Supporting Information

Mechanoenzymatic Hydrolysis of Cotton to Cellulose Nanocrystals

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Table of Contents

Materials

The cotton was purchased as non-sterile raw cotton balls (CB) and Whatman type 1 filter paper (FP). Both materials were milled to powders using a Thomas Scientific Wiley Mini Mill 475-A equipped with a 30-mesh screen, and thereafter used in reactions without further purification. MilliQ water was from a MilliQ system, with a conductivity of 1.8 µS cm^{-1} at 25°C. The 0.1 M sodium phosphate buffer was prepared from NaH_2PO_4 and Na_2HPO_4 (supplied by Riedel-de Haën and Sigma Aldrich), using MilliQ water and adjusting the pH of the buffer to 6 with HCl. The 0.05 M sodium citrate buffer was prepared from sodium citrate tribasic dihydrate and citric acid (Sigma Aldrich), using MilliQ water, resulting in a solution with pH 6 to which no adjustments was done. The 0.05 M sodium acetate buffer was prepared from sodium acetate (Sigma Aldrich) and acetic acid (J.T. Baker), resulting in a solution with pH 5 to which no adjustment was done. To each buffer 0.02 wt% of sodium azide was added as a bacteriostatic preservative. The cellulases from *Trichoderma reesei*, *Aspergillus niger* and the CTec2® blend were purchased from Sigma-Aldrich. The enzymes were stored at 4°C. The Glucose (HK) Assay Reagent was purchased from Sigma Aldrich and reconstituted in MilliQ water before use. The reconstituted reagent was stored at 4°C for up to 4 weeks. Calibration standards for glucose analysis (0 – 10 mg mL^{-1}) were prepared from D-(+)-Glucose purchased from Sigma Aldrich. The 0.05 M citrate washing buffer, to remove enzymes from cellulose at the end of the reaction, was prepared from sodium citrate dihydrate and citric acid, in MilliQ water and adjusted to pH 10 with NaOH. Solvents used for GPC analysis (acetone, dimethylacetamide) were purchased from Sigma Aldrich in HPLC grade.

Methods

Equipment

Sartorius Entris 224i-1S analytical balance (linearity 0.2 mg, repeatability 0.1 mg) and calibrated variable volume pipettors from Thermo Scientific (Finnpipette F1) and Sartorius were used for measuring out the reactants. Ball milling was carried out using a Retsch MM400 shaker mill, set at a frequency of 25 Hz. Stainless steel jars (15 mL) provided by InSolido Technologies d.o.o., were used as the reaction vessels, charged with one 10 mm stainless steel ball (4 g). Aging steps at 55°C were carried out in a Memmert Natural Convection Standard Incubator equipped with humidity box saturated with water vapors. A heating block, Branson 2510 ultrasound bath and VWR MicroStar12 Centrifuge were used in preparation of the reaction aliquots for glucose analysis. The glucose assay was performed using clear bottom 96-well microtiter plates and recording absorbance at 340 nm on either Biotek Synergy H1 or Biotek Cytation microplate readers. After washing the reaction solids of the hydrolysis products and enzyme, the remaining solids were collected on 0.22 um Nylon or PDVF membrane filters using vacuum filtration. The samples were freeze dried on a Christ Alpha 2-4 freeze dryer. Gel permeation chromatography analysis was performed on a Thermo Scientific Dionex Ultimate 3000 GPC system. Transmission Electron Microscopy imaging was performed on a FEI Tecnai 12 microscope, at an acceleration voltage of 120 kV. Atomic Force Microscopy (AFM) images were recorded on a Bruker MultiMode 8 instrument. Wide-angle X-ray scattering (WAXS) data were recorded using a Xeuss 3.0 C (Xenocs, France) scattering device, with a GeniX 3D microfocus Cu source (wavelength λ = 1.54 Å) and an EIGER2 R 1M hybrid pixel detector. Elemental analysis was carried out on a Thermo Scientific Flash Smart CHNS/O Elemental Analyzer, on 2.0 ± 0.2 mg samples with sulfanilamide was used as the standard. Zeta potentials were measured on the Malvern Zetasizer, and the thermal analysis was carried out with the Netzsch STA 449 F3 Jupiter system.

Characterization of the enzymes

Enzymes were used as the supplied powder (*Trichoderma reesei*, *Aspergillus niger*) or solution (CTec2). The protein content of the enzyme preparations was determined using the Bradford assay, and was 31 ± 1%, 1.7 ± 0.2% and 6.1 ± 0.8% w/w, respectively, for the *Trichoderma reesei*, *Aspergillus niger* and the CTec2 cellulase blend. The total protein content represents a consortium of cellulase enzymes: cellobiohydrolases, endoglucanases and β-glucosidases.[1] Cellulases produced by the

Trichoderma reesei fungus, for example, consist of cellubiohydrolases (20% Cel6A and 60% Cel7A) and endoglucanases (12% Cel5A). Commercial cellulase mixtures were preferred in this study over a combination of purified individual enzymes, to allow for the strong synergistic effect of cellulase enzymes, and eliminate cost of individual enzyme purification in the developed methods.

Previous experience has shown that commercial enzyme preparations can inherently contain some glucose, and/or carbohydrates from which glucose is produced during aging.[2] This inherent glucose was quantified in this work at two timepoints: straight from the bottle, after 24 h of aging in the reaction buffer. The analysis mixtures were prepared by mixing 10 mg of *Trichoderma reesei*, 10 mg of *Aspergillus niger* or 10 mg (8.7 µL) of the CTec2® with 210 uL of 0.1 M NaPB buffer (pH 6), to represent the concentrations used in the reaction setup. Each mixture was prepared in triplicate, which were aliquoted to two 40 uL samples for timepoint analysis (n = 3 for each timepoint). At the respective timepoints, the samples were diluted to 2 mg/mL, by bringing the volume of the sample to 952 uL (added 912 uL of milliQ water), and quickly heated for 30 min at 90°C to deactivate the enzymes. The centrifuged and the clear supernatant was analyzed by glucose assays. The commercial cellulase enzyme from *Trichoderma reesei* and CTec2® contained 0.6 ± 0.1% w/w and 21.1 ± 0.3% w/w glucose, respectively, which did not significantly change upon aging for 24 h (increased slightly to 0.8 ± 0.3% w/w and 23 ± 1% w/w glucose, respectively). The enzyme from *Aspergillus niger* contained 14 ± 1% w/w glucose, which was found to increase 64 ± 1% w/w upon aging. This shows that all the commercial enzyme preparations contain some glucose (up to *ca* 21%), and that in the case of cellulases from *A. niger* the commercial enzyme contains sugars that are converted to glucose upon aging. The amount of inherent glucose in milled and aged reaction mixtures was accounted for (subtracted) in the calculation of the glucose yield of cellulose.

The activity of the cellulase enzymes was determined by assaying the hydrolysis microcrystalline cellulose (Sigma Aldrich, average 51 µm particles) into glucose at 37°C. The assay was carried out using a 5% (w/v) suspension of MCC in 50 mM sodium acetate buffer (pH 5.0). The commercial enzyme stocks were prepared by diluting the powder or solution to 5 mg mL^{-1} with MilliQ water. The assay was started by mixing 400 µL of the MCC suspension with 100 µL of the 5 mg mL^{-1} enzyme solution (resulting enzyme concentration in sample 1 mg mL^{-1}) was added and the mixture and shaken on a tabletop shaker (300 rpm) at 37°C for 120 min. The samples were promptly centrifuged, and the clear supernatant was assayed for glucose content. The activity of cellulases from *Trichoderma reesei* was measured as 1.2 ± 0.1 U mg^{-1}, the activity of cellulases from *Aspergillus niger* was measured as 1.3 ± 0.1 U mg^{-1}, and the activity of CTec2® cellulases was measured as 2.0 ± 0.1 U mg^{-1} (1770 ± 120 U mL^{-1}). No loss of activity was observed within 18 months, with enzymes stored at 4°C.

