Enzymatic-mediated production of cellulose nanocrystals from recycled pulp

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Endoglucanase was used to hydrolyze recycled pulp to produce cellulose nanocrystals. The highest yields of cellulose nanocrystals were obtained by treatment with 84 EGU of endoglucanase per 200 mg recycled pulp at 50 °C for 60 min of microwave and conventional heating. Of the two modes of heating investigated, microwave heating at each treatment gave a higher yield than conventional heating. Transmission and scanning electron microscopic analysis of suspensions showed cellulose nanocrystals with widths of 30 nm to 80 nm and lengths of 100 nm to 1.8 μm). This was within range of the length of cellulose crystals (100 nm to 3.5 μm) obtained using dynamic light scattering analysis. The average zeta potential of cellulose nanocrystals was −31.37 mV. X-ray diffraction of cellulose nanocrystals, recycled pulp and residues of recycled pulp shows a gradual change in the particle size.

Introduction

Cellulose nanocrystals (nanofibers) represent a new emerging biological source of reinforcing biofillers. Various forms of lignocellulosic biomass are potential raw materials for the production of these new biofillers. These potential sources are also renewable, sustainable, abundant, and cheap. In addition, the new biofillers are of low density, high specific strength and modulus.[1] Due to the presence of hydroxyl groups on the surfaces of cellulose nanocrystals, their surfaces are reactive, making them suitable candidates as reinforcing material for the manufacture of composites.[2,3] Consequently, we have witnessed the increasing application of cellulose nanocrystals as reinforcing materials for reinforced polymer nanocomposites. These nano-biofillers enhance the strength of the resulting nanocomposites. To improve adhesion in nanocomposites containing hydrophobic matrices, hydroxyl groups on the surfaces of cellulose nanocrystals can be converted to hydrophobic groups through grafting and other methods.[3]

Cellulose nanocrystals are produced from several starting materials including microcrystalline cellulose,[4,5] valonia,[3,6] cotton,[7] wood pulp,[8] tunicin[9] and sugar beet pulp.[10] The most commonly used method for the preparation of cellulose nanocrystals is mineral acid hydrolysis of cellulosic materials using sulfuric acid *ca* 64% (w/w). Cellulose nanofibers have also been produced from hardwood by treatment with the 2,2,6,6-tetramethylpiperidine-1-oxyl radical in combination with sodium bromide and NaClO.[11] In the production of cellulose nanocrystals, acid hydrolysis of cellulosic materials is dependent on three factors: temperature, time and concentration of the mineral acid.[5] These factors affect the yield as well as the physical and mechanical properties of cellulose nanocrystals. The most commonly used protocol involves the hydrolysis of cellulosic materials with mineral acids at a temperature range of 45 to 50 °C depending on the time of hydrolysis and expected physical characteristics of the cellulose nanocrystals.

In the acid hydrolysis protocol, heating has traditionally been conventional heating. In conventional heating, energy is conveyed through convection, conduction and radiation.[12] However, the rate of heating with conventional heating is slow compared to microwave heating. In microwave heating, electromagnetic energy converts thermal energy through direct interaction of the incident radiation with molecules of a target material.[12] Microwave heating is selective and specific which results in a reduction of the processing time during hydrolysis thereby increasing the yield of expected products as shown by Venkatesh *et al.*[12]

Current methods for producing cellulose nanocrystals are characterized by low yields (*ca* 20%). To commercialize production of cellulose nanocrystals, it is important to address the question of low yields associated with current processes. Cellulose consists of amorphous and crystalline regions. Since cellulose crystals are produced from the crystalline region, we explored the selectivity of endoglucanase enzyme to hydrolyze the amorphous regions leaving the crystalline region. The latter is then fragmented into cellulose crystals using ultrasonic treatment. Ultrasonication of the crystalline region increases the yield of cellulose nanocrystals by dispersing and releasing the cellulose nanocrystals in the suspension.

