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PAPER

Integrated production of nano-fibrillated cellulose and cellulosic biofuel (ethanol) by enzymatic fractionation of wood fibers

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This study demonstrates the feasibility of integrating the production of nano-fibrillated cellulose (NFC), a potentially highly valuable biomaterial, with sugar/biofuel (ethanol) from wood fibers. Commercial cellulase enzymes were used to fractionate the less recalcitrant amorphous cellulose from a bleached Kraft eucalyptus pulp, resulting in a highly crystalline and recalcitrant cellulose (RC). The RC is difficult to hydrolyze to sugars but very suitable for producing biobased nanomaterials through mechanical homogenization. A range of fractionation yields of RC from 10–70% can be achieved by varying fractionation duration and enzyme dosage. The crystallinity of the RC was found to be as much as 24% higher than that of the original bleached pulp. The cellulase fractionation process facilitated mechanical homogenization by significantly reducing the degree of polymerization (DP) to about 400 and the length of the fibers to about 200 μm. The hydrolyzed sugars were found to be easily converted to ethanol through yeast fermentation with excellent efficiency of 92%. Films made from nano-fibrillated cellulose were found to be optically transparent, with opacity as low as 12%. The NFC films were mechanically strong and stiff, with tensile strengths and moduli approximately 10 and 6 times higher than film made from fibers that had not been nano-fibrillated.

1. Introduction

The production of cellulosic biofuels can mitigate climate change through the reduction of greenhouse gas emissions as a part of sustainable development.[1,2] However, cellulosic biofuel production is not economical using current technologies.[3] One of the key technical barriers to economical cellulosic biofuel production through biochemical conversion is the high cost and low efficiency of enzymatic cellulose saccharification of lignocellulosic biomass because of its strong recalcitrance.[4,5] It is very difficult to achieve near-complete cellulose saccharification even at high cellulase loadings and after a long period of hydrolysis. This is especially true when saccharification is conducted at the high cellulosic solid consistencies required to achieve the high biofuel titer necessary for reducing distillation/separation energy consumption.[6] Significant past research efforts in cellulosic biofuels have focused on overcoming the recalcitrance of cellulose and enhancing the saccharification of lignocellulose.[7] Instead, we propose to enzymatically fractionate the recalcitrant cellulose (RC) at high cellulosic solids consistency with low enzyme dosages. Because the hydrolysis rate of the amorphous cellulose is about 30 times faster than that of the crystalline cellulose,[8,9] we hypothesize that the fractionated recalcitrant cellulosic solids will be mainly crystalline cellulose. The crystalline nature of fractionated RC may be very suitable for nanocellulose production through mechanical or chemical means. Nanocellulose has exceptional optical and mechanical properties, and therefore can be used as a building block for a variety of high-performance cellulosic products through self-assembly or other means.[10–12] The dissolved cellulose can be further hydrolyzed and converted to biofuel/ethanol through fermentation or catalysis.[13] The proposed approach, by using low cellulase loadings while producing a potentially high value co-product, nanocellulose, has the potential to improve the economics of cellulosic biofuel production. It can be a viable shortcut to commercial production of both biofuels and biobased nanomaterials. This study also presents a green approach for bionanomaterial and biofuel production from lignocelluloses.

Cellulose as a structural material is extremely strong, with a theoretical modulus of around 250 GPa[14] and a specific tensile strength of about 5200 kN m kg^{-1}, about 18 times that of titanium. However, most cellulose is naturally present in plant lignocellulosic biomass as a biocomposite made of cellulose, hemicelluloses, lignin, *etc.*, with a hierarchical structure.[14] Advanced separation techniques are required to effectively liberate cellulose from lignocellulosic biomass in the forms of elementary fibrils and nano-fibrils made of elementary fibrils. There are

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several approaches for nanocellulose production from plant biomass. Acid hydrolysis was developed in the 1940s,[15] and remains a major process to produce stable colloid suspensions of nanocrystalline cellulose (NCC)[16–18] with negative charges.[19–21] The process for creating NCC has a very low yield of about 30–40%.[16] The use of strong sulfuric acid is a hazard, and also is an environmental concern for waste stream treatment.