Method for milling and milling + aging reactions

In a typical reaction, pre-milled cotton powder (400 mg) was weighed into a 15 mL stainless steel jar with a 4 g stainless steel ball, to which the commercial enzyme preparation (either 20 mg of *Trichoderma reesei*, 20 mg of *Aspergillus niger* or 20 mg (17.4 µL) the CTec2® blend and buffer (420 µL) were added, bringing the total liquid-to-solid ratio to 1.0 µL mg^{-1}, corresponding to a solid loading of 50% w/w. The CTec2 commercial enzyme preparation contains a high concentration of inherent glucose (*ca*. 30% w/w, see section ***Characterization of the enzymes***) and the volume of this enzyme solution was therefore counted as solids in the calculation of the total liquid-to-solid ratio. The milling jars were then closed and set to mill at 25 Hz for 15 or 30 minutes. The resulting uniform solids were aliquoted into 1.5 mL Eppendorf tubes affording analysis samples for glucose assays (20–30 mg) and for recovered cellulose isolation (200 mg) at different time points: immediately after milling, after subsequent aging for 24 h at 55°C, and after subsequent aging for 72 h at 55°C. Specific reaction conditions, together with the hydrolysis yields are compiled into **Table S1.**

Determination of glucose yield by the glucose assay

A commercial glucose assay reagent was used to quantify glucose in the reaction mixtures. The aliquot of the reaction mixture to be analyzed was suspended at 10 mg mL^{-1} in cold water, closed tightly and quickly heated to 90°C to denature the enzymes. After 30 minutes of heating, the sample was sonicated for 5 minutes to ensure the dissolution of glucose from solid aggregates, and the suspension was centrifuged at 12 300 × *g* for 5 minutes. An aliquot of the clear supernatant (2 µL) was mixed with the glucose assay reagent (150 µL) in a clear-bottom 96-well plate and incubated at room temperature. The absorption was monitored at 340 nm until the reading plateaued. Glucose calibration standards (0 – 10 mg mL^{-1}) were measured on each occasion alongside sample measurements, due to the gradual inactivation of the Glucose Assay Reagent. The resulting calibration curve, which relates the glucose concentration to absorbance at 340 nm was used to interpolate the glucose concentration in the reaction samples.

The yield of glucose (%) is calculated by dividing the experimentally determined yield of glucose (mg) with the theoretical yield at 100% conversion, calculated by approximating the cellulose in cotton to infinite linear chains of condensed glucose. The experimentally determined yield of glucose (mg) is extrapolated from the content of glucose in solids as determined by the glucose assay, with enzyme-inherent glucose subtracted (see further details in section ***Characterization of the enzymes***).

Isolation of hydrolyzed cellulose from mechanoenzymatic reactions

An aliquot of 200–250 mg of the reaction mixture (solids content 48–50%) was taken at the end of the reaction, suspended at 1 wt% in the washing buffer (see below) and incubated for 1 h with gentle magnetic stirring at 100 rpm to remove the enzyme. Thereafter, the solids were collected by vacuum filtration and washed on a 0.22 µm polypropylene or nylon membrane, once with the washing buffer and three times with milliQ water. The solids were collected from the membrane by rinsing with milliQ water into a Falcon tube and freeze dried. Some loss of solids could not be avoided upon flushing of the membrane, which is reflected in the recovery% of cellulose. The weight of the dry solids was recorded, from which the recovery% was calculated as follows:

$$recovery\% = \frac{m(dry\ solids, mg)}{m(aliquot, mg) \times 0.48} \times 100\% \qquad \text{(Equation S1)}$$

The washing buffer for the removal of the enzyme from the cellulose post-reaction was chosen based on the report from Shang et al.[3] Enzyme desorption was carried out at 1 wt% solids content in 50 mM citrate buffer, adjusted with NaOH to pH 10,[4] which was shown by Shang et al. to remove 85% of cellulases after enzymatic hydrolysis by 1 h of room-temperature incubation.[3] By measuring the protein content in the washing buffer filtrate after the solid-state reaction (by Bradford assay), less efficient removal of the enzymes was generally achieved in our reaction setting and a clear correlation can be observed between decreased removal efficiency and duration of the aging step (0 h, 24 h and 72 h), regardless of the enzyme preparation used. *Ca*. 7% of the *T. reesei* cellulases could be extracted to the washing buffer right after the milling step, and < 5% could be removed after 24 h or 72 h of aging. *Aspergillus niger* cellulases could be removed slightly more efficiently, with *ca*. 40% extractable to the washing buffer right after the milling step, *ca*. 25% could be removed after 24 h of aging and *ca*. 20% after 72 h of aging. Also cellulases from CTec2 could be removed slightly more efficiently than *T. reesei*, with *ca*. 15% extractable to the washing buffer right after the milling step, *ca.* 10% could be removed after 24 h of aging and only *ca*. 4% after 72 h of aging. When higher *T. reesei* loading (4.1 wt% or 8.2 wt%) was used in the reaction, *ca*. 50% enzyme could be removed even after 72 h of aging, which may indicate that some of the protein at high enzyme loading is not irreversibly bound to cellulose. Alternative washing buffer tested, 45% glycerol solution, did not improve the enzyme removal efficiency. Alternatively, enzymes could also be inactivated by heating at 90°C for 30 minutes, or by suspending and sonicating the solid reaction mixture in EtOH.

Size exclusion chromatography

The molecular mass distribution was determined by gel permeation chromatography (GPC). The washed and freeze-dried recovered cellulose samples were prepared for GPC analysis by the water-acetone-*N*,*N*-dimethylacetamide (DMAc) solvent exchange procedure. Namely, 50±2 mg of the sample was mixed on a polyethylene frit with 4 mL of MilliQ water, kept overnight and then dewatered by vacuum filtration and rinsing with 2 mL of acetone. Thereafter 4 mL of acetone was added, and the sample was kept for at least 6 hours, after which the acetone was removed and replaced with 4 mL of DMAc (from Sigma Aldrich, HPLC grade, purity > 99.9%). The sample was kept in DMAc overnight, after which the DMAc was removed, and the sample dissolved in 5 mL of 90 g L^{-1} LiCl/DMAc (LiCl from VWR, purity > 98.5%) at room temperature with slow speed overnight magnetic stirring. The resulting 10 mg mL^{-1} sample was diluted in pure DMAc to 1 mg mL^{-1} (9 g $^{-1}$ LiCl/DMAc) and filtered (0.2 μm) before GPC analysis. Dionex UltiMate 3000 HPLC System equipped with an isocratic pump, PLgel MIXED-A 7.5 x 300 mm column, Shodex RI-101 refractive index detector and a Viscotek/Malvern SEC/MALS 20 multi-angle light scattering detector, was used to elute the samples and pullulan standards with 9 g L^{-1} LiCl/DMAc at a flow rate of 0.75 mL min^{-1}, with the injection volume of 100 μL. Detector constants (MALS and DRI) were determined using narrow polystyrene sample (Mw = 96 000 g/mol, $Đ$ = 1.04) dissolved in 0.9% LiCl in DMAc. Broad polystyrene sample (Mw = 248 000 g/mol, $Đ$ = 1.73) was used for checking the detector calibration. The $\partial n/\partial c$ value of 0.136 mL g^{-1} was used for celluloses in 9 g L^{-1} LiCl/DMAc (Potthast et al., 2015). Viscotek OmniSEC software was used to determine the cellulose molar mass distribution, based on the pullulan standards (343 Da – 2500 kDa, Polymer Standard Service GmbH, Mainz, Germany; 1600 kDa from Sigma Aldrich) according to the equation $M_{cellulose} = 12.19 \times \left(M_{pullulan}\right)^{0.78}$, according to Berggren, et al.[5] Each sample was measured twice, with the average of two measurements reported in the Table S1. The deviation given for the DP_n, DP_w and $Đ$ values represents measurement uncertainty (generally 10-20%). Entry 3 was carried out in triplicate, therefore the average and standard deviation is calculated based on 6 measurements.

Crystallinity determination of cotton based on wide-angle X-ray scattering

About 15–20 mg of the recovered (hydrolyzed) cellulose was placed between two Kapton films on a sample holder. Small- and wide-angle X-ray scattering (SAXS, WAXS) data was recorded using a Xenocs Xeuss 3.0 SAXS/WAXS system (Xenocs SAS, Grenoble, France), with a microfocus X-ray source (sealed tube, operated at 50 kV and 0.6 mA) with a Cu target and a multilayer mirror which yields a parallel beam with a nominal wavelength of 1.542 Å (combined Cu K-α1 and Cu K-α2 characteristic radiation). The beam is collimated by a set of variable slits and the beam size at the sample was 0.4 mm during the experiment. The system does not include a beam stop, which enables direct measurement of sample transmission. The background scattering from sample holder is normalized and subtracted from the data according to sample transmission. The data is acquired using an area detector (Eiger2 R 1M, Dectris AG, Switzerland) that was in the evacuated chamber and set at 60 mm distance from the sample. The sample-to-detector distance was calibrated by measuring the diffraction from a known LaB6 standard sample. The scattering vector q is defined as $q = 4\pi\sin(\theta)/\lambda$ with scattering angle 2θ. The measurement was carried out in a vacuum to avoid scattering from the air. The two-dimensional scattering images were normalized by the transmitted intensity, and the scattering contribution from the background (empty chamber and sample holder with Kapton films) was subtracted.