Cellulases are composites of endoglucanases, exoglucanases and cellobiohydrolases. These enzymes act synergistically in the hydrolysis of cellulose.[13] Endoglucanase randomly attacks and hydrolyzes the amorphous region whilst exoglucanase attacks the cellulose polymer chain from either the reducing or non-reducing ends. Cellobiohydrolases hydrolyze cellulose from either the C1 or the C4 ends using a protein in each case, into cellobiose units.

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Recycled pulp is largely cellulose with low lignin and hemicellulose contents. Chemical and mechanical treatments of pulp in the recycling process increase the amorphous regions and reduce the cellulose chain length of cellulose molecules, which cumulatively decrease the crystallinity of the recycled pulp. Generally, recycled pulp makes poor quality paper. However, efforts to improve the physical and chemical properties,[14] as well as the crystallinity and surface properties[15] of recycled fibers have met with little success.

Overall, recycled pulp may be a viable raw material for the production of cellulose nanocrystals. The goal of this paper is to exploit endoglucanase enzyme to hydrolyze selectively the amorphous portion of the recycled pulp to produce cellulose nanocrystals using two modes of heating: conventional and microwave coupled to ultrasonication. In this paper, we report the method of isolation and characterization of cellulose nanocrystals from recycled pulp.

Experimental

Materials

Recycled pulp (1% lignin) was provided by American Fiber Resources (Fairmont, West Virginia, USA) (Fig. 1). Recycled pulp was produced from waste commercial wood pulp and used business papers. Endoglucanase, Celluclast 1.5 L FG was provided by AS Novozyme North America, Incorporated (Franklinton, North Carolina, USA) with a density of 1.20 g/ml and a declared activity of 700 EGU/g (endoglucanase units per gram). Sodium hydrogen phosphate buffer (1 M, pH 6.8) was used. Deionized water was obtained using Corning Megapure (distilled water) and Barnstead E-pure purification systems (deionized water).

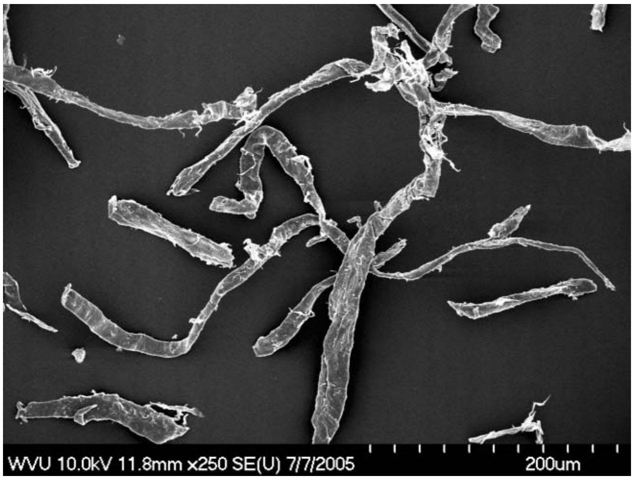


Fig. 1 Scanning electron micrograph showing recycled pulp before cellulose hydrolysis.

Heating methods

Two methods of heating: (1) conventional (Hybridization Incubator Combi-V12, FINEPCR, Yang-Chun, South Korea) and (2) microwave (MARS Xpress, CEM Corporation, Matthews, North Carolina) were used. The microwave system was programmed to ramp suspensions of recycled pulp to the desired temperature in 20 min and this was held constant for the times indicated in Table 1. An ultrasonic bath, Branson 2510 (Branson Ultrasonics Corporation, Danbury, Connecticut, USA), with water was maintained at 20 °C during sonication of suspensions of recycled pulp. This process further fragmented cellulose particles particularly unhydrolyzed large crystalline regions into cellulose nanocrystals.