Mechanical homogenization or shearing has been used to produce nano-fibrillated cellulose (NFC).[22–25] However, it is very energy-intensive. Energy consumption can be 20 000–30 000 kWh ton^{-1} (72–108 GJ ton^{-1})[10] or approximately 4–5 times the energy stored in wood. Chemical pretreatments[26,27] can significantly reduce this energy consumption.[28] For example, the 2,2,6,6-tetramethylpiperidine-1-oxyl radical (TEMPO)-mediated oxidation of cellulose fibers achieved some level of success in efficiently producing NFC.[29–32] However, TEMPO is a very expensive chemical, and effective methods for the recovery of TEMPO need to be developed.

Enzyme pretreatment is an environmentally friendly alternative to chemical pretreatment for nanocellulose production that is then followed by mechanical homogenization.[28,33,34] In these studies, both commercial endoglucanase,[28,33] such as Novozyme 476, and purified exoglucanase from commercial cellulase[34] were used. However, glucose concentrations in the enzymatic hydrolysate were not quantified, with glucose yields under 5%, and the investigations were conducted at very low cellulosic substrate consistencies.[33,34] To achieve high yields of cellulosic solids, most published studies on enzyme pretreatments used only endoglucanase for a very short period of time to avoid cellulose saccharification.[28,33] The recovery and utilization of hydrolyzed sugars in the enzymatic hydrolysates were not the objective, and were not considered. This study attempts to achieve effective fractionation of bleached Kraft eucalyptus fibers into hydrolyzed cellulose and crystalline RC using a commercial complex cellulase. The objective of the study is to demonstrate the feasibility of the proposed approach to integrate production of NFC and cellulosic biofuel (ethanol) using yeast fermentation and mechanical homogenization of the fractionated hydrolyzed cellulose and RC, respectively.

2. Experimental

2.1 Materials

Wet bleached Kraft eucalyptus pulp was complementarily provided by Fibria (Aracruz, Brazil). The pulp was frozen in a freezer at –4 °C before use. The major chemical components of the pulp were: glucan = 92.9%, xylan = 5.7%, Klason lignin = 1.2%, analyzed by the Analytical Chemistry and Microscopy Lab at the Forest Products Laboratory, U.S. Forest Service, Madison, WI. Endoglucanase of Novozym 476 (Novozymes, Franklinton, NC) and complex cellulase of Genencor Multifect B (Genencor, Palo Alto, CA) were used as received.

2.2 Enzymatic fractionation and fermentation

Enzymatic fractionation of the eucalyptus pulp was conducted in a 250-mL flask on a shaking bed (Thermo Fisher Scientific, Model 4450, Waltham, MA) at 10% (w/v) solid consistency; *i.e.*, 10 g of pulp in 100 mL cellulase solution. The loadings of cellulases of Novozym 476 and Genencor multifect B were 10 and 5 FPU per g substrate, respectively, based on a series of studies to maximize the crystallinity of the resultant RC. Fractionation experiments were all conducted at 50 °C and buffered at pH 4.8 using sodium acetate but with varied durations from 1–72 h. At the end of each experiment, the solid/liquid sample was separated by filtration through a filter paper (Sartonlon Polymid, pore size 0.45 μm, Sartorius Stedim Biotech GmbH, Goettingen, Germany). The liquid sample produced from the 72-h hydrolysis was stored in a freezer at –16 °C for subsequent fermentation to ethanol.

2.3 Determination of degree of polymerization and crystallinity of fractionated solids

The degree of polymerization (DP) of the cellulase-fractionated cellulosic solids were measured according to TAPPI Standard Test Method T230 om-99.[35] TAPPI standard methods have been well developed and proven effective by the pulp and paper industry and scientific communities, and therefore were used throughout this study for various testings. 0.1 g of oven-dry cellulosic solid was first dissolved into 20 mL of 0.25 M cupriethylenediamine solution. The viscosity of the resultant solution was determined by a capillary viscometer. The DP of the cellulose was calculated using the following expression:[36]

$$\mathrm{DP}^{0.905} = 0.75\,[954 \log X - 325] \quad (1)$$

where X is the measured viscosity.