Crystallinity indices, CrI and CI, were calculated according to the empirical Segal peak height method (Equation S2),[6] and by peak fitting methods, respectively. The CrI was calculated by comparing the intensity of the 2 0 0 diffraction peak at 2θ = 22° ($I_{2\,0\,0}$), representing the sum of crystalline and amorphous contributions, with the intensity at the minimum between the 1 1 0 and 2 0 0 peaks at 2θ = 18° (I_{AM}) which represents the amorphous-only component. Note, that the CrI based on Segal's peak height method reveals relative changes in crystallinity within the same material type (CB or FP) upon

different reaction conditions, and should not be taken as an absolute value of crystalline and amorphous fractions in the materials.[6–8]

$$Crystallinity\ index\ (CrI) = 100\ \times (I_{2\,0\,0} - I_{AM})/I_{2\,0\,0} \qquad \text{(Equation S2)}$$

The crystallinity index CI was calculated based on fitting of crystalline diffraction peaks of cellulose 1 $\bar{1}$ 0 (max 2θ = 14.7°), 1 1 0 (max 2θ = 16.5°), 1 0 2 (max 2θ = 20.4°) and 2 0 0 (max 2θ = 22.5°), and a broad peak at 2θ = 20.5° assigned as the amorphous contribution, with Gaussian functions to the experimental diffractogram, using OriginPro 2024 (OriginLab Corporation, Northampton, MA, USA), and calculating the ratio of the area of all crystalline peaks to the total area under the diffractogram.

The calculated CrI and CI do not represent the absolute quantity of crystalline fractions in these materials,[7,8] but is rather used here to evaluate relative changes in crystallinity brought about by the mechanoenzymatic process, compared to the respective starting material. The trends, showing increasing cellulose crystallinity with milling and with subsequent aging, is consistent with both calculation methods (Figure 3).

Elemental analysis

The recovered hydrolyzed cellulose, obtained at the reaction conditions indicated in Table S1 entries 3, 6 and 8, was analyzed by elemental analysis after the washing and drying protocol described in section ***Isolation of hydrolyzed cellulose from mechanoenzymatic reactions***. Samples after 72 hours of aging were chosen, as this represents the largest amount of potentially denatured and immobilized enzyme present in the samples. Each sample was measured in triplicate. The elemental analysis data (N%) was used as a qualitative evidence of protein present in the enzyme-treated hydrolyzed sample, while the quantification of protein removal was determined with a Bradford assay. Since the enzymes are adsorbed onto cellulose, the ratio of protein-to-cellulose is higher after washing, compared to the start of the reaction, due to the removal of soluble hydrolysis products. The yield of glucose for the analyzed samples can be found in Table S1, and the elemental analysis results are compiled in Table S5.

Zeta potential measurements

The zeta potential of the recovered (hydrolyzed) cellulose samples, obtained at the reaction conditions indicated in Table 1 (main text) entries 1, 5, 8 and 11 were analyzed using a Malvern Zetasizer instrument. These samples compare the isolated products after mechanoenzymatic treatment (*Tr*, *An* or CTec2 cellulases, 15 minutes of milling, no aging) to the starting material (pre-milled cotton). The samples were dispersed at 1 wt% in water, sonicated for 3 x 5 min and diluted with milliQ water to 0.1 wt%. As all the recovered cellulose samples were washed at the same conditions (see section *Isolation of hydrolyzed cellulose from mechanoenzymatic reactions*), we expect the to be the same for all samples. The measurement was performed using a ZEN1002 dip cell. Each sample was measured 3 times. The reported zeta potential gives the average, with the standard deviation representing the measurement error. The zeta potential of the starting material, the pre-milled cotton, was −24.9 ± 0.7 mV, and the enzymatically treated samples were −16.6 ± 0.2 mV (*Tr*), −26.3 ± 0.87 mV (*An*) and −22.2 ± 0.6 mV (CTec2). The particle count rate varied between the triplicate measurements, indicating poor dispersion quality in water (as expected for non-charged CNCs in water). The low deviation in zeta potential shows that the measurement was not significantly affected by the dispersion quality.

Atomic Force Microscopy

Dry isolated hydrolyzed cellulose samples were dispersed at 1 wt% in formic acid (purity 99.3%) by 15 min of ultrasonication. UV-cleaned (15 min) silicon wafers were soaked in 0.3% poly(ethyleneimine) (PEI) aqueous solution for 15 min, after which they were rinsed with milliQ water and dried with air. The PEI-functionalized wafers were spin coated (90 s, 4000 rpm) with 20 µL of the cellulose dispersion in formic acid and air-dried. All samples were imaged in tapping mode (512 scans per line, amplitude setpoint adjusted according to image, scan rate 1 Hz), using Bruker NCHV-A probes with a tip radius 8 µm, cantilever thickness 3.5 µm, reflective aluminum reflex coating, starting resonance of 320 kHz

and a spring constant of 40 N/m. AFM images were flattened with polynomial equations (0^{th}, 1^{st} and 2^{nd} order), where necessary, to remove scan line misalignment, tilt and bow of the surface prior to particle analysis. Particle analysis (height and length, see Figure S4) was carried out with the NanoScope Analysis 3.0 program.

Transmission Electron Microscopy

Sample preparation protocol was adopted from Meija, et al.[9] The recovered (hydrolyzed) cellulose was dispersed in milliQ water at 1 wt% by ultrasonication. The solution was diluted to the final concentration of 0.025 wt% with milliQ water, and deposited on plasma-treated (30 s, 25%/75% O_2-Ar at 40 W) carbon coated copper grids (Electron Microscopy Sciences), and stained with uranyl formate. The deposition protocol was as follows: 10 µL of the 0.025 wt% hydrolyzed cellulose dispersion was deposited on the grid, left for 4 minutes, after which the drop was carefully wicked with filter paper. The sample on the grid was washed by adding 10 µL of pure milliQ water onto the grid and wicking it away. Thereafter the sample was stained by depositing 10 µL of 2% (w/v) uranyl formate aqueous solution onto the grid for 4 minutes, wicked away and washed with another 10 µL of milliQ water. The excess water was wicked away and the grid was allowed to air-dry for at least 2 hours before imaging on the FEI Tecnai 12 miscroscope at 120 kV acceleration voltage. Particle analysis (length, see Figure S3) was carried out with the ImageJ program.

Thermal analysis

The recovered (hydrolyzed) cellulose samples were obtained at the reaction conditions indicated in Table 1 (main text) entries 5, 8, 11 and 16. The thermal behavior of the samples, *Tr*, *An*, CTec2 and blank was analyzed by a Netzsch STA 449 F3 Jupiter system using the following settings: heating rate of 20 K/min; helium atmosphere; temperature from 40 to 900°C. The data was analysed via NETZSCH Proteus Thermal Analysis 8.0 software, and OriginPro 2024.

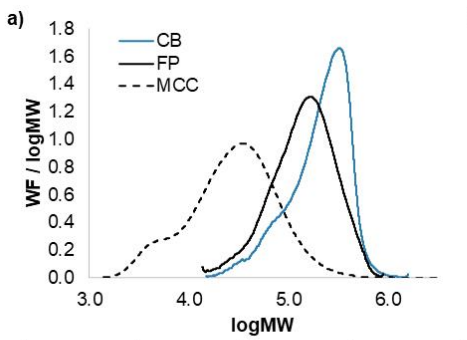
Green metrics calculations

To evaluate this method in the context of sustainability,[10] we calculated the following green metrics (Table S6) – process mass intensity (PMI)[11] and space-time-yield (STY), and compared with previously developed processes reporting enzymatic hydrolysis as the main step for cellulose nanocrystal production. We excluded comparisons with reported processes where relevant reaction parameters were missing (cellulose solid loading, reaction time or amount of recovered hydrolysed cellulose), or where the enzymatic step was preceded by acid hydrolysis. We limited our comparisons to processes using pure (or purified) cellulose sources. Washing water is generally not included in the calculation of PMI, therefore the calculated metrics compare the enzymatic hydrolysis up to the washing step. The volume of reaction media (solvents, water) strongly influences the efficiency of a process, therefore the amount of water (or buffer) used in the reaction is included in the PMI calculation. Following equations were used:

$$PMI = \frac{Total\ mass\ used\ in\ the\ process\ (kg)}{Mass\ of\ product\ (kg)} \qquad \text{(Equation S3),}$$

$$STY = \frac{Mass\ of\ product\ (g)}{Reaction\ volume\ (L) \times duration\ (h)} \qquad \text{(Equation S4).}$$

Supplementary Figures



Material	DP_n	DP_w	Đ (M_w/M_n)
CB	924	1518	1.6
FP	656	1089	1.7
MCC	102	282	2.8

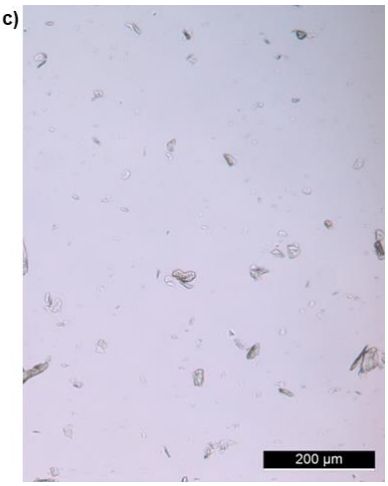


Figure S1.
a) Molecular weight distribution graphs, degree of polymerization (DPn, DPw) and dispersity (Đ) of the cotton starting materials (pre-milled CB and FP), in comparison to commercial microcrystalline cellulose (MCC). MCC was not chosen as a starting material for enzymatic hydrolysis in this study, since the powder represents an acid-processed, low DP cellulose. b) Optical microscopy image of the pre-milled CB material (Wiley cutting mill, mesh 30). c) Optical microscopy image of the as-purchased MCC.