Table 1 Overview of hydrolysis conditions for recycled pulp

Heating method	Temperature (°C)	Time (min)
Conventional		
C-1	50	60
C-2	50	45
C-3	60	60
C-4	60	45
Center-point (CP-C)	55	52
Microwave		
M-1	50	60
M-2	50	45
M-3	60	60
M-4	60	45
Center-point (CP-M)	55	52

Design of experiment

Three primary factors: (1) hydrolysis temperature, (2) hydrolysis time and (3) ratio of endoglucanase enzyme concentration to mass of pulp were explored. To reduce the number of treatments, a full factorial experimental design with two factors, hydrolysis temperature and hydrolysis time, were nested with two endoglucanase enzyme concentrations per known mass of recycled pulp. Response factors evaluated were (1) flow birefringence, (2) appearance of turbidity in suspension, (3) shape and (4) size of cellulose particles.

Three hydrolysis temperatures: (1) 50 °C (lower limit), (2) 60 °C (upper limit) and (3) 55 °C (center-point) and three hydrolysis times: (1) 45 min (lower limit), (2) 60 min (upper limit) and (3) 52 min (center-point) were selected (Table 1).[12,13] Two endoglucanase enzyme concentrations (42 and 84 EGU) per 200 mg of pulp respectively were used. Sixty treatments were generated by this experimental design.

Production of cellulose nanocrystals from recycled pulp

Twenty five ml of deionized water and phosphate buffer were added to 200 mg of recycled pulp in a 125 ml beaker; this was mixed with the help of a magnetic stirrer for 2 h to soften the pulp. Then 100 μl of endoglucanase enzyme (42 EGU) was added. Microwave heating and conventional heating were performed in a MARS Xpress laboratory microwave and hybridization incubator respectively. The resulting suspensions of recycled pulp specimens were placed in 75 ml vessels in a MARS Xpress microwave and in an 80 ml vessel in the hybridization chamber and heated to the target temperature (Table 1). Another set of experiments was carried out using 200 μl (84 EGU) of endoglucanase enzyme per 200 mg of recycled pulp maintaining the same heating conditions and time as in the earlier experiment.

A control experiment for each treatment was carried out using deionized water and recycled pulp without endoglucanase enzyme. At the end of the treatment procedure, 25 ml of 95% w/v ethanol was added to each resulting suspension and stirred vigorously with magnetic stirring bars for 20 min to terminate the action of endoglucanase.

The resulting suspensions were centrifuged at 12,000 rpm at 10 °C for 10 min using a Sorvall RP-5B refrigerated superspeed centrifuge (DuPont Instruments, Chadds Ford, Pennsylvania, USA) and the supernate was decanted. Cellulose particles (mass) from each suspension were washed repeatedly with deionized water to remove endoglucanase enzyme from the solute until the supernate turned turbid. Turbidity is an indication of the presence and release of the cellulose nanocrystals. The turbid solutions were collected after 50 ml deionized water was added to the slurry of recycled pulp and sonicated for 2 min in a water bath. The water bath was kept at 20 °C by adding ice blocks continuously to maintain the temperature during sonication. Each suspension was allowed to stand for 30 min after sonication. The top turbid layer was then decanted. This process was repeated several times until there was no indication of turbidity in each suspension. Half the initial amount of endoglucanase enzyme was applied to the cellulose residues from each treatment. The resulting suspensions were treated per the above protocol until there was no turbidity. The total turbid solution collected for each treatment was filtered through a 200 mesh sintered glass filter and evaporated at 50 °C until the volume of the suspension was reduced to about 25% of the initial volume. The remaining suspension was freeze-dried and the cellulose nanocrystals vacuum-dried to a constant dry mass. The yield was calculated as the percentage of the ratio of the dry mass of cellulose particles to the initial dry mass of recycled pulp.