The crystallinities of these residual solids were measured by a FT-Raman spectroscopic method.[37] Approximately 0.25 g of air-dried sample was pressed into a pellet that was analyzed using a Bruker MultiRam spectrometer (Bruker Instruments Inc., Billerica, MA). The sample crystallinity index was calculated using the Raman spectral intensities at two wavenumbers (cm^{-1}) as follows, and was corrected for the instrument-dependent intensity differences between RFS-100 and MultiRam (both FT-Raman instruments).

$$\mathrm{Cr}_{\mathrm{Raman}} = [(I_{380}/I_{1096}) - 0.0286]/0.0065 \quad (2)$$

2.4 Nano-fibrillated cellulose (NFC) production

The RC collected at the end of 48 h of hydrolysis was used for mechanical homogenization to produce NFC. The RC from enzymatic fractionation was diluted to approximately 0.3 g L^{-1} in deionized water, and mixed in a household Waring blender for 5 min. A microfluidizer processor (M-110EH-30 Microfluidics, Newton, MA) was used to homogenize the RC into NFC. The 0.3% suspension was passed through the microfluidizer up to 50 times using a 200-μm chamber and 10 additional times using an 87-μm chamber to produce highly nano-fibrillated cellulose. The homogenized cellulosic fibers were collected and characterized after 10, 20, 50, and 60 total passes.

2.5 Electron microscopy

Specimens for scanning electron microscopy (SEM) were prepared by drying drops of the aqueous slurry on aluminium mounts. All SEM specimens were sputter-coated with gold to provide adequate conductivity for examination in a Zeiss

EVO 40 SEM (Carl Zeiss NTS, Peabody, MA) under ultra-high vacuum conditions. For transmission electron microscopy (TEM), aqueous NFC suspensions (0.2 g L^{-1} were further diluted in water by a factor of 300 and sonicated. Drops of approximately 2–3 μL were deposited and dried on TEM sample grids containing ultrathin carbon films about 3 nm thick supported by thicker carbon films with holes. A transmission electron microscope (Philips CM120, Netherlands) with an accelerating potential of 80 keV was used to image these fibers.

2.6 Production and testing of NFC films

Films of fibers resulting from various degrees of mechanical homogenization were formed by ultrafiltration of fiber slurries using a 142-mm Millipore ultrafiltration apparatus with polytetrafluoroethylene (PTFE) membranes of 0.1-μm pore size (Millipore JVWP14225, Bedford, MA). Filter paper was placed below the ultrafiltration membranes to provide support. Fiber slurries of approximately 0.2 g L^{-1} were added to the ultrafiltration apparatus to make sheets with a target weight of 1.0 g. After dewatering, individual films were blotted and placed between filter and blotter papers. The films and filter papers were placed between smooth metal caul plates and allowed to dry at room temperature under a pressure of approximately 14–20 kPa for three days.

The mechanical and optical properties of the films were measured according to TAPPI Standard Test Methods.[35] The films were first conditioned according to TAPPI Method T 402, *i.e.*, preconditioning at 22–40 °C under 10–35% relative humidity for at least 24 h, followed by conditioning under 50 ± 2.0% RH at 23 ± 1.0 °C for at least 24 h. Two sheets for each experimental condition were prepared and sampled for physical and mechanical testing. Three tensile specimens and eight zero-span specimens were obtained from each sheet. Tensile tests were performed using TAPPI T 494 in which a span of 6.4 cm, a width of 15 mm, and an elongation speed of 0.2 mm min^{-1} were used. Zero-span tensile tests were performed using a Zero Span Tensile tester (model ZST-15, Pulmac) according to TAPPI T 231 with sample specimens at least 20 mm wide. Reported zero-span tensile data were normalized to a basis weight of 60 g m^{-2}. The opacities of the films were performed according to TAPPI T 519.