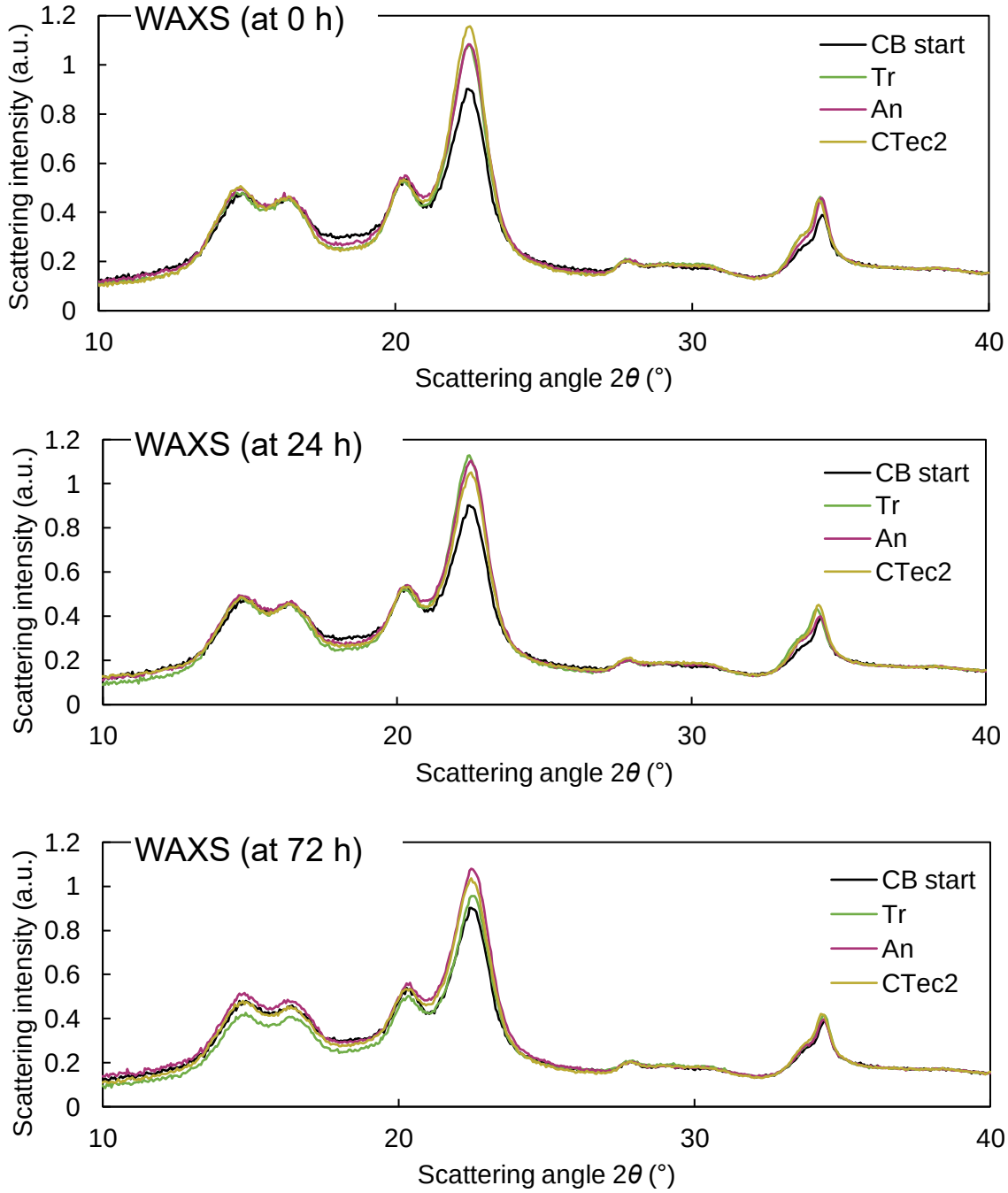


Figure S2.
Wide-angle X-ray scattering data for the isolated hydrolysed cellulose, right after milling (top), and after subsequent aging for 24 (middle) or 72 h (bottom) at 55°C, comparing Trichoderma reesei (Tr), Aspergillus niger (An) and Cellic CTec2 enzymes to the starting material (CB start).

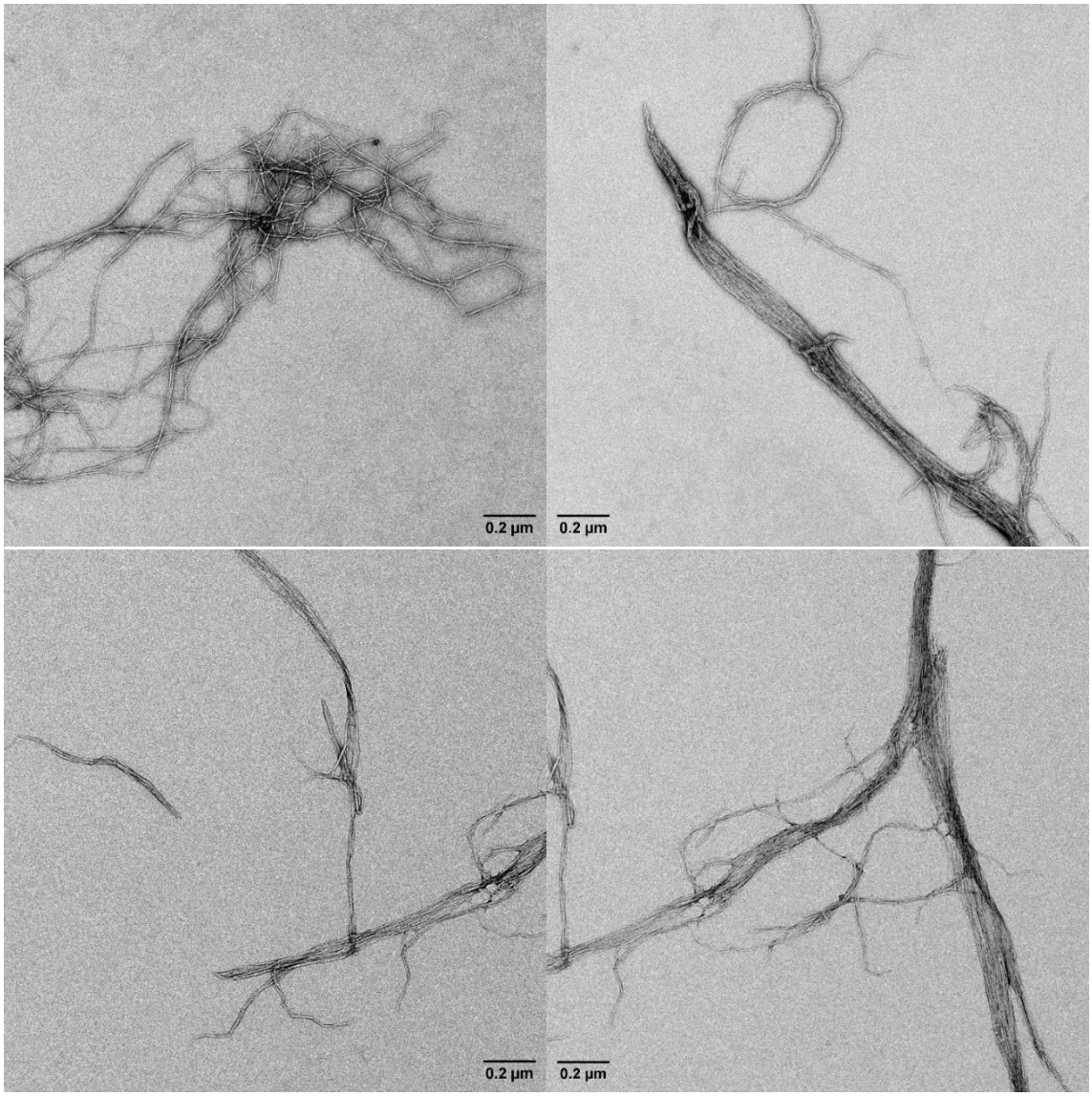


Figure S3.
Additional TEM images of cotton cellulose isolated from a blank reaction (no enzyme). The reaction conditions can be found in Table S1 entry 1 (72 h).