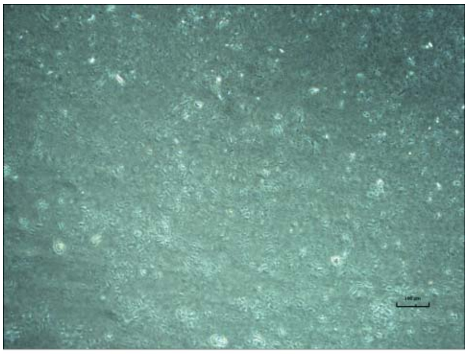
Microscopic methods

Images of flow birefringence of suspensions of the cellulose nanocrystals (0.1% w/v) between two cross polarizers were taken using a Canon EOS digital camera (Fig. 2). A droplet of 0.01% of each resulting cellulose nanocrystals suspension from each treatment was placed in a glass well slide and covered with a thin glass slide and imaged using an Olympus bright field polarizing microscope at magnifications of 100× and 200× (Fig. 3).

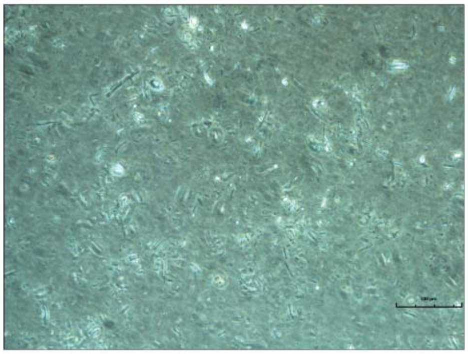
A droplet of cellulose nanocrystals suspension (0.001% w/v) was placed on a clean cut silicon surface and allowed to dry overnight. The silicon surfaces were imaged using an Ultra-high resolution Hitachi S-4800 field emission scanning electron microscope (UHRFESEM) with an operating accelerating voltage of 10 kV. The UHRFESEM was also used to image the initial recycling pulp fibers and the residues from each treatment to study cellulosic particles at each stage of endoglucanase enzyme mediated hydrolysis. A sample of cellulose nanocrystals in each suspension were imaged as per the protocol of Filson and Dawson-Andoh.[16]

About 0.5 µl of each suspension of cellulose nanocrystals per treatment was loaded onto a 300-mesh carbon coated formvar copper grids (Electron Microscopy Sciences, Hatfield, PA, USA) using a Labnet micropipette. Water in the suspensions on the carbon coated grids was allowed to evaporate. Cellulose nanocrystals coated grids were examined using a JEOL 100CXII transmission electron microscope at an operating voltage of 80 kV. The dimensions of the imaged cellulose nanocrystals were determined using the transmission electron microscope software.

Fig. 2 Flow birefringence of cellulose nanocrystals from recycled pulp between two cross polarizing films. (Container is 20 mm in diameter).



(a)



(b)

Fig. 3 Cellulose nanocrystals seen under a polarizing microscope at (a) 100× and (b) 200×. Cellulose nanocrystals agglomerate.

Measurement of dimension of cellulose nanocrystals using light scattering

Particle size distribution, average molecular weight and zeta potential of the various suspensions of cellulose nanocrystals from recycled pulp (0.01% w/v) from each treatment were determined using Nanotrac Ultra/Zetatrac instruments, which operate on the theory of light scattering. Nanotrac Ultra/Zetatrac instruments determine particle size and polydispersity on the principle that particles in dispersion are in Brownian motion. When these particles scatter photons of light, exchanges of small amounts of energy between particles and photons occurs. The diffusion coefficient of particles is measured and used to calculate the apparent hydrodynamic diameter of particles using the Stokes–Einstein equation.[17] Zeta potential and apparent average molecular weights of particles in suspension were determined based on the principle of electrophoretic and static light scattering respectively.[17]

X-ray diffraction studies of cellulose nanocrystals

Samples of freeze-dried cellulose nanocrystals and residues from endoglucanase mediated hydrolysis of recycled pulp were first made into powder by grinding them using an agate mortar and pestle. The resulting powders of cellulose nanocrystals, as well as the residue of recycled and initial recycled pulp were placed separately on quartz with a rectangular depression (dimensions 1 mm × 20 mm × 15 mm). Ethanol (95%) was added to samples on quartz glass and allowed to evaporate. The sample was later pressed with enough pressure to make the surface flush with the plane of quartz before mounting on a stage to record the X-ray diffraction patterns. Wide-angle X-ray diffraction patterns of each sample of cellulosic material were recorded with a Rigaku D/Max-B diffractometer using CuKα radiation generated at 40 kV and 30 mA. The range (2θ) of X-ray reflections was varied from 10° to 50° in steps of 0.06°.