3. Results and discussion

3.1 Enzymatic fractionation of amorphous and crystalline cellulose

Effective fractionation of the less recalcitrant amorphous segments from the recalcitrant crystalline segments of wood pulp cellulose is critical to integrate the productions of NFC and sugar for cellulosic ethanol through fermentation. A range of desired yields of cellulosic solid substrate from 60–90% were possible by varying the duration of enzymatic hydrolysis (Fig. 1). Similar degrees of enzymatic fractionation were also achieved by varying the loading of Multifect B cellulase. The cellulase also significantly reduced the degree of polymerization (DP) of the fractionated solid cellulose from 1400 to 400 after 48 h of hydrolysis (Fig. 2). Such depolymerization is important to facilitate NFC production through mechanical means.[38] This

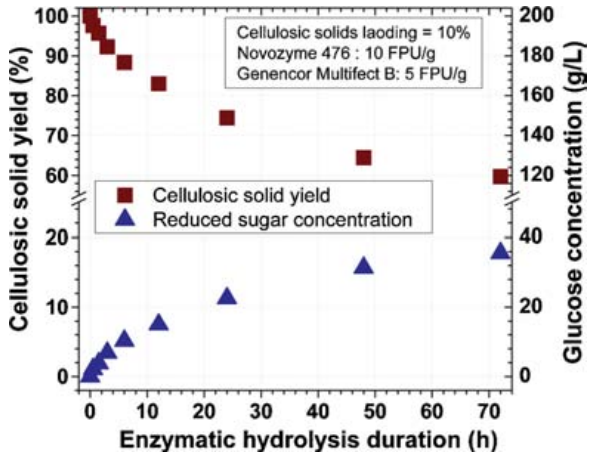

Fig. 1 Effect of enzymatic pretreatment duration on recalcitrant cellulose (RC) yield and reduced sugar concentration in the hydrolysate from bleached eucalyptus pulp.

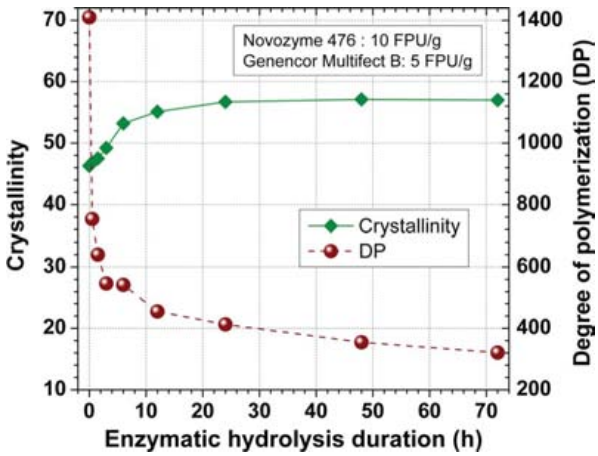

Fig. 2 Effect of enzymatic hydrolysis duration on DP and crystallinity of the resultant recalcitrant cellulose (RC) from bleached eucalyptus pulp.

can be verified from images of the microfluidized suspensions. The suspension of the enzyme fractionated cellulosic solids after 60 passes through a microfluidizer (50 passes through a 200-μm chamber followed by 10 passes through an 87-μm chamber) was transparent, suggesting the resultant cellulose is highly nanofibrillated. However, the suspension of the original bleached eucalyptus fibers remained opaque after the same amount of processing. The measured crystallinity index of the fractionated RC increased from 46 to 57 (an increase of 24%) after 48 h of hydrolysis, which is very significant and validates our hypothesis that the fractionated RC solids contain primarily crystalline cellulose. Pure cellulose nanocrystal samples are reported to be 66% crystalline by the Raman method.[39]

The morphology of the fractionated RC solids can reveal the physical destruction of the cellulosic fibers by the cellulase, which is important to facilitate NFC production using the microfluidizer in this study. Cellulase not only cuts the chain length of cellulose, as evidenced by the significant reductions in DP throughout the hydrolysis process (Fig. 2), but also significantly shortens the fiber length as hydrolysis proceeds. The average fiber length was reduced to approximately 200 μm

(Fig. 3a) from 1.2 mm (Fig. 3b) measured by a commercial fiber quality analyzer (HiRes FQA, OpTest Equipment, Hawkesbury, ON, Canada) after 24 h hydrolysis. Interestingly, cellulase not only reduced the length of long fibers, but also completely hydrolyzed small cellulosic particles and fines, to produce a substrate with good uniformity in length (Fig. 3a).