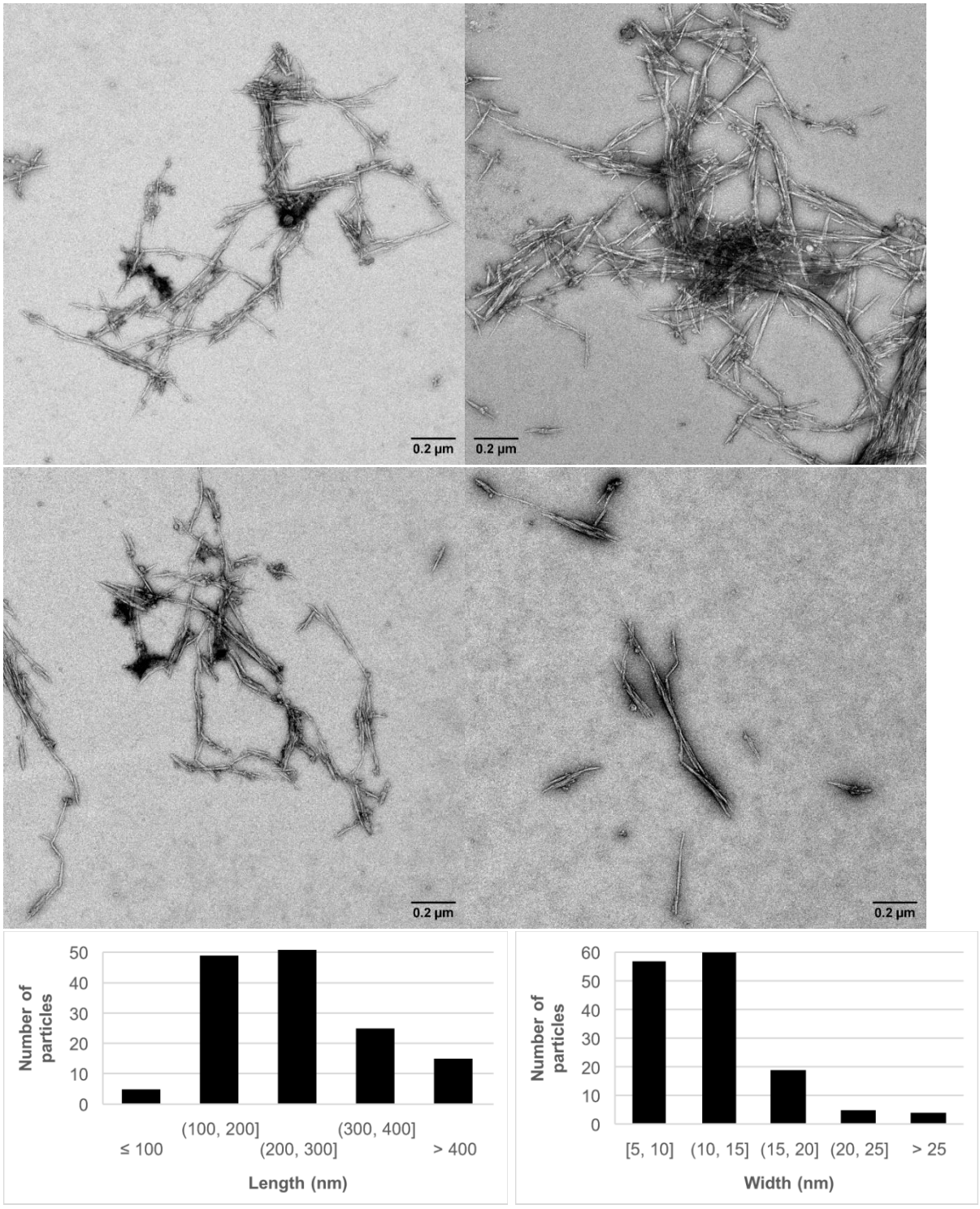


Figure S4.

Additional TEM images of cellulose isolated from a hydrolysis reaction with Tr cellulases. The reaction conditions can be found in main text Table 1 entry 7 (72 h). Histograms show the distribution in the length and width of particles (145 particles total). Since longer particles are more likely to be entangled, these values may be skewed towards shorter, more individualized particles. Side-to-side aggregation of CNCs can also skew the width measurements.

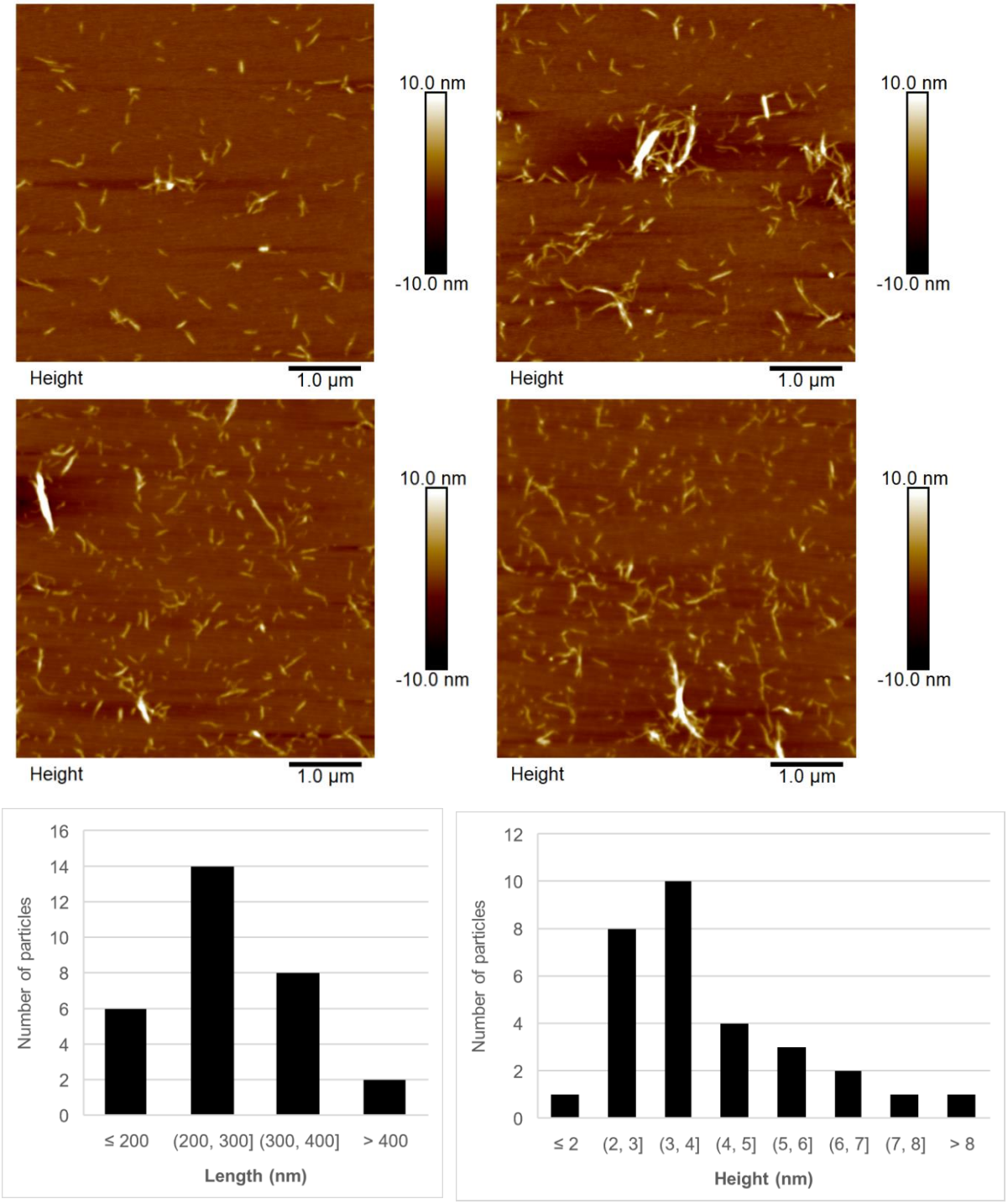


Figure S5.
AFM images of cellulose isolated from a hydrolysis reaction with Tr cellulases. The reaction conditions can be found in main text Table 1 entry 5 (0 h). Histogram showing the distribution in the length (left)and height (right) of particles (30 particles total).

Supplementary Tables

Table S1.

The varied conditions and results for the milling + aging reactions. Same for all reactions: the substrate was pre-milled cotton balls (Thomas Scientific Wiley Mini Mill 475-A equipped with a 30-mesh screen), the buffer was 100 mM sodium phosphate (pH 6), introduced at 1.0 µL mg^{-1}.