Results and discussion

Particle size distribution of cellulose nanocrystals

The presence of cellulose nanocrystals after each treatment was indicated by the presence of birefringence in the suspensions. This is due to polarization of plane light by nematic orientation of the cellulose nanocrystals. Birefringence of the cellulose nanocrystals suspension was imaged by first shaking the suspension for a short time before placing the suspension between two polarizing films in a dark box (Figs. 2 and 3). Transmission and scanning electron micrographs (Figs. 4 and 5) showed cellulose nanocrystals with widths between 30–80 nm and lengths between 100 nm to 1.8 μm. The dimensions of the cellulose nanocrystals closely match previously reported work of several authors.[3-10] However, the transmission electron microscopy images only a small area of surface containing particles. Consequently, scanning electron microscopy was further used to image a larger surface area on the substrate to give a better report of the sizes of the particles in the suspensions. The sizes and shapes of the cellulose nanocrystals imaged using both TEM and SEM were in good agreement.

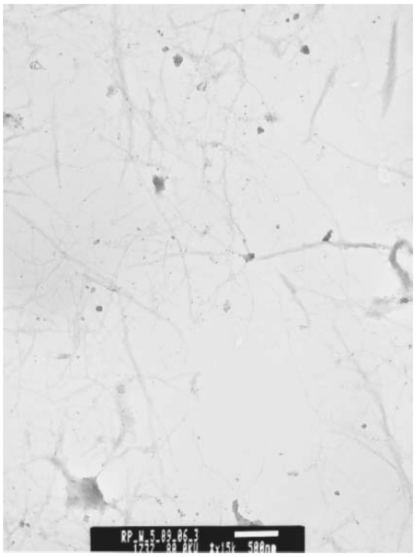


Fig. 4 TEM images of cellulose nanocrystals from recycled pulp ((Scale bar = 500 nm).

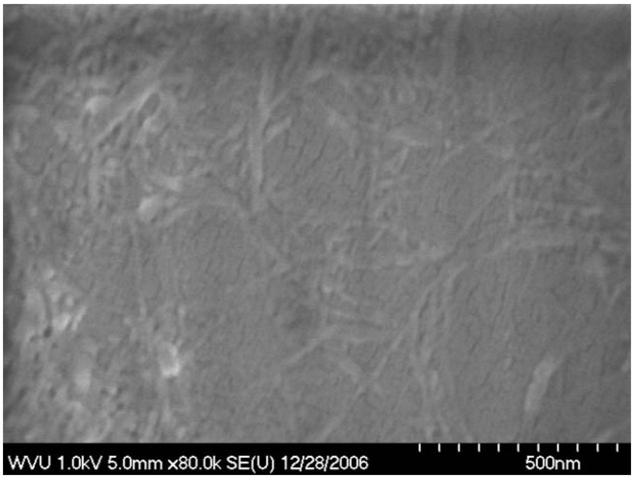


Fig. 5 SEM images of cellulose nanocrystals from recycled pulp (Scale bar = 500 nm).

The results of dynamic light scattering studies on the sizes and distribution of cellulose nanocrystals in suspensions were compared with that of electron microscopy. The volume of cellulose nanocrystals suspension used for particle size and size distribution is large enough and provides a better representation of the total volume of the suspensions than that used for the electron microscopy study. The average length of cellulose nanocrystals ranged from about 100 nm to 3.5 μm (Fig. 6). The cellulose nanocrystals size distribution exhibited a trimodal frequency distribution with apparent average lengths of 154.9, 820 and 3540 nm for each peak. This was comparable to lengths obtained using the electron microscope. Finally, the apparent average molecular weight of the cellulose nanocrystals was determined to be 1.65×10^{11} Daltons. The unusually high apparent average molecular weight can partly be ascribed to aggregation of the cellulose nanocrystals in water.

