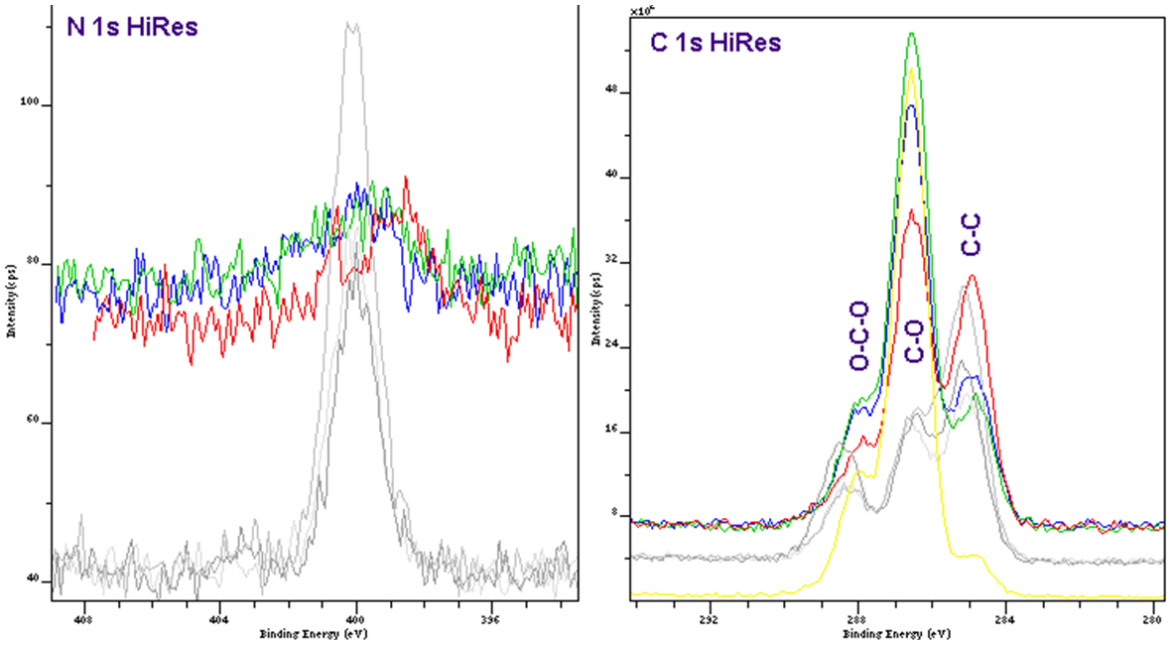

Figure S2. Surface chemical composition by XPS analyses of LCNF films before (colored lines) and after (greys lines) enzymatic hydrolysis: nitrogen N1s spectra after enzymatic hydrolysis indicating the presence of protein on the surface (a). The carbon C1s spectra shown in (b) include the three typical peaks of cellulose shown in the case of surfaces before enzymatic treatment (colored lines); however no cellulose can be detected after hydrolysis (grey spectra).

References

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(2) Johansson, L. S.; Campbell, J.; Koljonen, K.; Stenius, P., *Applied Surface Science*, **1999,** *144*, 92-95.

(3) Miller, G. L., *Analytical Chemistry*, **1959,** *31* (3), 426-428.