Entry	Cotton (mg)	Enzyme	Enzyme loading	Milling duration	**Aging duration**	**Glucose yield (%)** [i]	**Recovered cellulose (%)**	DP_n [ii]	DP_w [ii]	$Đ$ (M_w/M_n)[ii]
1	400	blank	0% w/w	15 min	**0 h** **24 h** **72 h**	**0.03** **0.03** **0.0**	**91** **90** **94**	**1300±200** **1070±40** **1130±90**	**1720±90** **1565±1** **1620±20**	**1.3±0.1** **1.5±0.1** **1.4±0.1**
2	400	blank	0% w/w	30 min	**0 h** **24 h** **72 h**	**0.5** **0.4** **0.4**	**92** **92** **94**	**1290±10** **1100±120** **1000±160**	**1760±20** **1580±70** **1570±30**	**1.4±0.1** **1.5±0.1** **1.6±0.2**
3	400	T. R.	1.56% w/w	15 min	**0 h** **24 h** **72 h**	**2.8** **13.7** **24.5**	**91** **77** **68**	**290±40**[iii] **280±50**[iii] **260±30**[iii]	**1100±130** **950±105** **1050±230**	**4.0±0.3** **3.5±1** **4±1**
4	400	T. R.	1.56% w/w	30 min	**0 h** **24 h** **72 h**	**2.9** **13.9** **27.4**	**100** **80** **68**	**340±70** **185±5** **205±5**	**980±60** **645±5** **620±5**	**2.9±0.5** **3.5±0.1** **3.0±0.1**
5	300[v]	T. R.	1.56% w/w	15 min	**72 h**	**27.8**	**69**	**290±10**	**1130±30**	**3.9±0.2**
6	300	T. R.	4.12% w/w	15 min	**72 h**	**37.1**	**61**	**360±50**	**1280±50**	**3.6±0.4**
7	300	T. R.	6.24% w/w	15 min	**0 h** **24 h**	**5.0** **32.6**	**79** **62**	**470±60** **280±70**	**1300±30** **1000±125**	**2.8±0.3** **3.7±0.4**
8	300	T. R.	8.2% w/w	15 min	**24 h** **72 h**	**26.3** **45.2**	 **49**[iv]	 **510±70**	 **1410±50**	 **2.8±0.3**
9	400	A. N.	0.085% w/w	15 min	**0 h** **24 h** **72 h**	**0.6** **3.5** **6.8**	**100** **86** **93**	**290±50** **240±30** **300±50**	**1220±60** **1040±10** **1010±50**	**4.2±0.5** **4.4±0.6** **3.4±0.4**
10	400	CTec2	0.3% w/w	15 min	**0 h** **24 h** **72 h**	**1.6** **12.1** **20.4**	**94** **80** **76**	**450±20** **250±5** **290±20**	**1290±5** **820±5** **1150±70**	**2.9±0.1** **3.3±0.1** **4.0±0.1**
Comparisons										
Starting material: pre-milled cotton balls								**920±20**	**1520±20**	**1.6±0.1**

[i] The amount of glucose added with the enzyme preparation (see section ***Characterization of the enzymes***) was subtracted from the experimentally determined total glucose before calculating this yield. [ii] The given variation for DP_n, DP_w and $Đ$ represents the standard deviation of duplicate measurements. [iii] For entry 3 triplicate reactions were carried out to estimate experimental variation. The given deviation represents 6 measurements from three independent reactions, which shows that experimental variation does not exceed measurement uncertainty. [iv] Reducing the scale of the reaction by 25% did not significantly impact the hydrolysis, as the glucose yield and DP at 72 hours are very close to entry 3. [iv] Remaining weight determined from 45% glycerol wash.

Table S2.

The varied conditions and results for the milling + aging reactions. Same for all reactions: the substrate was pre-milled cotton balls (Thomas Scientific Wiley Mini Mill 475-A equipped with a 30-mesh screen), the enzyme was *T. reesei*, introduced at 1.56% w/w loading.

Entry	Cotton (mg)	Buffer	Eta	Milling duration	Milling frequency	**Aging duration**	**Glucose yield (%)** [i]
1	200	water	1.0 μL mg^{-1}	5 min	25 Hz	**0 h** **24 h** **72 h**	**1.4** **13.8** **18.4**
2	200	50 mM citrate, pH 6	1.0 μL mg^{-1}	5 min	25 Hz	**0 h** **24 h** **72 h**	**1.6** **11.9** **21.7**
3	200	50 mM NaOAc, pH 5	1.0 μL mg^{-1}	5 min	25 Hz	**0 h** **24 h** **72 h**	**2.5** **15.1** **20.4**
4	200	100 mM PB, pH 6	1.0 μL mg^{-1}	5 min	25 Hz	**0 h** **24 h** **72 h**	**1.7** **12.4** **23.8**
5	200	100 mM PB, pH 6	1.0 μL mg^{-1}	15 min	25 Hz	**0 h** **24 h** **72 h**	**2.8** **13.7** **24.5**
6	200	100 mM PB, pH 6	1.5 μL mg^{-1}	15 min	25 Hz	**0 h** **24 h** **72 h**	**1.6** **14.5** **26.6**
7	200	100 mM PB, pH 6	2.0 μL mg^{-1}	15 min	25 Hz	**0 h** **24 h** **72 h**	**1.4** **15.1** **27.2**

[i] The amount of glucose added with the enzyme preparation (see section ***Characterization of the enzymes***) was subtracted from the experimentally determined total glucose before calculating this yield.

Table S3.

The varied conditions and results for the milling + aging reactions. Same for all reactions: the substrate was pre-milled **Whatman 1 filter paper** (Thomas Scientific Wiley Mini Mill 475-A equipped with a 30-mesh screen), the buffer was 50 mM NaOAc (pH 5), introduced at 1.0 µL mg^{-1}.

Entry	Cotton (mg)	Enzyme	Enzyme loading	Milling duration	Milling frequency	**Aging duration**	**Glucose yield (%)** [i]
1	200	blank	0% w/w	5 min	25 Hz	**0 h** **24 h** **72 h**	**1.0** **0.3** **0.2**
2	200	T. R.	1.56% w/w	5 min	25 Hz	**0 h** **24 h** **72 h**	**2.8** **15.7** **21.6**
5	200	A. N.	0.085% w/w	5 min	25 Hz	**0 h** **24 h** **72 h**	**1.4** **7.2** **9.0**
6	200	CTec2	0.305% w/w	5 min	25 Hz	**0 h** **24 h** **72 h**	**1.1** **7.9** **11.8**

[i] The amount of glucose added with the enzyme preparation (see section ***Characterization of the enzymes***) was subtracted from the experimentally determined total glucose before calculating this yield.

Table S4.

The varied conditions and results for the milling + aging reactions. Same for all reactions: the substrate was pre-milled **Whatman 1 filter paper** (Thomas Scientific Wiley Mini Mill 475-A equipped with a 30-mesh screen), the buffer was 50 mM NaOAc (pH 5), introduced at 1.0 µL mg^{-1}.

Entry	Cotton (mg)	Enzyme	Enzyme loading	Milling duration	Milling frequency	**Aging duration**	**Glucose yield (%)** [i]	**Recovered cellulose (%)**	DP_n [ii]	DP_w [ii]	$Đ$ (M_w/M_n)[ii]
1	200	blank	0% w/w	15 min	25 Hz	**0 h** **24 h**		**92** **92**	**580±70** **500±40**	**1000±50** **970±20**	**1.7±0.1** **1.9±0.1**
2	200	T. R.	1.56% w/w	15 min	25 Hz	**0 h** **24 h**		**88** **74**	**250±30** **360±30**	**720±10** **1040±50**	**2.9±0.3** **2.9±0.1**
Comparisons											
Starting material: pre-milled Whatman 1 filter paper									**655±10**	**1090±20**	**1.7±0.1**
Whatman 1 filter paper, hydrolyzed by pressurized HCl gas (100 kPa, 24 h), according to Pääkönen et al.[12]									**250±5**	**2250±500**	**8.9±1.7**

[i] The amount of glucose added with the enzyme preparation (see section ***Characterization of the enzymes***) was subtracted from the experimentally determined total glucose before calculating this yield. [ii] The given error represents the standard deviation of duplicate measurements.

Table S5.

Elemental analysis results, comparing the starting material (CB) to the recovered and washed cellulose from hydrolysis with *Tr* cellulases, revealing (qualitatively, based on recorded N%) the persistent protein impurity in these samples.