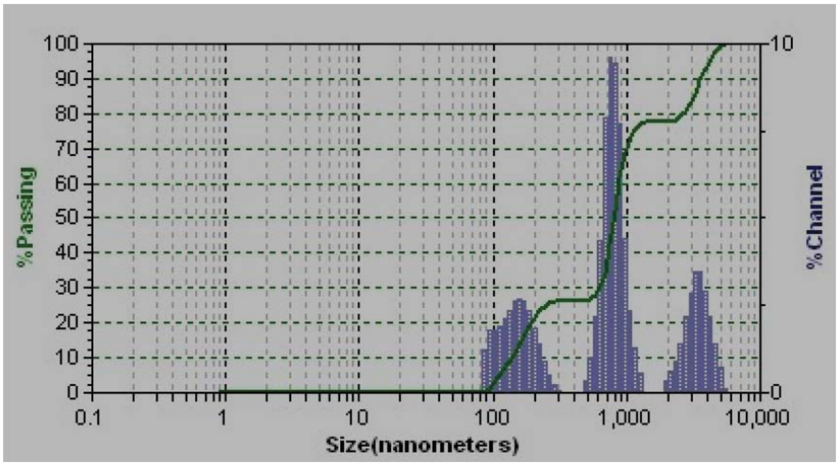


Fig. 6 Particle size distribution of cellulose nanocrystals from recycled pulp.

Crystallinity of cellulose nanocrystals, recycled pulp and residue

X-ray diffraction patterns show an increase in the crystalline domains in the recycled pulp (residue) as recycled pulp (starting) undergoes endoglucanase mediated hydrolysis (Fig. 7) with a decrease in the amorphous region. This is evidenced by the presence of stronger diffraction peaks at $2\theta = 16.8°$ and $22.6°$ as compared to weak or no reflection at 18°. Diffraction peaks at $2\theta = 16.8°$ and $22.6°$ are ascribed to cellulose I (native cellulose) and $2\theta = 18°$ to amorphous. This suggests that endoglucanase mediated hydrolysis of recycled pulp does not change the polymorphism of cellulose I in the residue as well as in the cellulose nanocrystals produced. However, the average crystallite sizes show progressive increase in size from cellulose nanocrystals through recycled pulp (starting) to recycled pulp (residue) using Scherrer's equation below. Furthermore, X-ray diffraction patterns (Fig. 7) show that cellulose nanocrystals have broader X-ray diffraction peaks, which further supports their smaller particle size compared to both recycled pulp (initial), and recycled pulp (residue).[18]

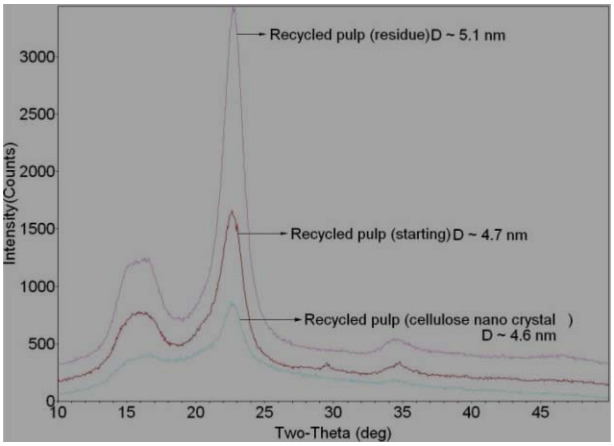


Fig. 7 X-ray diffraction patterns of cellulose nanocrystals, recycled pulp (initial) and recycled pulp (residue).