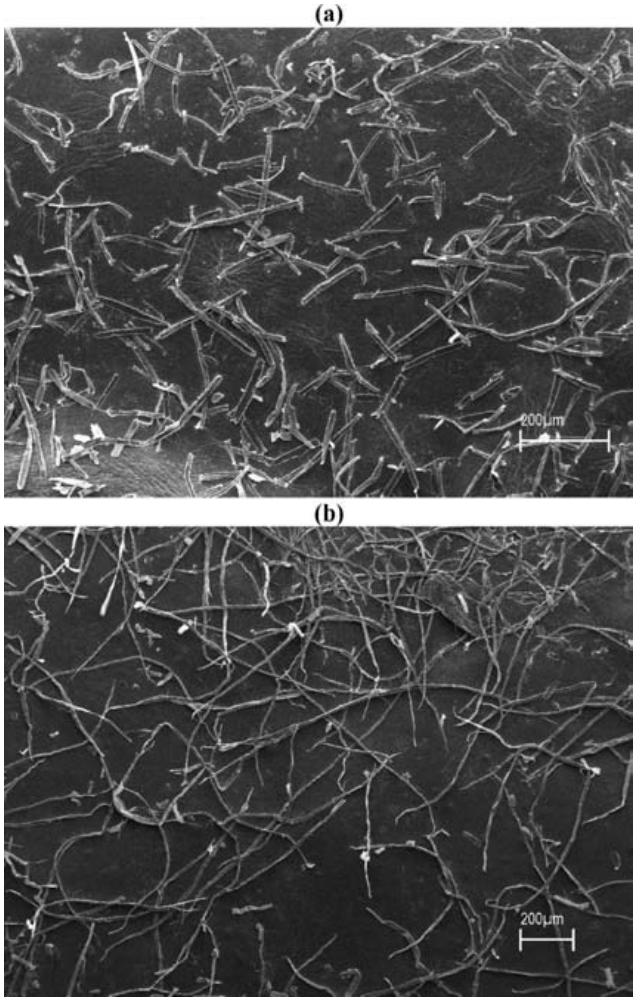

Fig. 3 Scanning electron microscope image of: (a) fractionated recalcitrant cellulose (RC) resulting from 48 h of enzymatic hydrolysis under enzyme loadings of 5 FPU per g substrate of Genencor Multifect B; (b) the original bleached Kraft eucalyptus pulp fibers (control).

3.2 Ethanol production from hydrolyzed amorphous cellulose

The sugar streams from cellulase fractionation can be further hydrolyzed using β-glucosidase to glucose for ethanol production through fermentation. Without the application of xylanase, the resultant sugar stream is simply glucose, and was found to be easily fermentable to ethanol using *Saccharomyces cerevisiae* D5A (ATCC® number: 200062) as demonstrated by the measured time-dependent glucose and ethanol concentrations in the fermentation broth. Glucose consumption and ethanol production in the first 48 h were 0.72 g L^{-1} h^{-1} and 0.31 g L^{-1} h^{-1}, respectively. The fermentation efficiency was 91.6% based on the measured initial glucose concentration of 41 g L^{-1} and the terminal ethanol concentration of 19.2 g L^{-1} at 72 h.

3.3 Morphology of nano-fibrillated cellulose (NFC)

Enzymatically fractionated fibers were refined using the microfluidizer and a specialized homogenizer, and the morphology of the homogenized fibers was examined after incremental levels of homogenization corresponding to discrete numbers of passes through the microfluidizer. After 20 passes through the microfluidizer with a chamber size of 200 µm, a significant portion of the fibers were not yet nano-fibrillated, and fibers with micrometre-sized diameters and with lengths of 10 µm or longer were still present. However, after a total of 60 passes (50 passes through a chamber of 200 µm and 10 passes through a chamber of 87 µm), large micrometre-sized fibers were no longer present, and nano-fibrillated cellulose with diameter of about 20 nm and lengths of 500 nm or longer was produced (Fig. 4).