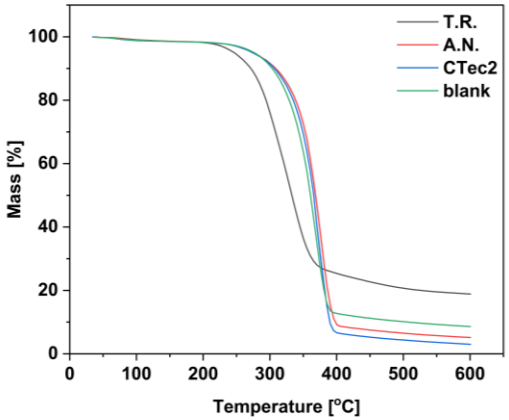
Reaction	Enzyme loading	C (%)	H (%)	N (%)	S (%)
Measured					
Cotton, pre-milled	N/A	44.08 ± 0.16	6.30 ± 0.02	0.00 ± 0.00	0.00 ± 0.00
Denatured *T. reesei* [a]	Only enzyme	45.08 ± 0.10	6.32 ± 0.00	12.42 ± 0.03	ND [b]
Table S1, entry 3 (72 h)	1.56 % w/w	44.03 ± 0.03	6.27 ± 0.01	0.27 ± 0.01	0.0 ± 0.0
Table S1, entry 6 (72 h)	4.12 % w/w	44.78 ± 0.27	6.36 ± 0.09	1.60 ± 0.40	0.0 ± 0.1
Table S1, entry 8 (72 h)	8.2 % w/w	44.15 ± 0.34	6.30 ± 0.03	1.05 ± 0.46	0.0 ± 0.0
Calculated					
Cellulose [c]	N/A	44.48	6.22	0	0
No enzyme removed [d]	1.56 % w/w	44.10	6.30	0.25	0.02
No enzyme removed [d]	4.12 % w/w	44.14	6.30	0.76	0.05
No enzyme removed [d]	8.2 % w/w	44.21	6.30	1.62	0.10

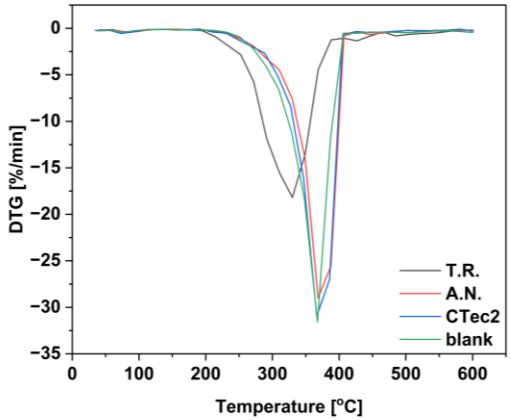
[a] An aliquot of the commercial protein solution was precipitated with ethanol, dried, and analyzed. For the calculation of the theoretical mass balances, the precipitate of *T. reesei* is assumed to contain only protein, thus representing the elemental balance of the enzyme. [b] Sulfur amount was below the detection limit. [c] Calculated for infinite chains of cellulose with the sum formula $(C_6H_{10}O_5)_n$. [d] Calculated assuming that all of the protein introduced into the reaction mixture gets immobilized on the cellulose remaining after the removal of the hydrolysis products (glucose), based on the respective glucose yield.

Table S6.

Thermal stability analysis of the recovered washed cellulose from hydrolysis with *Tr*, *An* and CTec2 cellulases (Table 1, entries 5, 8 and 11), compared to a sample treated without enzymes present (blank, Table 1, entry 16).

Reaction	Transformations	Temperature at maximum loss per min (°C)	Onset temperature (°C)	Mass loss (%)	Yield at 600°C (%)	Degradation enthalpy (J/g)
Tr	1 (main)	330	276	71	19	297
An	1 (main)	369	334	90	5	712
CTec2	1 (main)	368	335	92	3	385
blank	1 (main)	367	323	86	9	281





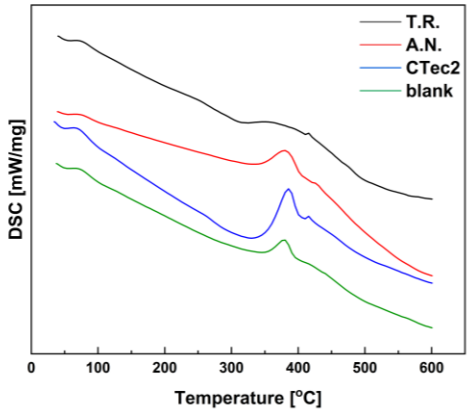


Table S7.

Process mass intensity (PMI) and space-time-yield (STY), comparing the developed method with previously reported processes using enzymatic hydrolysis as the main step for cellulose nanocrystal production. Optimal reactions minimize the PMI (minimum value 1) and maximize the STY.

Reaction	Enzyme	Cellulose source	Recovered hydrolysed cellulose (g)	Reaction volume (L)[i]	Reaction duration (h)	PMI	STY (g L^{-1} h^{-1})
This work	*Trichoderma reesei (Tr)*, 1.56 wt%	Cotton (cotton balls, filter paper)	**0.364** (0.308) (0.272)	**0.00042**	**0.25** (24) (72)	**2.3** (2.7) (3.0)	**3470** (31) (9)
This work	*Aspergillus niger* (*An*), 0.085 wt%	Cotton (cotton balls)	**0.4** (0.344) (0.372)	**0.00042**	**0.25** (24) (72)	**2.1** (2.4) (2.2)	**3810** (34) (12)
This work	Cellic CTec2®, 0.3 wt%	Cotton (cotton balls)	**0.376** (0.32) (0.304)	**0.00042**	**0.25** (24) (72)	**2.2** (2.6) (2.7)	**3580** (32) (10)
Bauli, et al.[13]	Cellic CTec2® and Cellic HTec®, 6 wt%	Wood flour (pre-treated)	0.0456 [ii]	0.008	48	184	0.12
Teixeira, et al.[14]	Optimash™BG, 345 U/g	Celish® KY-100G pure cellulose	0.256 [iii]	0.035	72	101	0.14
Teixeira, et al.[14]	Endoglucanase from *P. horikoshii* (EGPh) and *β*-glucosidase from *P. furiosus* (BGPf), 345 U/g	Celish® KY-100G pure cellulose	0.14 [iii]	0.035	72	253	0.14
Filson, et al.[15]	Endoglucanase, Celluclast 1.5 L FG, 210 U/g	Recycled pulp (1% lignin)	0.076 [iv]	0.025	0.75	330	4.08
Aguiar, et al.[16]	Cellic CTec3® 1 wt%	Treated sugarcane bagasse	0.0452 [v] (0.0648)	0.004	96	97 (68)	0.12 (0.17)
Aguiar, et al.[16]	Cellic CTec3® 1 wt%	Treated wheat straw	0.048 [v] (0.07)	0.004	96	92 (63)	0.13 (0.18)
Anderson, et al.[17]	*Aspergillus niger*, 10 wt%	Fully bleached kraft pulp	0.04 [vi]	0.04	62	1010	0.016

Yarbrough, et al.[18]	*Trichoderma reesei*, 0.75 wt%	Softwood bleached kraft pulp	0.12 [vii]	0.04	24	337	0.125
Yarbrough, et al.[18]	*Caldicellulosiruptor bescii*, 0.75 wt%	Softwood bleached kraft pulp	0.12 [vii]	0.04	24	337	0.125
Pere, et al.[19]	EcoPulp Energy (*Thermoascus aurantiacus*), 0.6 wt%	Softwood bleached pulp	261[viii]	1.2	8	5.7	27.2
Pere, et al.[19]	EcoPulp Energy (*Thermoascus aurantiacus*), 0.6 wt%	Softwood bleached pulp with residual lignin	273 [ix]	1.2	8	5.5	28.4
Alonso-Lerma, et al.[20]	Ancestral endoglucanase + CBM, 0.5 wt%	Whatman 1 filter paper	0.06 [x]	0.04	24	673	0.063

[i] Since water or dilute buffers are generally used in enzymatic hydrolysis, density of 1 g mL^{-1} is assumed for the calculation of the PMI (see Equation S3). [ii] Yield of 11.43 ± 2.6% is reported, with main material loss coming from close to 50% yield of glucose from cellulose at 48 h. These values are calculated for a reaction in the same scale as in this work (0.4 g of cellulose). [iii] Calculated based on the reported reaction scale (0.350 g of cellulose) and the reported glucose yields from the pure cellulose source. [iv] Calculated based on the reported reaction scale (0.200 g of cellulose) and the reported highest CNC yield 38.2 ± 2.5%. [v] These values are calculated for a reaction in the same scale as in this work (0.4 g of cellulose), with the reported CNC yields for bagasse 11.3% and straw 12%. In brackets, the same calculation is done using the total recovered cellulose solid yield, 16.2% for bagasse and 17.5% for wheat, respectively. [vi] This value is calculated for a reaction in the same scale as in this work (0.4 g of cellulose), with the reported CNC yield of 10%.[vii] Calculated based on the reported reaction scale (0.400 g of cellulose) and the reported nanocellulose yield 30%. [viii] Calculated based on the reported reaction scale (300 g of cellulose) and the reported nanocellulose yield 87%. [ix] Calculated based on the reported reaction scale (300 g of cellulose) and the reported nanocellulose yield 91%. [x] Calculated based on the reported reaction scale (0.4 g of cellulose) and the highest reported nanocellulose yield 15% at 24 h.

Table S8.

Comparison of costs for transforming 1 g of cotton to CNCs, comparing the developed method with previously reported processes using acid hydrolysis. Please note, that the pricing of the reaction components is based on the current prices (as of August 2024) from a chemical vendor for laboratory scale reagents (Sigma Aldrich), not industrial scale, and therefore should only be taken as an estimate for comparison purposes.