Scherrer's equation:

$$D = \frac{k\lambda}{B\cos} \tag{1}$$

where D = Average crystallite (particle) size perpendicular to the direction of the X-rays

θ = Bragg's angle
λ = wavelength of X-rays used
B = full width at half maximum of the diffraction peak.

Zeta potential and stability of cellulose nanocrystals suspensions

The stability of suspensions of nanoparticles is critical in the preparation of nanocomposites and derived from the absolute magnitude of zeta potential of suspensions. The average zeta potential was determined to be −31.37 mV, which reflects the favorable stability of the suspensions for an extended time. The voltage recorded is an indication that the suspension of cellulose nanocrystals from recycled pulp was fairly stable,[19] exhibited by the stability of suspensions of cellulose nanocrystals for over a month before they aggregated and settled at the bottom of containers (Fig. 8).

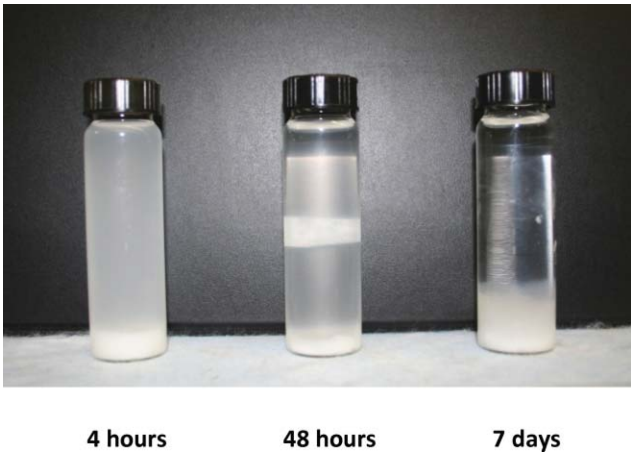


Fig. 8 Suspensions of cellulose nanocrystals showing gradual aggregation of cellulose nanocrystals with time.

Table 2 The properties and yield of cellulose nanocrystals from recycled pulp

	Factor responses			
Conventional heating	Characteristics of suspension	Flow birefringence	Shape of particles	Yield (%)
C-1-E	Turbid	Yes	Cylindrical	10.7 ± 0.8
C-1-B	Clear	No	—	—
C-1-W	Turbid	Yes	Cylindrical	7.4 ± 1.2
C-2-E	Turbid	Yes	Cylindrical	29.0 ± 0.9
C-2-B	Turbid	Partial	Cylindrical	25.7 ± 1.9
C-2-W	Turbid	Yes	Cylindrical	7.1 ± 0.9
C-3-E	Turbid	Yes	Cylindrical	15.0 ± 1.2
C-3-B	Clear	Partial	Cylindrical	21.0 ± 1.4
C-3-W	Turbid	Yes	Cylindrical	3.7 ± 0.4
C-4-E	Turbid	Yes	Cylindrical	3.4 ± 0.3
C-4-B	Turbid	Partial	Cylindrical	18.0 ± 2.5
C-4-W	Turbid	Yes	Cylindrical	1.5 ± 0.3
CC-E	Turbid	Yes	Cylindrical	16.0 ± 3.3
CC-B	Clear	Partial	Cylindrical	11.0 ± 3.1
CC-W	Turbid	Yes	Cylindrical	4.9 ± 0.7
Microwave heating				
M-1-E	Turbid	Yes	Cylindrical	34.9 ± 1.6
M-1-B	Clear	Partial	Cylindrical	10.9 ± 1.4
M-1-W	Turbid	Yes	Cylindrical	12.8 ± 1.9
M-2-E	Turbid	Yes	Cylindrical	38.2 ± 2.5
M-2-B	Turbid	Yes	Cylindrical	26.4 ± 1.7
M-2-W	Turbid	Yes	Cylindrical	4.9 ± 1.1
M-3-E	Turbid	Yes	Cylindrical	22.9 ± 2.6
M-3-B	Clear	Partial	Cylindrical	15.9 ± 2.5
M-3-W	Turbid	Yes	Cylindrical	6.2 ± 2.3
M-4-E	Turbid	Yes	Cylindrical	20.5 ± 4.1
M-4-B	Turbid	Yes	Cylindrical	15.0 ± 3.1
M-4-W	Turbid	Yes	Cylindrical	6.6 ± 1.5
MC-E	Turbid	Yes	Cylindrical	18.2 ± 1.9
MC-B	Clear	Partial	Cylindrical	13.0 ± 2.3
MC-W	Turbid	Yes	Cylindrical	6.0 ± 1.3