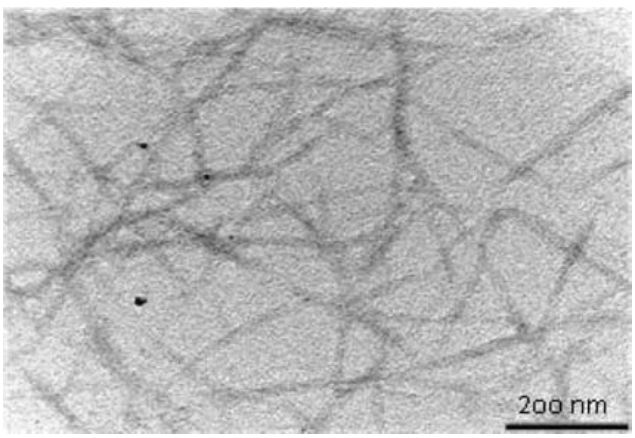

Fig. 4 Transmission electron microscopy (TEM) image of the nano-fibrillated cellulose (NFC) produced from the fractionated recalcitrant cellulose (RC) resulting from 48 h of enzymatic hydrolysis followed by 60 passes through the microfluidizer (50 passes using the 200 µm chamber followed by 10 passes using the 87 µm chamber).

3.4 Properties of NFC films

Films of the fractionated and microfluidized fibers were produced and their optical and mechanical properties were measured as an initial evaluation of the properties and utilities of the NFC. The opacity of the films clearly decreases with the number of passes through the microfluidizer (Fig. 5). The NFC films, such as the one shown in Fig. 6, produced from highly nano-fibrillated cellulose (60 passes) had opacity values of only approximately 12%. Zero-span tensile strength was markedly improved for sheets made of fibers that had been through the microfluidizer as few as 10 times, compared to purely hydrolyzed fibers without microfluidization (control). The zero-span of the NFC film is 5.0 ± 0.2 kN m^{-1}, or a more than a 5-fold increase over films produced from the control fibers (0.9 ± 0.1 kN m^{-1}). The tensile strength (maximum stress) of the NFC film is 45 ± 5 MPa, or more than an order of magnitude greater than sheets made from the control fibers (3.7 ± 0.3 MPa). The modulus of the NFC film (Fig. 6) was 5400 ± 180 MPa, or approximately 6 times of the sheet made from the control fibers (900 ± 60 MPa).

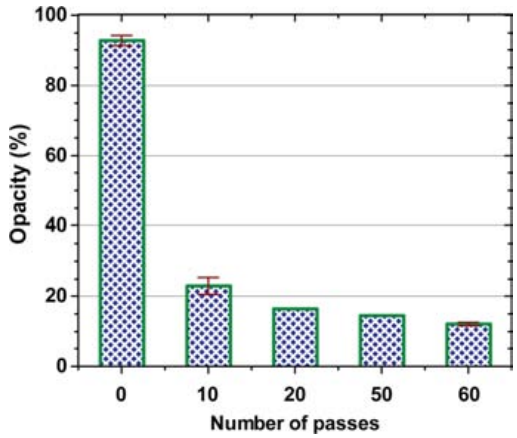

Fig. 5 Opacity of nanocellulose sheets as a function of number of passes through the microfluidizer.

Fig. 6 Image of a nanocellulose film produced in this study. The transparent nature of the film can be seen from the U.S. Forest Service logo laid beneath the film.

4. Conclusions

This study has successfully demonstrated a novel concept to integrate the production of nano-fibrillated cellulose (NFC), a highly valuable bionanomaterial and sugar/cellulosic biofuel (ethanol). Cellulase fractionation produced a high-quality glucose stream for biofuel production through yeast fermentation, with efficiency of over 90%. It also resulted in a recalcitrant cellulosic solid fraction for NFC production by mechanical homogenization. The NFC-produced film has high optical opacity and mechanical strength. The study demonstrated a feasible and green approach to merge two separate fields of science and technology to develop one integrated manufacturing industry for a future biobased economy. Future studies will focus on novel enzyme technologies for effective fractionation of lignocellulosic biomass.

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