Reaction	Enzyme/Acid for g of cellulose (Cost)	Buffer (Cost, EUR/g)	Total cost of reactants (EUR for 1 g of cellulose)	Reaction temperature	Reaction duration (h)	Energy consumption [ii]	Post-processing steps (cost not calculated)
This work	*Trichoderma reesei (Tr)*, 1.56 wt% (1.910 EUR/g cellulose)	1 mL of 0.1M NaPB buffer, pH6 (0.001 EUR/g)	1.911 EUR/g	RT[i]	0.25	Ball-milling 0.016 kWh	Removal or deactivation of enzymes
This work	*Aspergillus niger* (*An*), 0.085 wt% (0.225 EUR/g cellulose)	1 mL of 0.1M NaPB buffer, pH6 (0.001 EUR/g)	0.226 EUR/g	RT[i]	0.25	Ball-milling 0.016 kWh	Removal or deactivation of enzymes
This work	Cellic CTec2®, 0.3 wt% (0.125 EUR/g cellulose)	1 mL of 0.1M NaPB buffer, pH6 (0.001 EUR/g)	0.126 EUR/g	RT[i]	0.25	Ball-milling 0.016 kWh	Removal or deactivation of enzymes
Sulfuric acid hydrolysis[21]	17.5 mL 64% sulfuric acid (0.55 EUR/g cellulose)	–	0.919 EUR/g	45°C	0.75	Heating (mantle), stirring 0.024 kWh	Neutralization and removal of acid
HCl hydrolysis[22]	60 mL 6 M HCl (1.74 EUR/g cellulose)	–	1.74 EUR/g	110°C	3	Heating (mantle), stirring *Ca*. 0.250 kWh	Neutralization and/or removal of acid
HCl-gas hydrolysis[23,24]	100 kPa pressure of HCl gas (ND)	–	ND	RT	1.5	None	Neutralization and/or removal of acid
Phosphoric acid hydrolysis[25]	*Ca*. 85 mL of 10.7 M H_3PO_4 (6.405 EUR/g cellulose)	–	6.405 EUR/g	100°C	1.5	Heating (mantle), stirring *Ca*. 0.160 kWh	Neutralization and/or removal of acid

[i] At the operating frequency of 25 Hz, using a single 10 mm stainless steel ball (ball-to-vessel filling ratio 0.03) and sample filling ratio up to 0.15 in the 15 mL stainless steel vessel, 15 minutes of ball milling should not raise the temperature within the jar above 25°C.[26] [ii] The energy consumption (kWh) was measured with a Waldsee Electronic LVM 605 power meter, for the Retsch MM400 mixer mill used for the 15-minute reaction as described *Method for milling and milling + aging reactions*. Energy consumption of a heating and stirring setup was measured with the same device, for a comparable scale acid hydrolysis reaction,

simulated by heating either 17.5 mL of water to 45°C and maintaining this temperature for 45 minutes (sulfuric acid hydrolysis); or by heating 50 mL of water to 100°C and maintaining this temperature for 15 minutes (HCl and phosphoric acid hydrolysis). The energy consumption of heating 17.5 mL of water to 45°C was 0.010 kWh, and maintaining it for 45 minutes was X kWh. The energy consumption of heating 50 mL of water to 100°C was 0.062 kWh, and maintaining it for 15 minutes was 0.016 kWh. Note that the reaction times for HCl and phosphoric acid hydrolysis are 90 and 180 minutes, not 15 minutes, therefore the energy consumption for these was extrapolated from the energy required to maintain the temperature at 100°C (0.016 kWh / 15 minutes).

References

1 B. Henrissat, *Cellulose*, 1994, **1**, 169–196.

2 S. Kaabel, J. Arciszewski, T. H. Borchers, J. P. D. Therien, T. Friščić and K. Auclair, *ChemSusChem*, 2023, **16**, e202201613.

3 Y. Shang, R. Su, R. Huang, Y. Yang, W. Qi, Q. Li and Z. He, *Appl Microbiol Biotechnol*, 2014, **98**, 5765–5774.

4 D. E. Otter, P. A. Munro, G. K. Scott and R. Geddes, *Biotechnol Bioeng*, 1989, **34**, 291–298.

5 R. Berggren, F. Berthold, E. Sjöholm and M. Lindström, *J Appl Polym Sci*, 2003, **88**, 1170–1179.

6 L. Segal, J. J. Creely, A. E. Martin and C. M. Conrad, *Textile Research Journal*, 1959, **29**, 786–794.

7 K. S. Salem, N. K. Kasera, Md. A. Rahman, H. Jameel, Y. Habibi, S. J. Eichhorn, A. D. French, L. Pal and L. A. Lucia, *Chem Soc Rev*, 2023, **52**, 6417–6446.

8 P. Ahvenainen, I. Kontro and K. Svedström, *Cellulose*, 2016, **23**, 1073–1086.

9 J. Meija, M. Bushell, M. Couillard, S. Beck, J. Bonevich, K. Cui, J. Foster, J. Will, D. Fox, W. Cho, M. Heidelmann, B. C. Park, Y. C. Park, L. Ren, L. Xu, A. B. Stefaniak, A. K. Knepp, R. Theissmann, H. Purwin, Z. Wang, N. De Val and L. J. Johnston, *Anal Chem*, 2020, **92**, 13434–13442.

10 N. Fantozzi, J.-N. Volle, A. Porcheddu, D. Virieux, F. García and E. Colacino, *Chem Soc Rev*, 2023, **52**, 6680–6714.

11 C. Jimenez-Gonzalez, C. S. Ponder, Q. B. Broxterman and J. B. Manley, *Org Process Res Dev*, 2011, **15**, 912–917.

12 T. Pääkkönen, P. Spiliopoulos, A. Knuts, K. Nieminen, L.-S. Johansson, E. Enqvist and E. Kontturi, *React Chem Eng*, 2018, **3**, 312–318.

13 C. R. Bauli, D. B. Rocha, S. A. de Oliveira and D. S. Rosa, *J Clean Prod*, 2019, **211**, 408–416.

14 R. S. S. Teixeira, A. S. A. Da Silva, J. H. Jang, H. W. Kim, K. Ishikawa, T. Endo, S. H. Lee and E. P. S. Bon, *Carbohydr Polym*, 2015, **128**, 75–81.

15 P. B. Filson, B. E. Dawson-Andoh and D. Schwegler-Berry, *Green Chemistry*, 2009, **11**, 1808.

16 J. De Aguiar, T. J. Bondancia, P. I. C. Claro, L. H. C. Mattoso, C. S. Farinas and J. M. Marconcini, *ACS Sustain Chem Eng*, 2020, **8**, 2287–2299.

17 S. R. Anderson, D. Esposito, W. Gillette, J. Y. Zhu, U. Baxa and S. E. McNeil, *Tappi J*, 2014, **13**, 35–42.

18 J. M. Yarbrough, R. Zhang, A. Mittal, T. Vander Wall, Y. J. Bomble, S. R. Decker, M. E. Himmel and P. N. Ciesielski, *ACS Nano*, 2017, **11**, 3101–3109.

19 J. Pere, T. Tammelin, P. Niemi, M. Lille, T. Virtanen, P. A. Penttilä, P. Ahvenainen and S. Grönqvist, *ACS Sustain Chem Eng*, 2020, **8**, 18853–18863.

20 B. Alonso-Lerma, L. Barandiaran, L. Ugarte, I. Larraza, A. Reifs, R. Olmos-Juste, N. Barruetabeña, I. Amenabar, R. Hillenbrand, A. Eceiza and R. Perez-Jimenez, *Commun Mater*, 2020, **1**, 57.

21 M. S. Reid, M. Villalobos and E. D. Cranston, *Langmuir*, 2017, **33**, 1583–1598.

22 H. Yu, Z. Qin, B. Liang, N. Liu, Z. Zhou and L. Chen, *J Mater Chem A Mater*, 2013, **1**, 3938–3944.

23 E. Kontturi, A. Meriluoto, P. A. Penttilä, N. Baccile, J. Malho, A. Potthast, T. Rosenau, J. Ruokolainen, R. Serimaa, J. Laine and H. Sixta, *Angewandte Chemie International Edition*, 2016, **55**, 14455–14458.

24 T. Pääkkönen, P. Spiliopoulos, A. Knuts, K. Nieminen, L.-S. Johansson, E. Enqvist and E. Kontturi, *React Chem Eng*, 2018, **3**, 312–318.

25 S. Camarero Espinosa, T. Kuhnt, E. J. Foster and C. Weder, *Biomacromolecules*, 2013, **14**, 1223–1230.

26 R. Schmidt, H. Martin Scholze and A. Stolle, *International Journal of Industrial Chemistry*, 2016, **7**, 181–186.