C = conventional heating; M = microwave heating; CC = conventional heating at center-point temperature; MC = microwave heating at center-point temperature; E = Enzyme in deionized water; B = Enzyme in sodium phosphate buffer and W = water only.

Effect of conventional and microwave heating on the yield cellulose nanocrystals

Table 2 shows various treatments of recycled pulp with endoglucanase enzyme at different temperatures (50 °C, 55 °C and 60 °C) in different media: (1) (water and endoglucanase), (2) phosphate buffer and endoglucanase), and (3) water only and response factors for each treatment condition for preparation of cellulose nanocrystals. The yield of cellulose nanocrystals in the various treatments using conventional and microwave ranged 3.4–29.0% and 4.9–38.2% respectively. The yield of cellulose nanocrystals in water and endoglucanase were higher at all modes of heating (Table 1). Additionally, endoglucanase treatments at 50 °C in water at both heating modes gave the highest yield of cellulose nanocrystals (29% for conventional and 38.2% for microwave heating). This may therefore represent the optimum temperature for endoglucanase hydrolysis of the amorphous regions of recycled pulp in the reaction media. Comparatively, the yield of cellulose nanocrystals was higher for the microwave mode of heating. This may be a reflection of the relative efficiency and specificity of microwaves in heating materials.[12]

The yield of cellulose nanocrystals from recycled pulp at the optimum temperature for both conventional and microwave heating modes were higher than the yield reported by several authors.[2,5] Though the yield may not appear significantly higher than that reported by several authors,[2,5] it suggests that there is the potential to improve the yield if feedstocks with higher crystallinity than recycled pulp are used. Additionally, this method of production of cellulose nanocrystals leads the way to a process that is devoid of environmentally hazardous wastes unlike the use of sulfuric acid to hydrolyze cellulosic materials to produce cellulose nanocrystals.

Conclusions

The treatment of recycled pulp with endoglucanase enzyme at all the experimental conditions of temperature, media and pH produced cellulose nanocrystals at different yields. The yield of cellulose nanocrystals were higher in water and endoglucanase at both modes of heating. For both modes of heating, endoglucanase enzyme treatment in water gave the highest cellulose nanocrystals yield.

In all treatments, the presence of cellulose nanocrystals was confirmed by flow birefrigerence. Transmission electron microscopy showed that cellulose nanocrystals had widths between 30–80 nm and a length of 100 nm to 1.80 µm. Dynamic light scattering studies showed that cellulose nanocrystals exhibited a length of 100 nm to 3.5 µm. Size distribution of cellulose nanocrystals showed a trimodal frequency distribution with an average length of 154.9, 820 and 3540 nm for each peak. X-ray diffraction indicated an increase in crystallinity as the amorphous domains in the recycled pulp is reduced by endoglucanase enzyme hydrolysis.

Cellulose nanocrystals had an average zeta potential of −31.37 mV, an indication of favorable stable cellulose nanocrystals for an extended time. The stability of suspensions of cellulose nanocrystals in deionised water suggests their suitability as nanofillers in the making of cellulose nanocrystals reinforced polymer nanocomposites. The success of this study provides a potential green method for the production of cellulose nanocrystals using endoglucanase.

Acknowledgements

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